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Effects of salinity on cell growth and docosahexaenoic acid content of the heterotrophic marine microalga *Crypthecodinium cohnii*

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The effects of salinity on cell growth and docosahexaenoic acid (DHA) content of three marine microalgal strains, *Crythecodinium cohnii* ATCC 30556, *C. cohnii* ATCC 50051 and *C. cohnii* RJH were investigated. The lag phases of the three strains increased with increasing salinity in Porphyridium medium. The specific growth rate of *C. cohnii* ATCC 30556 was the highest at 9 g L^{-1} NaCl while the other two strains had their highest specific growth rates at 5 g L^{-1} NaCl. The highest cell dry weight concentrations of 2.51 g L^{-1} and 1.56 g L^{-1} were achieved at 9 g L^{-1} NaCl for *C. cohnii* ATCC 30556 and ATCC 50051, respectively, while the highest dry weight concentration of 2.49 g L^{-1} was achieved at 5 g L^{-1} NaCl for *C. cohnii* RJH. The highest cell growth yield coefficient on glucose was 0.5 g g^{-1} for both *C. cohnii* ATCC 30556 and *C. cohnii* RJH and 0.45 g g^{-1} for *C. cohnii* ATCC 50051. All three strains responded to the change of salinity by modifying their cellular fatty acid compositions. At 9 g L^{-1} NaCl, *C. cohnii* ATCC 30556 had the highest total fatty acid content and DHA (C22:6) proportion. In contrast, *C. cohnii* ATCC 50051 and *C. cohnii* RJH had the highest DHA content at 5 g L^{-1} NaCl. *C. cohnii* ATCC 30556 and ATCC 50051 had the highest DHA yield (131.55 and 68.24 mg L^{-1} respectively) at 9 g L^{-1} NaCl while *C. cohnii* RJH had the highest DHA yield (128.83 mg L^{-1}) at 5 g L^{-1} NaCl.

Keywords: microalgae; *Crypthecodinium cohnii*; salinity; fatty acid composition; docosahexaenoic acid; heterotrophic production

Introduction

Recent clinical and epidemiological studies have demonstrated that certain ω-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA), are important in the development and functioning of brain, retina and reproductive tissues for both adults and infants [17]. DHA is currently produced commercially from fish oil. However, fish oil is an insufficient source of this product. Furthermore, fish oil possesses a number of problems including peculiar odour, taste, instability and inclusion of impurities [20] which make separation and purification of DHA from fish oil extremely difficult and expensive. All these problems have significantly hindered commercial production of DHA from fish oil. It is now clear that fish obtain ω-3 fatty acids from zooplankton that consume algae. Consequently, the possibility of using microalgae as producers for this valuable polyunsaturated fatty acid is being explored [20,21].

Crypthecodinium cohnii is a heterotrophic marine dinoflagellate in which nearly 30–50% of its constituent fatty acids is C22:6 (DHA, ω-3) fatty acid, and other polyunsaturated fatty acids are present in trace amounts [12]. *C. cohnii* can grow rapidly on glucose medium [20,21]. These properties indicate that *C. cohnii* may represent a potential microalgal source for industrial production of DHA. We have recently obtained several high DHA-yielding strains as a result of extensive screening.

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Lipids are vital to cell functions acting as structural components of cell membranes. Microalgae usually modify their biochemical composition in response to environmental changes, including nutrient availability, light intensity, pH, temperature and salinity. As *C. cohnii* is a marine microalga whose growth is favoured by supplementing certain concentrations of NaCl to the culture medium, a knowledge of the effects of salinity on cellular growth and fatty acid production is thus necessary. The primary aim of this study was to investigate the effects of various salinity levels of the culture medium on growth and fatty acid production by selected strains of *C. cohnii*. This information could be used to assist in determining the optimum salinity for growth and production of DHA by these *C. cohnii* strains.

