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Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*

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Abstract

The effect of the carbon to nitrogen (C/N) ratio of the medium and the aeration rate on the lipid content and fatty acid composition of *Chlorella sorokiniana* was investigated using heterotrophic, batch culture. Both parameters had a significant effect. A C/N ratio of approximately 20, was found to indicate a change from carbon to nitrogen limitation for *C. sorokiniana*. Cell lipid content was at a minimum at this value and increased at both higher and lower C/N values. Low C/N ratios favoured a high proportion of trienoic fatty acids at the expense of monoenoic acids. Aeration enhanced cell growth, fatty acid yield and the synthesis of unsaturated dienoic and trienoic fatty acids, but reduced cell lipid content. The results demonstrate that the fatty acid composition and lipid content of heterotrophically-grown microalgae can be favourably manipulated by varying culture conditions.

Introduction

Microalgae can produce large quantities of unsaturated fatty acids (Cohen, 1986; Behrens *et al.*, 1989), some of which have received growing attention for the prevention and treatment of human disease (Tamura *et al.*, 1986), and as growth promoters in the mariculture industry (Yone, 1978; Langdon & Waldcock, 1981). A major obstacle to the commercialisation of such products from microalgae is their poor productivity compared to other microbial systems. This is due to their low growth rates and the meagre cell densities obtained in photosynthetic culture (Johns *et al.*, 1989).

Some microalgae are capable of heterotrophic growth on monosaccharides or organic acids. This mode of growth offers the possibility of greatly improving the productivity of microalgal culture through the use of fed-batch and high cell density techniques, which can not be applied to photosynthetic systems. These techniques are routinely applied to bacterial and yeast cultures to product cell densities in the order of 150–200 g l^{-1} dry weight (Suzuki *et al.*, 1985; Lee & Chang, 1987).

The production of fatty acids by photosynthetic microalgal cultures has been studied by many workers and it has been demonstrated that yields are sensitive to a number of environmental factors, including temperature, nitrogen concentration, light intensity etc. in most species (Piorreck *et al.*, 1984; Pohl & Wagner, 1972; Cohen *et al.*, 1988; Lee & Tan, 1988). In contrast, fatty acid

production in heterotrophic cultures of microalgae has received little attention.

To evaluate the possibility of using heterotrophic culture to produce fatty acids, the effect of glucose and nitrogen concentrations and aeration rate on fatty acid production by a heterotrophic microalga, *Chlorella sorokiniana*, was studied.

Materials and methods

Cultures

Chlorella sorokiniana (UTEX 1602) was maintained heterotrophically in liquid culture at 25 °C on K2N medium (Kuhl medium (Kuhl & Lorenzen, 1964) with a doubled nitrate concentration (2 g l^{-1}) and supplemented with 10 g l^{-1} glucose). For heterotrophic cultivation, 800 ml of K2N medium contained in a 1-litre glass fermentor was inoculated with approximately 5% (v/v) of a 5-day-old heterotrophic preculture which was grown on the same medium in 250 ml static flasks at 25 °C. Medium and fermentors were sterilised by autoclaving at 121 °C for 15 min.

The culture was agitated by magnetic stirrer and maintained at 25 °C by a waterbath. Air was supplied to the culture via a rotameter, a hydrophobic filter (0.2 μm) and sparged into the fluid through a sintered glass diffuser. The initial pH of the medium was adjusted to 6.8, and varied only slightly during the cultivation. The dissolved oxygen concentration was monitored by a dissolved oxygen meter (LH Fermentation Ltd, Stoke Poges, UK) with a galvanic electrode (Uniprobe Instruments Ltd, Cardiff, UK).

Determination of lipid content and fatty acid composition

Twenty ml of culture fluid was centrifuged at 2300 rpm for 30 min, the supernatant was discarded, and the cell pellet was resuspended in distilled water to a volume of 1.6 ml. Six ml of a methanol: chloroform mixture (2:1 v/v) were added and the mixture was shaken for several minutes and centrifuged at 2300 rpm for another 30 min. The chloroform phase was carefully withdrawn and transferred to a glass vial, diluted with benzene (2 ml), and evaporated to dryness under a stream of nitrogen in order to avoid oxidation of unsaturated fatty acids. The residue was immediately weighed to give total lipid content (Ben-Amotz *et al.*, 1985).

