

# Long-chain $n - 3$ polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of isolates and optimisation of docosahexaenoic acid production

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## Abstract

Following an isolation programme for thraustochytrids (marine fungoid protists) from three different locations, 57 isolates were screened for biomass, oil and docosahexaenoic acid production (DHA). Although a common fatty acid profile for the thraustochytrid isolates emerged, there was considerable variation in the DHA content of the oil. In some isolates from a cold temperate environment, DHA represented almost 50% of the total fatty acids present. Although isolates from a sub-tropical environment produced higher levels of biomass, with up to 37% (w/w) oil, the DHA fraction of the fatty acids was low. Cool temperate isolates gave intermediate values. Studies to optimise biomass and DHA production by manipulation of growth medium composition were carried out on a tropical strain. Results indicated that medium with a high C:N ratio stimulated DHA production. The use of such media in bioreactor cultivations gave maximum biomass, lipid and DHA content of 14 g l<sup>-1</sup>, 78 and 25% (w/w), respectively. Optimum DHA production was 2.17 g l<sup>-1</sup> after 107 h cultivation. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Docosahexaenoic acid; Eicosapentaenoic acid; Marine;  $N - 3$  polyunsaturates; Protist; Thraustochytrid

**Abbreviations:** ASW, artificial seawater; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; PUFA, polyunsaturated fatty acids; SCO, single cell oil.

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## 1. Introduction

Increasing awareness and understanding of the importance of the  $n - 3$  polyunsaturates, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), in human development, health and disease (Dratz and Deese, 1986; Simopoulos, 1991) emphasises the need for sources of these molecules, other than from fish oils (Yongmanitchai and Ward, 1989). A range of au-

totrophic and heterotrophic microbes has been assessed for potential commercial sources of EPA and DHA by various workers including Barclay et al. (1994), Lewis et al. (1998) and Vazhappilly and Chen (1998), and the results of many studies in this area have been reviewed by Singh and Ward (1997) and Ratledge (1998). Particular attention has been given to the thraustochytrids, a group of fungoid protists generally considered obligately marine with a specific requirement for  $\text{Na}^+$  ions (Siegenthaler et al., 1967). Recent work by Nakahara et al. (1996), Yaguchi et al. (1997), Yokochi et al. (1998) examined the production of DHA by a species of *Schizochytrium* and the ultrastructure and lipid production of a species of *Thraustochytrium* have been investigated by Weete et al. (1997).

Thraustochytrids have attracted attention since their lipid contains proportionately large quantities of DHA; in some strains over 50% of the fatty acid content can consist of DHA (Yokochi et al., 1998). Assessment of EPA and DHA synthesis by thraustochytrids (Kendrick and Ratledge, 1992; Bajpai et al., 1991a,b; Li and Ward, 1994) indicated some potential, but both biomass and lipid yields were low. Two patents (Barclay, 1992, 1994) have however been filed, detailing the cultivation of thraustochytrid strains to produce lipids containing EPA and DHA, and indeed PUFA derived from these organisms are now available commercially in aquaculture products and chicken feed.

The largest potential market for microbial oils containing DHA is perceived to be as an additive to infant formulae as an essential fatty acid for brain and retinal development (Horrobin, 1995). Ratledge (1998) considered that the presence of significant quantities of EPA in the thraustochytrid oils so far assessed, precluded its use for this purpose. Eicosapentaenoic acid is considered contra-indicatory in breast milk substitutes, but Ratledge suggested strain selection as a means of overcoming this difficulty.

Previous studies on polyunsaturated fatty acid (PUFA) production have involved experimentation with only a fraction of the potential diversity of thraustochytrids which are ubiquitous microorganisms and common members of marine pi-

coplankton communities (Naganuma et al., 1998). This study has therefore focused on two aspects. A sampling programme to obtain thraustochytrid cultures from a range of habitats and ecological niches was carried out; the biomass production and PUFA profiles of these strains were assessed. Secondly, experimentation, including growth in bioreactors was undertaken to further optimise the production of both biomass and DHA.