Materials and methods

Microalgae and culture conditions

Crypthecodinium cohnii strains ATCC 30556 and ATCC 50051 were obtained from the American Type Culture Collection (Rockville, MD, USA). *C. cohnii* strain RJH was a gift from Dr RJ Henderson (University of Stirling, UK). Cultures were maintained axenically in the dark at 20°C on liquid Porphyridium medium [18] consisting of: 1 g L^{-1} yeast extract, 1 g L^{-1} tryptone, 100 ml of soil water, 500 ml of artificial seawater without NaCl, and 400 ml of distilled water supplemented with 5 g L^{-1} glucose, and subcultured every 7 days. An inoculum was prepared in a 100-ml Erlenmeyer flask containing 20 ml of Porphyridium medium, and grown at 25°C for 48 h (log-phase growth) with orbital shaking at 150 rpm in darkness. Erlenmeyer flasks (250-ml), each containing 50 ml of the medium, were

inoculated with 5% (v/v) of an exponentially growing inoculum and incubated at 25°C in an orbital shaker at 200 rpm in the dark.

Cell concentration

Cell concentration (optical density) in the culture fluids was determined at 520 nm. Cell dry weight was determined by drying the cells at 80°C in a vacuum oven to constant weight.

Specific growth rate

Specific growth rate was determined by plotting the natural logarithm of culture optical density or dry weight concentration against time. Readings within the exponential phase were then used to obtain correct values of the specific growth rate by linear regression [5].

Determination of glucose concentration

Glucose concentration in the culture fluids was determined by a DX 500 HPLC system (Dionex Co, Sunnyvale, CA, USA) according to the method of Shi *et al* [16]. The system comprised an ED 40 electrochemical detector, a GP 40 gradient pump and an LC 20 chromatography enclosure, equipped with a 4 × 250-mm CarboPac™ PA1 column (Dionex) and an LC30 stainless steel automated Rheodyne injection valve with a 25-μl fixed loop. PeakNet software and a DX LAN™ computer interface card (Dionex) were used. The mobile phase consisted of 200 mM NaOH-distilled water (8:92, v/v). The flow rate was set at 2.0 ml min^{-1}. The column was kept at room temperature (22–25°C). The samples were filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA) before injection. Standard glucose (Sigma Chemical Co, St Louis, MO, USA) was used for identification and quantification.

Analysis of fatty acids

Fatty acid methyl esters were prepared from lyophilized cells by trans-methylation with methanol-acetyl chloride [7]. The esters were extracted with hexane and analyzed by HP 6890 capillary gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a flame-ionization detector (FID) and a Supelco Omegawas™ 250 capillary column (30 m × 0.25 mm). Nitrogen was the carrier gas. The initial column temperature was 170°C and was subsequently raised to 225°C at 1°C min^{-1}. The injector was kept at 250°C with an injection volume of 3 μl under splitless mode. The FID detector temperature was set at 270°C. Fatty acid methyl esters were identified by chromatographic comparison with authentic standards (Sigma). The quantities of fatty acids were estimated from the peak areas on the chromatogram using C17:0 fatty acid as the internal standard.

Results and discussion

Cell growth

For the majority of microalgae, sodium is required to facilitate photosynthesis, silicate uptake, intracellular pH regulation as well as alkalotolerance [15]. It is also reported that both ammonium and amino acid uptake depend on external Na^+ concentrations [22]. In marine microalgae, certain concentrations of NaCl are required for maintaining the osmotic balance of the cells. Marine unicellular algae are generally considered to be tolerant and adaptable to a wide range of salinity [9], but little information is available about the effect of salinity on cell growth and fatty acid content of the marine dinoflagellate *C. cohnii*. In this study, NaCl concentrations in the range of 0–35 g L^{-1} were investigated. *C. cohnii* ATCC 30556 had its highest specific growth rate of 0.090 h^{-1} at an NaCl concentration of 9.0 g L^{-1}, whereas *C. cohnii* ATCC 50051 and *C. cohnii* RJH had their highest specific growth rates of 0.049 and 0.067 h^{-1}, respectively, at an NaCl concentration of 5 g L^{-1} (Table 1). The specific growth rate decreased with increasing salinity when the salinity was higher than the optimum value (Table 1). Reduced growth at high salinities might be attributed to excessive ions in the cells [13]. There was an increase in lag phase with increasing NaCl concentration (data not shown). This may be due to salinity adaptation, and these adaptation phenomena have been reported to be accompanied by phase shifts of the rhythm of cell development which reach a new constant state after one cell cycle [11,19]. The longer lag-phase, the lower growth rate and the longer generation time at higher or lower salinities might be caused by the delay in cell division as a result of inhibition in DNA synthesis [8].