Lipid residue was converted to fatty acid methyl esters with BF_3/methanol (Morrison & Smith, 1964) and quantitated by capillary GC-MS (HP 5995A with 3392 integrator, Hewlett Packard, Avondale, PA) equipped with a flame ionisation detector. A 25 m × 0.2 mm I.D. BP21 column (SGE, Ringwood, VIC) was used with helium carrier gas and temperature programming consisting of 3 min at 200 °C, and subsequently increasing to 220 °C at 5 °C min^{-1}. Authentic standards (Alltech Associates, Inc., Deerfield, IL) were employed for the identification of fatty acids. The total fatty acids and individual fatty acids were estimated from the peak areas.

Assay of glucose concentration

Glucose concentration in the culture fluids was determined by HPLC. Culture fluid was filtered through a 0.2 μm cellulose acetate membrane, diluted as required with distilled water and held for analysis at −12 °C. HPLC analysis was performed using a Waters Associates liquid chromatograph equipped with a R401 differential refractometer (Waters Associates, Milford, MA). Fifteen μl of sample was injected onto a 300 mm × 7.8 mm Bio-Rad HPX-87H$^+$ column (Bio-Rad Labs, Richmond, CA) with a micro-guard ion exclusion cartridge.

Analyses were conducted at a flow rate of 0.6 ml min^{-1} corresponding to a pressure of 1000–1200 psi, a column temperature of 65 °C with a mobile phase comprising 0.008 N sulphuric acid. Glucose concentration (retention time, generally 8.7 min) was determined by comparison of peak height with that of a standard injected after every 5 samples.

Assay of nitrogen concentration

Nitrate levels were determined using a Dionex Ion Chromatograph (2010i) equipped with a Dionex Ion Pac HPLC AS4 anion separator, an Ion Pac AG4 Guard column and a packed anion fibre suppressor (Dionex Corp., Sunnyvale, CA). Detection was achieved using a Dionex conductivity detector with a temperature of 20–23 °C. An injection volume of 50 μl was used. The mobile phase comprised 0.0022 M Na_2CO_3 and 0.0028 M $NaHCO_3$ with 0.025 N H_2SO_4 as regenerant at a flow rate of 2.0 ml min^{-1}. Nitrate concentration (retention time, 3.25 min) was estimated by comparison of peak height with that of a standard (1 g l^{-1} NO_3) injected after every five samples.

Measurement of dry cell weight

Ten ml of sample was transferred to a pre-weighed centrifuge tube and centrifuged at 2300 rpm for 30 min. After rinsing the pellet twice with distilled water, it was dried overnight at 70 °C in a vacuum oven, cooled over silica gel in a desiccator and weighed. The dry weight of cells was determined in duplicate.

Results

Effect of C/N ratio

Experiments were performed using heterotrophic, batch cultures of *C. sorokiniana*, in which glucose and nitrate concentrations in the medium were varied to give initial C/N ratios in the range from 7–35. Exhaustion of the growth-limiting nutrient generally occurred by 5 days of culture, which corresponded to the end of growth. The critical C/N ratio at which the transition from carbon to nitrogen limitation of growth occurred was found to be C/N = 20–25 (Fig. 1). There was good agreement between the critical C/N values determined for cultures in which initial glucose (Fig. 1a) or nitrate concentrations (Fig. 1b) were varied. The fermentation parameter which most strongly reflected the limiting nutrient was the cellular yield coefficient based on nitrogen $Y_{x/N}$), which approximately doubled when nitrogen was limiting (Table 1).