## 2. Materials and methods

### 2.1. Isolation and screening programme

#### 2.1.1. Isolation and maintenance of cultures

Three distinct ecological and climatic environments were selected for the isolation programme: (1) cold temperate littoral (59–61°N); (2) cool temperate littoral (50–51°N); and (3) sub-tropical mangroves (25–27°N). Thraustochytrids were isolated from water samples, sediment samples, mangrove leaf tissue, algae and marine angiosperms onto a medium comprising: yeast extract (1 g l<sup>-1</sup>), peptone (1 g l<sup>-1</sup>) and agar (10 g l<sup>-1</sup>) prepared in 70% seawater. A 2-ml solution of streptomycin and penicillin G (300 mg of each antibiotic per litre) was added to the isolation medium to prevent bacterial contamination during isolation. Methods for isolation were direct plating and baiting with  $\gamma$ -irradiated (246 kGy) pine pollen as previously described by Gaertner (1968), Fell and Master (1973), Raghukumar et al. (1992) and Bremer (1995). The axenic cultures derived from the isolation programme were maintained on nutrient agar slopes overlaid with sterile autoclaved seawater.

#### 2.1.2. Biomass production

Isolates were grown in 250-ml Erlenmeyer flasks with 50 ml of cultivation medium comprising: yeast extract (2 g l<sup>-1</sup>), peptone (2 g l<sup>-1</sup>) and glucose (5 g l<sup>-1</sup>) in 70% natural seawater. The flasks were inoculated with 1 ml of a zoospore suspension harvested from the seawater overlaying cultures on agar plates. Three replicate flasks of each isolate were incubated at 25°C on an orbital shaker at 150 rpm for 72 h. The biomass

from each flask was harvested separately by centrifugation, washed with phosphate-buffered saline and re-centrifuged. Each biomass pellet was lyophilised overnight and the dry weight determined.

## 2.2. Lipid extraction and fatty acid analysis

### 2.2.1. From biomass

For the screening programme, biomass from the thraustochytrid isolates was analysed by taking 15–25 mg of dried cells, weighed in 5 ml Reacti-vials. Lipids were extracted with 1 ml chloroform and esterified overnight in 2 ml  $\text{BCl}_3$ /methanol (12%, w/w) reagent at 65°C. Deionised water (1 ml) was added and the chloroform layer removed and dried under nitrogen. The residue was re-extracted in 1.5 ml hexane and 1 ml of deionised water was added. The solvent layer was removed and filtered through a 0.45- $\mu\text{m}$  filter with the syringe containing  $\text{Na}_2\text{SO}_4$ . Fatty acid methyl esters (FAME) were analysed using gas chromatography (HP5890, series II GC with HP7673 injector and HP3365 Chemstation) on polar column (DB23 30 m length, 0.25 mm diameter) using flame ionisation detection with helium as the carrier gas (14.5 psi). FAMEs were identified and quantified using standard fatty acids. Nonadecanoic acid was used as an internal standard. Chloroform and hexane used for extraction contained 10 ppm butylated hydroxy-toluene. Extraction and determination of lipids from the optimisation studies was carried out using cells harvested from flask cultures. Cells were weighed (usually 2–3 g wet weight or 1 g dry weight) and lipids were extracted using chloroform:methanol (2:1) following the method of Bligh and Dyer (1959).