The highest biomass concentration of 2.51 g L^{-1} was achieved at 9 g L^{-1} NaCl for *C. cohnii* ATCC 30556; at this NaCl concentration, *C. cohnii* ATCC 50051 also achieved its highest biomass concentration (1.56 g L^{-1}) although it is much lower (Table 1). For *C. cohnii* RJH, the highest biomass concentration of 2.49 g L^{-1} was obtained at 5 g L^{-1} NaCl. As far as cell growth yield is concerned, a growth yield coefficient of approximately 0.5 g g^{-1} (g dry cells g^{-1} glucose) was obtained for *C. cohnii* ATCC 30556 over the entire range of NaCl concentrations investigated. For *C. cohnii* RJH, the growth yield coefficient was also stable, although its average value was slightly lower than that for *C. cohnii* ATCC 30556. In contrast, *C. cohnii* ATCC 50051 had relatively lower values of growth yield coefficient, and the highest value was 0.45 g g^{-1} corresponding to the highest biomass concentration (Table 1). Almost no growth was observed for all the strains when the medium did not contain NaCl, and at extremely high NaCl concentrations, growth was inhibited, and the cells were elongated. This observation was in agreement with published data on other strains of *C. cohnii* [1]. Algae tend to maintain an internal osmotic pressure above that of the medium. The degree of osmotic stress that can be tolerated depends ultimately on the ability of the cell protoplasm and membrane to function when its salt concentration is altered. The enlargement of the cell might be an indication of high external ionic concentrations that resulted in inhibition of cell growth.

Fatty acid composition

The major fatty acids found in *C. cohnii* were C14:0; C16:0; C18:0 and C22:6. In this study, the DHA percentage of all three *C. cohnii* strains ranged from 26.6 to 56.9% of the total fatty acids (Table 2). At 9 g L^{-1} NaCl, *C. cohnii* ATCC 30556 had the highest DHA proportion (56.9% of total fatty acids), and the highest degree of unsaturation

Table 1 Effect of salinity on cellular growth of three strains of *C. cohnii*[a,b]

NaCl concentration (g L^{-1})	Specific growth rate (h^{-1}) of strain:			Cell dry weight concentration (g L^{-1}) of strain:			Cell growth yield (g L^{-1}) of strain:		
	ATCC 30556	ATCC 50051	RJH	ATCC 30556	ATCC 50051	RJH	ATCC 30556	ATCC 50051	RJH
0	nd	nd	nd	0.13 ± 0.01	0.21 ± 0.01	0.22 ± 0.02	nd	nd	nd
2	0.057 ± 0.004	0.011 ± 0.002	0.052 ± 0.004	2.10 ± 0.14	1.17 ± 0.10	2.41 ± 0.15	0.50 ± 0.04	0.31 ± 0.02	0.49 ± 0.02
5	0.087 ± 0.005	0.049 ± 0.003	0.067 ± 0.005	2.45 ± 0.13	1.43 ± 0.10	2.49 ± 0.16	0.50 ± 0.03	0.40 ± 0.02	0.50 ± 0.04
9	0.090 ± 0.006	0.045 ± 0.002	0.061 ± 0.005	2.51 ± 0.16	1.56 ± 0.07	2.40 ± 0.18	0.50 ± 0.03	0.45 ± 0.02	0.49 ± 0.03
15	0.086 ± 0.005	0.037 ± 0.002	0.051 ± 0.004	2.47 ± 0.14	1.38 ± 0.08	2.30 ± 0.12	0.49 ± 0.04	0.38 ± 0.02	0.46 ± 0.03
23	0.085 ± 0.006	0.028 ± 0.002	0.059 ± 0.004	2.46 ± 0.15	1.18 ± 0.08	2.41 ± 0.15	0.49 ± 0.03	0.31 ± 0.02	0.48 ± 0.02
30	0.085 ± 0.005	nd	nd	2.35 ± 0.12	nd	nd	0.50 ± 0.02	nd	nd
35	0.081 ± 0.041	nd	nd	2.31 ± 0.12	nd	nd	0.50 ± 0.02	nd	nd