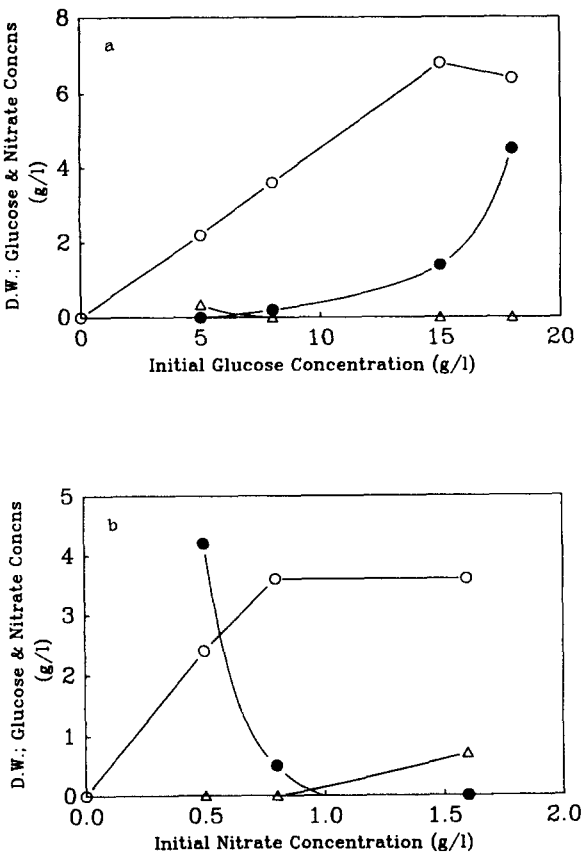

Fig. 1. Determination of critical C/N ratio in heterotrophic cultures of *C. sorokiniana*. (A) varying initial glucose concentration. (B) varying initial nitrate concentration. Symbols: ○, dry weight concentration; ●, glucose concentration; ▽, nitrate concentration.

Table 1. Effect of the initial C/N ratio on cellular yield coefficients, for *C. sorokiniana* grown heterotrophically at 25 °C. $Y_{x/s}$ is the dry cell weight yield based on glucose consumed $Y_{x/N}$ is the dry cell weight yield based on nitrate consumed.

Initial C/N ratio	7	11	21	28
$Y_{x/s}$ (g g^{-1})	0.43	0.49	0.49	0.5
$Y_{x/N}$ (g g^{-1})	2.2	2.3	5.0	5.0

The effect of initial glucose and nitrate concentration on the lipid content and fatty acid composition of *C. sorokiniana* cells in late growth/early stationary phase is given in Tables 2 and 3 respectively. These data are plotted as a function of the initial C/N ratio in Fig. 2. The initial C/N ratio of the medium has a clear impact on the lipid content and fatty acid composition of the microalgae. Cellular lipid content was increased at C/N ratios higher or lower than the critical value.

Fatty acid composition was also sensitive to C/N ratio (Fig. 2) with the percentage of trienoic acid increasing with decreasing C/N ratio at the expense of monoenoic acids. At all C/N ratios tested, the fatty acid profile of heterotrophic *C. sorokiniana* cells was characterised by C16 and C18 acids, of which palmitic acid, C16:0, and linoleic acid, C18:2, predominated. Palmitic acid content (expressed as %TFA) was particularly consistent with a mean ($n = 7$) of 22.5 ± 1.5 ($\pm$ S.D.), compared to 29.2 ± 2.7 for linolenic acid. The C18:3 acid was identified as the α-linolenic acid isomer.

Table 2. Effect of initial glucose concentration on lipid content any fatty acid composition of 7-day-old cells of *C. sorokiniana* in hetertrophic culture with an aeration rate of 62 ml min^{1} and at 25 °C.