### 2.2.2. From culture liquids and bioreactor broths

Total lipid extraction of the biomass from the bioreactor cultivation broths was based on the method of Christie (1989), which is in current commercial use. A known volume (minimum 100 ml) of medium was passed at a pressure of 800 bar, through a Soavi pressure homogeniser kept chilled on ice. The broth was then homogenised using an Ultra-turrax homogeniser together with

three times the volume of hexane. The resulting emulsion was centrifuged at 600 rpm at 4°C for 5 min. The upper layer of hexane was removed, filtered through Whatman IPS paper into a pre-weighed flask the method then followed that of Bligh and Dyer (1959). The fatty acids were esterified to their corresponding methyl esters and analysed using a modification of the method described above. Petroleum ether was used as the suspending solvent and heptadenoic acid was used as an internal standard. Peroxide values of the lipids extracted from cells of strain G13 were assessed using a method based on British Standard BS684, section 2.14. Of the three extraction methods used in this study, chloroform followed by re-extraction with hexane, chloroform:methanol and hexane, the first of these methods is being followed in current studies.

## 2.3. Optimisation and cultivation studies

### 2.3.1. Optimisation of biomass and DHA yield

The organism selected for these studies was strain G13 from the University of Portsmouth culture collection, a thraustochytrid strain resembling *Schizochytrium mangrovei* Raghu Kumar isolated in the tropics (2°N) from recently fallen leaves of the mangrove tree *Sonneratia alba* L.

The effects of carbon (as glucose) and nitrogen (as yeast extract and peptone) on lipid and DHA synthesis by G13, were examined using batch cultures in 250-ml Erlenmeyer flasks. Inocula of the test strain were prepared from a dense suspension of zoospores derived from a culture grown in liquid medium in shaken flasks at 25°C for 12 h. Experimental media and conditions are detailed with the relevant results.

### 2.3.2. Cultivation

Cultivation studies were carried out using B. Braun (Germany) bench-top bioreactors (2 l capacity). Inocula were prepared as described for flask cultures and a total of 300 ml of inoculum was used to inoculate 1500 ml of the experimental medium in the bioreactor. Artificial seawater (Tropic Marin, Aquarientechnik, Wartenburg, Germany) was used throughout, except for the series of cultivations where 20 g l<sup>-1</sup> sodium sul-

phate was used as a substitute source of sodium. The media constituents and the conditions employed in the bioreactor for each separate investigation are detailed with the relevant results.

### 3. Results and discussion

#### 3.1. Screening programme

Biomass produced under standardised culture conditions by 57 *thraustochytrid* isolates obtained

from the three distinct climatic environments, is presented in Fig. 1a. The 20 isolates from a sub-tropical location produced biomass values ranging between 710 and 3140 mg l<sup>-1</sup> dry weight after 72 h cultivation. These values were considerably greater than the biomass produced by *thraustochytrid* isolates from both the cool temperate and cold temperate locations. Variability in biomass production between individual isolates from each climatic environment was a consistent feature.