[a]Data are expressed as mean ± standard deviation of two to three replicates.
[b]nd, Not determined; cell growth yield, g cell dry weight produced per g glucose used.

Table 2 Effect of salinity on the fatty acid composition (% of total fatty acids) of three strains of *C. cohnii*[a,b]

Fatty acids	NaCl 0 g L^{-1}			NaCl 2 g L^{-1}			NaCl 5 g L^{-1}			NaCl 9 g L^{-1}			NaCl 15 g L^{-1}			NaCl 23 g L^{-1}		
	30556	50051	RJH	30556	50051	RJH	30556	50051	RJH	30556	50051	RJH	30556	50051	RJH	30556	50051	RJH
12:0	3.6 ±0.2	14.6 ±0.6	2.8 ±0.2	1.8 ±0.1	5.1 ±0.3	1.2 ±0.1	1.6 ±0.1	0.9 ±0.1	1.7 ±0.1	1.5 ±0.1	1.3 ±0.1	2.1 ±0.2	2.1 ±0.1	0.5 ±0.0	2.5 ±0.2	2.6 ±0.2	0.6 ±0.0	2.2 ±0.2
14:0	15.5 ±0.7	11.8 ±0.5	21.8 ±0.7	12.8 ±0.5	15.4 ±0.4	12.5 ±0.6	13.0 ±0.5	13.5 ±0.5	12.4 ±0.5	12.3 ±0.5	16.7 ±0.6	14.0 ±0.5	15.4 ±0.5	13.7 ±0.5	13.5 ±0.4	14.4 ±0.4	17.3 ±0.6	13.7 ±0.5
16:0	30.6 ±1.4	27.1 ±1.1	25.2 ±0.9	20.8 ±0.7	31.5 ±0.8	20.1 ±0.8	22.1 ±0.8	20.3 ±0.9	20.7 ±0.7	20.3 ±0.6	19.1 ±0.7	21.5 ±0.6	19.4 ±0.6	22.4 ±0.9	20.7 ±0.7	19.8 ±0.8	19.5 ±0.7	21.1 ±0.8
18:0	17.1 ±0.5	15.1 ±0.6	11.7 ±0.4	9.7 ±0.4	16.2 ±0.6	15.4 ±0.6	7.1 ±0.3	10.4 ±0.4	9.1 ±0.3	7.8 ±0.3	9.6 ±0.4	8.7 ±0.4	8.1 ±0.4	10.1 ±0.4	9.2 ±0.3	9.9 ±0.3	9.9 ±0.4	10.6 ±0.3
18:1	2.4 ±0.2	1.3 ±0.1	1.6 ±0.1	1.2 ±0.1	0.8 ±0.1	0.2 ±0.0	0.7 ±0.1	0.1 ±0.0	0.1 ±0.0	0.9 ±0.0	0.1 ±0.0	0.1 ±0.0	1.7 ±0.2	0.2 ±0.0	0.1 ±0.0	0.4 ±0.1	0.1 ±0.0	0.0 ±0.0
22:5	1.3 ±0.1	3.4 ±0.2	1.6 ±0.1	0.2 ±0.0	1.9 ±0.2	0.3 ±0.0	tr	0.2 ±0.0	0.3 ±0.1	0.3 ±0.1	0.3 ±0.1	0.4 ±0.1	0.3 ±0.1	0.3 ±0.0	0.3 ±0.1	0.1 ±0.0	0.3 ±0.0	0.2 ±0.1
22:6	29.4 ±1.2	26.6 ±1.0	35.4 ±1.1	53.5 ±1.3	29.1 ±1.0	50.5 ±1.2	55.4 ±1.3	54.6 ±2.5	55.7 ±1.4	56.9 ±1.5	52.8 ±1.2	53.8 ±1.3	52.9 ±1.3	52.8 ±1.4	53.7 ±1.4	52.9 ±1.4	52.3 ±1.4	52.1 ±1.4
TSA	66.9 ±3.9	68.7 ±3.7	61.5 ±3.2	45.1 ±1.9	68.2 ±3.0	49.2 ±2.9	43.9 ±2.3	45.1 ±2.4	43.9 ±2.3	41.9 ±2.1	±46.8 ±2.4	46.2 ±2.2	45.1 ±2.2	46.7 ±2.5	46.0 ±2.2	46.8 ±2.1	47.3 ±2.2	47.6 ±2.0
TFA	9.2 ±0.4	4.5 ±0.2	3.6 ±0.2	6.3 ±0.3	10.3 ±0.5	9.2 ±0.5	7.4 ±0.4	6.8 ±0.4	8.9 ±0.4	10.1 ±0.5	8.3 ±0.4	9.5 ±0.5	8.6 ±0.4	6.0 ±0.3	9.0 ±0.4	8.6 ±0.4	6.7 ±0.3	7.4 ±0.4
▽ mol^{-1}	1.8 ±0.1	1.8 ±0.1	2.0 ±0.3	3.2 ±0.2	1.8 ±0.1	3.0 ±0.1	3.3 ±0.1	3.3 ±0.2	3.4 ±0.1	3.4 ±0.1	3.2 ±0.1	3.2 ±0.1	3.2 ±0.1	3.2 ±0.1	3.2 ±0.1	3.2 ±0.1	3.1 ±0.1	3.1 ±0.1