	Glucose concentration ($g\,l^{-1}$)			
	5	8	15	18
Lipids (% dry wt.)	45.5	28.2	20.1	22.5
Total FAs (% lipids)	14.0	18.2	21.5	18.4
Fatty acid (% of total fatty acids)				
16:0	22.7	21.5	21.1	22.0
16:1	trace	2.5	4.0	4.3
16:2	9.0	10.7	12.2	11.5
16:3	8.8	10.2	7.8	5.1
18:0	4.7	4.5	4.0	3.5
18:1	3.9	2.8	7.0	11.3
18.2	27.8	26.9	28.4	31.1
18:3	18.7	17.3	12.7	9.1
Degree of lipid unsaturation (∇/mole)*	1.60	1.63	1.54	1.43

* The degree of unsaturation (∇/mole) in lipids was calculated according to Kates & Baxter (1962). ∇/mole = [1.0 (% monoene) + 2.0 (% diene) + 3.0 (% triene)]/100.

Table 3. Effect of nitrogen concentration on lipid content and fatty acids of 6-day-old cells of *C. sorokiniana* in hetertrophic culture, with an aeration rate of 62 ml min^{-1} and 25 °C.

	Nitrate concentration ($g\,l^{-1}$)		
	0.5	0.75	1.50
Initial C/N ratio	35.4	23.5	11.8
Lipids (% dry wt.)	29.1	14.9	28.8
Total FAs (% lipids)	18.4	31.1	18.2
Fatty acid (% total fatty acids)			
16:0	25.4	23.3	21.5
16:1	3.1	4.6	2.5
16:2	10.7	11.7	10.7
16:3	4.1	6.2	10.2
18:0	1.4	2.3	4.5
18:1	12.4	10.9	2.8
18:2	34.4	28.9	26.9
18:3	7.1	10.0	17.3

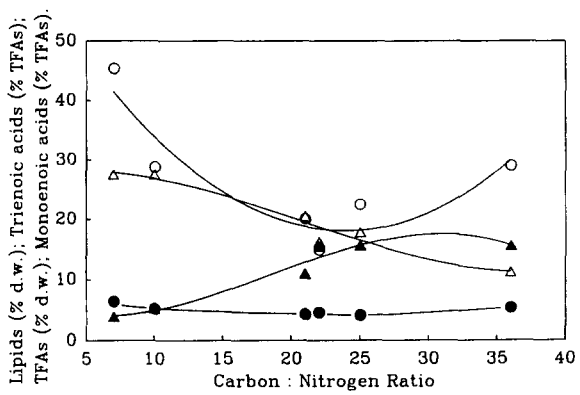

Fig. 2. Effect of initial C/N ratio on the lipid content and fatty acid composition of heterotrophic *C. sorokiniana* at 25 °C. Symbols: ○, cellular lipid content; ●, fatty acid content (% dry wt.); ▾, total monoenoic fatty acid content (% total fatty acids); ∇, total trienoic fatty acid content (% total fatty acids).

Effect of aeration

Batch cultures of *C. sorokiniana* were grown on K_2N medium containing $10\,g\,l^{-1}$ glucose (C/N = 11) under different aeration rates. Four-day-old cells were sampled and analysed for lipid content and fatty acid composition (Table 4). At this

Table 4. Effect of aeration rate on lipid content and fatty acids of 4-day-old cells of *C. sorokiniana* in heterotrophic culture at 25 °C. The ungassed liquid volume of the vessels was 800 ml.

Aeration rate (ml min^{-1})	0	57	85	140
Lipids (% dry wt.)	46.0	35.5	21.6	13.0
Total FAs (% lipids)	7.1	16.7	17.4	25.0
Fatty acid (% of total fatty acids)				
16:0	24.3	21.1	24.0	24.5
16:1	6.6	5.2	trace	trace
16:2	7.4	7.9	11.8	12.6
16:3	trace	7.3	6.7	6.5
18:0	21.5	1.8	4.1	3.1
18:1	16.3	18.1	7.3	5.8
18:2	20.1	18.2	33.1	35.4
18:3	3.9	18.3	11.3	10.8
Degree of lipid unsaturation (∇/mole)	0.90	1.52	1.51	1.54

age, the cells were not nutrient limited and any differences in composition were assumed to be the result of changes in the aeration rate.