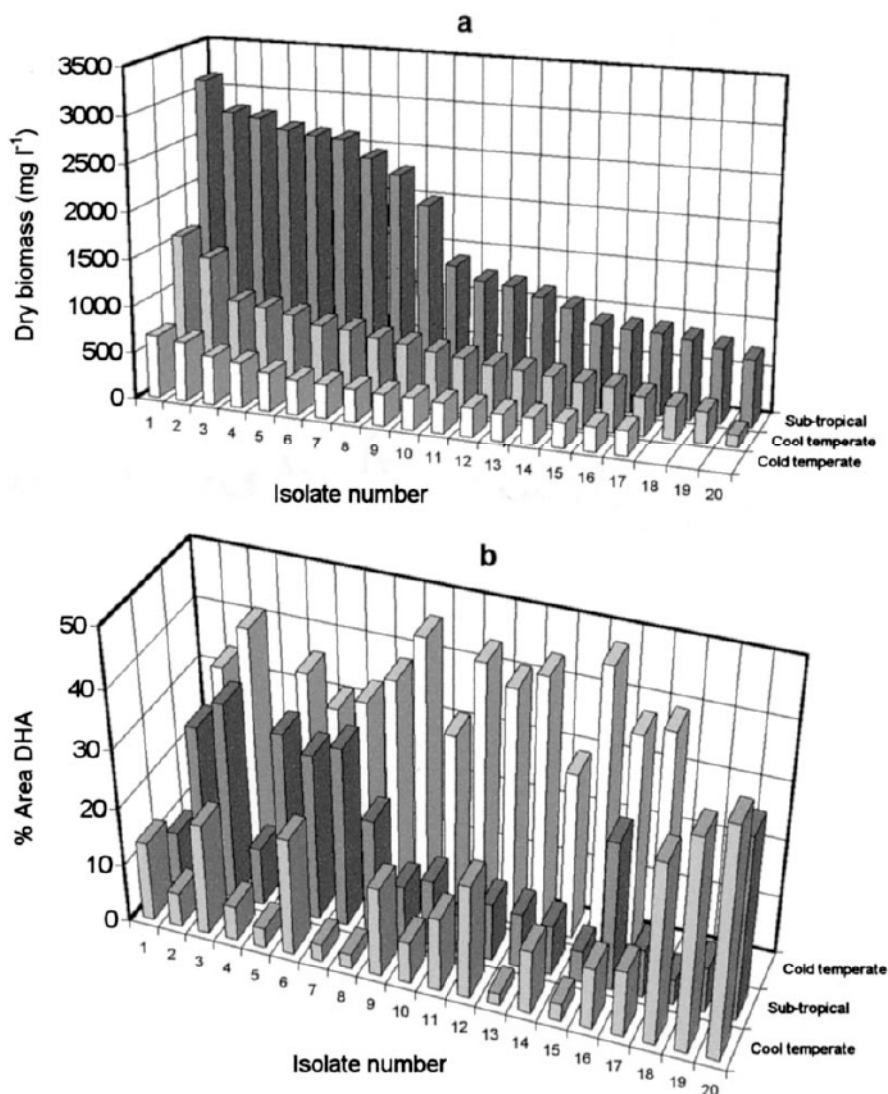


Fig. 1. Biomass and DHA produced by 57 selected *thraustochytrid* isolates collected from cold temperate, cool temperate and sub-tropical sites. Isolates were grown at 25°C for 3 days at 150 rpm in a medium containing 5 g l<sup>-1</sup> glucose, 2 g l<sup>-1</sup> yeast extract and 2 g l<sup>-1</sup> peptone prepared in 70% seawater. Mean biomass values (a) were measured as mg l<sup>-1</sup>. Mean DHA values (b) were measured as percent area of DHA in the fatty acid profile. The position of columns in (b) is identical to the biomass values for isolates in (a).

The percentage of DHA in the total fatty acids present in each of the isolates is shown in Fig. 1b. The sub-tropical isolates gave a range of 4–34% (mean 16.6%) of total fatty acids, the cool temperate isolates 2–39.6% (mean 13.7%) and the cold temperate isolates 17–47% (mean 35.9%). Wide differences in values were recorded, but overall the mean percentage of DHA in the oil was higher in thraustochytrid isolates from cold temperate locations. In general it would appear that the cold water isolates contained the largest amounts of PUFA, a feature that has been noted for other organisms from low-temperature environments (Erwin, 1973). Although baseline biomass values for these isolates were low (Fig. 1a), they were grown at 25°C, which is a temperature far higher than would be encountered in their natural habitat. Further exploratory cultivations will be carried out at temperatures below 25°C, and these will include studies to compare the DHA content in the cells with different phases of growth.

The oil and DHA content of the biomass from the 57 isolates was calculated and these results are presented in Fig. 2a–c. Although the range of values for total lipid in dried cells from the sub-tropical isolates was 1.8–37.3% (w/w), the maximum value (37.3%, w/w) was well in excess of that of the other sub-tropical isolates, where the range was 1.8–18.0% (w/w) (Fig. 2c). In contrast there was less total lipid extracted from the cold temperate isolates (2.0–7.1%, w/w) (Fig. 2a) and the cool temperate isolates (1.6–13.6%, w/w) (Fig. 2b).