[a]Data ae expressed as mean ± standard deviation of two to three replicates. [b] tr, Trace; –, not determined; TSA, percentage of total saturated fatty acids of total fatty acids; TFA, percentage of total fatty acids of cell dry weight; ▽ mol^{-1}, the degree of unsaturation. This value was calculated according to Chen and Johns [4]. ▽ mol^{-1} = [1.0(% monoene) + 2.0(% diene) + 3.0(% triene)]/100.

Table 3 Effects of salinity on DHA content and yield of three strains of *C. cohnii*[a,b]

NaCl concentration (g L^{-1})	DHA content (% of dry cells) in strain:			DHA yield (mg L^{-1}) in strain:		
	ATCC 30556	ATCC 50051	RJH	ATCC 30556	ATCC 50051	RJH
0	2.6 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	3.8 ± 0.2	2.5 ± 0.2	2.8 ± 0.2
2	3.4 ± 0.2	3.0 ± 0.2	4.6 ± 0.3	71.5 ± 3.2	35.0 ± 2.1	113.9 ± 6.3
5	4.1 ± 0.2	3.7 ± 0.2	4.9 ± 0.2	104.3 ± 4.6	52.6 ± 2.6	122.8 ± 5.9
9	5.3 ± 0.3	4.4 ± 0.2	4.9 ± 0.2	131.5 ± 6.4	68.2 ± 3.2	120.6 ± 6.2
15	4.5 ± 0.2	3.2 ± 0.1	4.8 ± 0.2	108.6 ± 5.1	43.6 ± 2.0	111.0 ± 5.2
23	4.5 ± 0.3	3.5 ± 0.2	3.9 ± 0.2	111.4 ± 5.2	41.2 ± 2.2	93.0 ± 4.8
30	2.9 ± 0.1	nd	nd	72.7 ± 3.0	nd	nd
35	2.6 ± 0.1	nd	nd	63.1 ± 2.7	nd	nd

[1]Data are expressed as mean ± standard deviation of two to three replicates.
[b]nd, Not determined.