Cultures in which no air was sparged, were oxygenated only by gas transfer at the liquid surface. Cells from these cultures exhibited a markedly different fatty acid composition to those cultures into which air was sparged. Non-sparged cells had a high lipid content, a significantly reduced degree of lipid unsaturation (59.2% of sparged cultures) and a high proportion of stearic acid (C18:0). The proportion of total fatty acids as palmitic acid was largely unaffected by the extent of aeration and it appears to be independent of C/N ratio and aeration.

Sparging air into the cultures led to a linear fall in lipid content with increasing aeration, whereas the fatty acid content of the lipids increased. Fatty acid composition showed less dependence on the extent of aeration. At all aeration rates, the concentration stearic acid was greatly reduced compared to the non-sparged culture, and C18:2, C16:3 and C18:3 acids were increased. However, as aeration rate increased, linoleic acid (C18:2) synthesis was favoured over α-linolenic acid (C18:3).

Discussion

The fatty acids produced by *C. sorokiniana* are not of commercial interest. Those that are so, particularly the ω3-polyunsaturated fatty acids (PUFA), appear to be produced only by marine *Chlorella* and other marine microorganisms. Nevertheless the data from this study demonstrate that the lipid content and the ratio of unsaturated to saturated fatty acids can be manipulated by the proper optimisation of conditions in heterotrophic microalgal cultures.

A high cellular lipid content is promoted by a growth medium which is either carbon or nitrogen limited. The former is preferred, since a low C/N ratio boosts the proportion of unsaturated acids, especially trienoic acids, significantly. At higher C/N ratios, carbon is supplied in excess, nitrogen becomes limiting and the increase in lipid content of the cell may be explained as a carbon storage mechanism. This behaviour has been well documented for other microorganisms (Ratledge, 1987). More surprising, is the increase in cell lipids at C/N ratios below the critical value, at which carbon is limiting. This phenomenon was also reported for *C. saccharophila* when cultured on a glucose-nitrate medium with an initial C/N ratio of 6 (Tan & Johns, 1990). The reason for this behaviour is uncertain. The phenomenon has not been reported for other heterotrophic microorganisms such as yeast or fungi, but this may be due to the common use of high C/N ratios to promote lipid formation in these organisms (Turcotte & Kosaric, 1989).

The effect of the C/N ratio on fatty acid composition may result from its influence on lipid composition. Behrens *et al.* (1989) observed a decline in the proportion of the polyunsaturated fatty acid (PUFA), eicosapentanoic acid (EPA; C20-5), in the lipids of the diatom *Phaeodactylum tricornautum* as the cultures became nitrogen limiting. This was due to increased production of triglycerides, which contained less unsaturated fatty acids under these conditions. This explanation may also fit our results.

A similar balance is required with aeration. Trienoic acids and lipid content were favoured by

aeration of the culture, but at a low level. In cultures without air-sparging, it would appear that the dissolved oxygen concentration was low and fatty acid desaturation was effected. Desaturation of fatty acids would be expected to be sensitive to dissolved oxygen levels, as the desaturase enzymes have a requirement for molecular oxygen (James *et al.*, 1965). However, aeration of the cultures above a relatively low rate of 57 ml min^{-1} led to a decrease in the lipid content of the cells, presumably due to the increased lipid catabolism (White *et al.*, 1978), which is stimulated under these conditions. Furthermore, the conversion of dienoic to trienoic acids was inhibited.

In conclusion, the production of fatty acids, or other microalgal metabolites, by heterotrophic culture offers the ability to achieve volumetric productivities well in excess of those obtained in photosynthetic cultures provided suitable microalgae, which have high concentrations of PUFAs, can be obtained. *C. sorokinana* concentrations of up to 9 g l^{-1} dry weight were obtained in these experiments, at good efficiencies of substrate utilisation. This advantage is significant as the move to large-scale culture of microalgae for valuable metabolites grows.

Research is continuing in this Department to identify heterotrophic marine *Chlorella* and diatoms for ω3-PUFA production and to develop techniques to achieve high cell densities of heterotrophic microalgae.

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