Fig. 2a–c also depicts the DHA content of the dried thraustochytrid biomass (solid bar). The maximal values for DHA in dried cell biomass of isolates from the three environments ranged between 2.5% (w/w) for cool temperate isolates and 7.8% (w/w) for sub-tropical isolates. The mean values for DHA and EPA content of the dried cells of isolates from the three environments are summarised in Table 1.

From these data, the isolate which had the highest oil content was identified from each geographical area. These isolates and their fatty acid profiles are summarised in Table 2. Docosahexaenoic acid constituted 45.7% of the fatty acid in the cold temperate isolate number 9 (SW4/4)

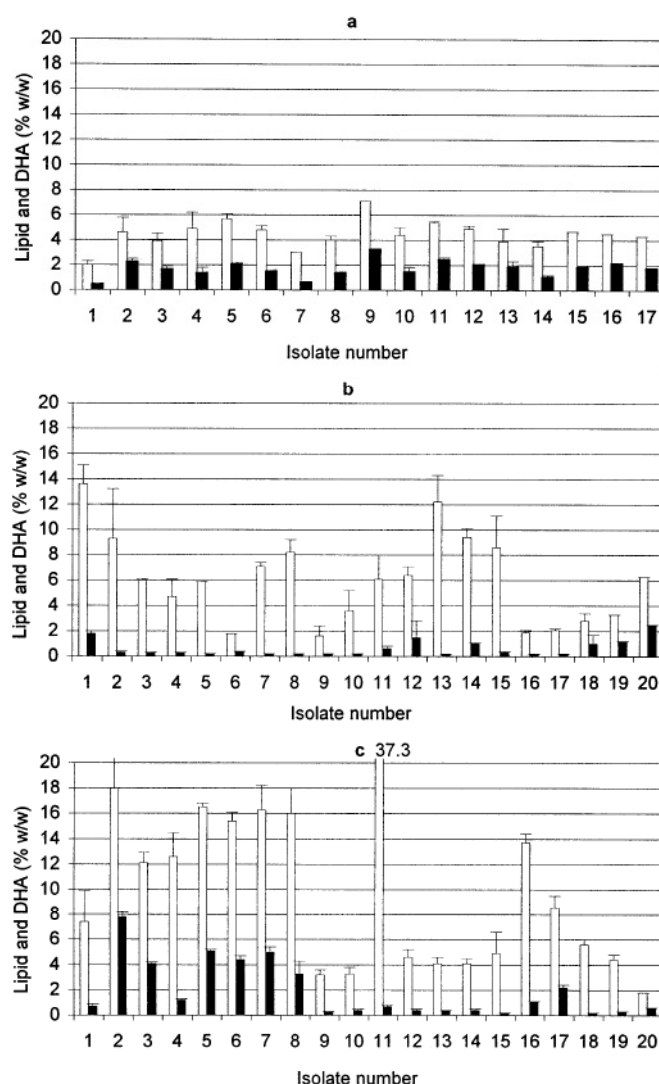


Fig. 2. Mean percentage total oil and DHA in dried cells of thraustochytrid isolates collected from (a) cold temperate, (b) cool temperate and (c) sub-tropical sites, grown at 25°C for 3 days at 150 rpm in a medium containing 5 g l<sup>-1</sup> glucose, 2 g l<sup>-1</sup> yeast extract and 2 g l<sup>-1</sup> peptone prepared in 70% seawater. (□) % w/w total oil; (■) % w/w DHA. Isolate numbers in (a), (b) and (c) correspond to isolate numbers in Fig. 1.

which was close to the maximum (47%) recorded for any isolate from this environment. The DHA from the other two isolates constituted significantly lower fractions of the fatty acid, 13.6% in the cool temperate isolate number 1 (HSED/14) and 23.8% in sub-tropical isolate number 2 (M6W/2), respectively. In addition, isolate HSED/14 contained large amounts of palmitic acid (38.8% of the total fatty acids present).