(∇ mol^{-1} = 3.4). There was an increase in DHA proportion to increasing NaCl concentration for *C. cohnii* ATCC 30556 when the salinity was below 9 g L^{-1}. At salinities of 9 g L^{-1} and above, no changes were observed in DHA percentage for *C. cohnii* ATCC 30556. As for the other two strains, *C. cohnii* ATCC 50051 and *C. cohnii* RJH, although their DHA contents reached the highest (54.6% and 55.7% of total fatty acids, respectively) at a salinity of 5 g L^{-1}, the proportion of DHA did not increase as the salinity was increased above 5 g L^{-1}. However the DHA proportion decreased when the salinity was below 5 g L^{-1}. At 5 g L^{-1} NaCl, the degree of unsaturation (∇ mol^{-1}) was the highest at 3.3 and 3.4 for *C. cohnii* ATCC 50051 and *C. cohnii* RJH, respectively. This difference may be strain-specific. Beach [1] reported that the proportion of DHA for *C. cohnii* decreased when the salinity was above 30 g L^{-1}. The main roles of fatty acids in algae are generally related to cell membrane functions and to metabolic processes. The degree of fatty acid unsaturation is important in maintaining the fluidity of the membrane and in providing the appropriate environment for membrane functions [3]. Reducing polyunsaturated fatty acids (ie the degree of unsaturation) with increasing salinity suggests a reduction in membrane fluidity and permeability. Such changes would improve the performance of the alga at high salinity by preventing leakage of a compatible solute, such as glycerol, out of the cell and diffusion of potential growth inhibitory ions into the cell. *C. cohnii* ATCC 30556 and ATCC 50051 had the highest DHA contents of 5.3% and 4.4% of dry cells at a salinity of 9 g L^{-1}, while *C. cohnii* RJH had the highest DHA content of 4.9% of dry cells at a salinity of 5 g L^{-1} (Table 3).

In *C. cohnii* ATCC 30556, the percentages of C12:0, C14:0, C16:0 and C18:0 were within the ranges of 1.5–3.6%, 12.3–15.5%, 19.4–30.6% and 7.1–17.1%, respectively. In the culture containing no added NaCl, the C16:0 and C18:0 fatty acids were the highest being 30.6% and 17.1% of the total fatty acids, respectively. In contrast, the percentages of C14:0 and C12:0 did not show apparent changes over the entire range of salinities investigated. For all three strains, when the salinity was over 9 g L^{-1}, the degree of unsaturation decreased with increasing salinity. The content of total fatty acids did not show an obvious relation to the salinity, but it decreased when the culture was at a higher salinity.

Without NaCl in the medium, the cells grew very slowly and accumulated more storage lipid such as triglycerol, which contains abundant saturated fatty acids [1]. With increasing salinity, the cell internal osmotic pressure would increase with the external salinity and would involve the accumulation of high concentrations of glycerol [2]. Fontana and Haug [10] observed a decrease in fluidity of the plasma membrane of *Dunaliella primolecta* cells adapted to a high NaCl concentration, whereas Xu and Beardall [23] reported a similar decrease in *Dunaliella* species. Cohen *et al* [6] found that *Porphyridium cruentum* produced less polyunsaturated fatty acids when growth was retarded by means of decreased light, increased salinity, increased cell concentration, sub-optimal pH or sub-optimal temperature. These suggested that the polyunsaturated fatty acid content was related to cell growth rate. Our results of variations in total saturated fatty acid and polyunsaturated fatty acid contents support the above findings. However, Molitor *et al* [14] demonstrated that the ratio of unsaturated to saturated fatty acids was higher in plasma membranes of salt-adapted *Anacystis nidulans*, a freshwater alga. Freshwater and marine algae might respond differently to the change in salinity. This might be dependent on the species and probably the habitat of origin.

The results indicate that salinity is an important factor influencing the growth and chemical composition of the heterotrophic marine dinoflagellate, *C. cohnii*. The highest DHA content (56.9% of total fatty acids and 5.3% of dry cells) and yield (131.6 mg L^{-1}) were obtained from *C. cohnii* ATCC 30556 at 9 g L^{-1} NaCl. Research is continuing in this laboratory to further enhance DHA production by optimising the medium salinity together with other nutrient and environmental factors.

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