The results of the isolation and screening programme confirmed earlier findings (Ellenbogen et al., 1969; Findlay et al., 1986; Barclay, 1992; Weete et al., 1997) that thraustochytrids have characteristic fatty acid profiles with  $n-3$  PUFA as a significant component. Docosahexaenoic acid represented the major component of the PUFA content in most isolates, particularly those from the cold temperate environment. All of the thraustochytrids synthesised the  $n-6$  PUFA arachidonic acid in varying amounts, mainly as a minor component of the PUFA but in other organisms levels may be higher (Singh and Ward, 1997). Although the  $n-3$  PUFA EPA was present in the oil produced by all the isolates except two, the EPA content was generally low, varying from 0.2 to 0.6% (w/w) of the dried thraustochytrid cells.

### 3.2. Optimisation and cultivation studies

#### 3.2.1. Optimisation studies

When grown in liquid basal medium at 24°C in shaken flask cultures, strain G13 exhibited a

growth rate (max) of  $0.2 \text{ h}^{-1}$  and a doubling time ( $t_d$ ) of 3.4 h. The yields of biomass, the DHA content of that biomass and the yield of DHA achieved over a 4-day growth cycle are shown in Table 3. These data represent the baseline for subsequent experimentation to improve biomass and DHA yields.

Raising biomass levels by medium manipulation is an obvious first step in optimising DHA yields. Previous studies reviewed by Singh and Ward (1997) and Ratledge (1998), using batch cultures in shake flasks and bioreactors, indicated biomass production was stimulated by increased glucose levels in the medium. Table 4 provides data on the effect of increasing glucose concentration while keeping organic nitrogen sources low. The results indicate that an increase in glucose concentration up to  $10 \text{ g l}^{-1}$  leads to a linear increase in biomass production, suggesting a limiting factor other than glucose concentration beyond that point. The highest production of lipid (65–66%, w/w) was observed in concentrations of 20 and  $30 \text{ g l}^{-1}$  of glucose. Although the DHA

Table 1

Mean biomass production, lipid, DHA and EPA content and percent area DHA in fatty acid profiles of selected isolates of thraustochytrids

| Isolate group  | Biomass production ( $\text{mg l}^{-1}$ ) | Lipid in biomass (% w/w) | DHA in biomass (% w/w) | EPA in biomass (% w/w) | % Area DHA in fatty acid profile |
|----------------|-------------------------------------------|--------------------------|------------------------|------------------------|----------------------------------|
| Cold temperate | 113–673                                   | 2.0–7.1                  | 0.4–3.2                | 0.2–0.5                | 17–47                            |
| Cool temperate | 30–1587                                   | 1.6–13.6                 | 0.2–2.5                | 0.2–0.6                | 2–40                             |
| Sub-tropical   | 710–3140                                  | 1.8–37.3                 | 0.2–7.8                | 0.2–0.6                | 4–34                             |

Table 2

Mean values for fatty acids in three thraustochytrid isolates measured as a percent of fatty acid profile<sup>a</sup>

| Fatty acid          | Cold temperate isolate (SW4/4) | Cool temperate isolate (HSED/14) | Sub-tropical isolate (M6W/2) |
|---------------------|--------------------------------|----------------------------------|------------------------------|
| 13:0                | 3.05                           | 1.51                             | 1.28                         |
| 14:0                | 1.60                           | 7.59                             | 8.43                         |
| 16:0                | 19.28                          | 38.88                            | 17.71                        |
| 20:4                | 3.30                           | 4.67                             | 16.04                        |
| 20:5                | 8.19                           | 4.36                             | 1.48                         |
| 22:6                | 45.73                          | 13.6                             | 23.79                        |
| Others <sup>b</sup> | 18.85                          | 29.39                            | 31.27                        |

<sup>a</sup> 13:0, tridecanoic acid; 14:0, myristic acid; 16:0, palmitic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid.

<sup>b</sup> Other fatty acids included: C15:0, C16:1, C18:0, C18:1, C18:2, C20:2 and C22:4.

Table 3

Mean biomass and DHA production of thraustochytrid strain G13 grown at 24°C for 4 days at 120 rpm in a medium containing 1 g l<sup>-1</sup> yeast extract, 1 g l<sup>-1</sup> peptone, 1 g l<sup>-1</sup> glucose and 27 g l<sup>-1</sup> ASW

| Time (h) | Biomass (mg l <sup>-1</sup> ) | DHA in biomass (% w/w) | DHA production (mg l <sup>-1</sup> ) |
|----------|-------------------------------|------------------------|--------------------------------------|
| 24       | 35 ± 9128                     | 0.51 ± 0.06            | 1.83                                 |
| 31       | 870 ± 29                      | 1.68 ± 0.08            | 14.58                                |
| 48       | 809 ± 99                      | 1.17 ± 0.65            | 9.47                                 |
| 55       | 525 ± 18                      | 0.95 ± 0.26            | 5.00                                 |
| 96       | 769 ± 30                      | 1.31 ± 0.09            | 10.08                                |

Table 4

Mean biomass production, total lipid and DHA content of thraustochytrid strain G13 grown in a medium adjusted with different glucose concentrations, 1 g l<sup>-1</sup> yeast extract, 1 g l<sup>-1</sup> peptone and 27 g l<sup>-1</sup> ASW at 24°C for 4 days at 120 rpm

| Glucose (g l <sup>-1</sup> ) | Dry biomass (g l <sup>-1</sup> ) | Lipid in biomass (% w/w) | DHA in lipid (% w/w) | DHA production (g l <sup>-1</sup> ) |
|------------------------------|----------------------------------|--------------------------|----------------------|-------------------------------------|
| 30                           | 2.93 ± 0.02                      | 65.0 ± 14.01             | 30.2 ± 0.2           | 0.575                               |
| 20                           | 2.39 ± 0.37                      | 66.0 ± 0.0               | 31.2 ± 0.1           | 0.492                               |
| 10                           | 2.27 ± 0.10                      | 36.0 ± 20.5              | 32.8 ± 0.4           | 0.268                               |
| 5                            | 1.16 ± 0.06                      | 3.7 ± 0.10               | 35.8 ± 2.3           | 0.015                               |
| 1                            | 0.12 ± 0.0                       | Insufficient biomass     | —                    | —                                   |
| 0                            | No growth                        | —                        | —                    | —                                   |

Table 5

Mean biomass production, lipid and DHA content of thraustochytrid strain G13 grown at 24°C for 4 days at 120 rpm in media containing different concentrations of glucose, yeast extract (YE) and peptone: all media contained 27 g l<sup>-1</sup> ASW

| Medium (g l <sup>-1</sup> ) | Peptone (g l <sup>-1</sup> ) | Dry biomass (g l <sup>-1</sup> ) | Lipid in biomass (% w/w) | DHA in lipid (% w/w) | DHA production (g l <sup>-1</sup> ) |
|-----------------------------|------------------------------|----------------------------------|--------------------------|----------------------|-------------------------------------|
| 40 glucose/40 YE            | 20                           | 8.93 ± 0.25                      | 5.60 ± 0.0               | 18.8 ± 0.0           | 0.094                               |
| 40 glucose/20 YE            | 20                           | 5.87 ± 0.45                      | 3.90 ± 0.76              | 38.5 ± 4.2           | 0.088                               |
| 20 glucose/40 YE            | 20                           | 7.48 ± 0.89                      | 11.23 ± 0.54             | 18.7 ± 0.5           | 0.157                               |
| 20 glucose/20 YE            | 20                           | 5.83 ± 0.45                      | 7.27 ± 0.58              | 29.4 ± 1.2           | 0.124                               |
| 40 glucose/40 YE            | 0                            | 7.02 ± 0.13                      | 6.13 ± 1.52              | 18.9 ± 0.0           | 0.081                               |
| 40 glucose/20 YE            | 0                            | 5.91 ± 0.38                      | 5.03 ± 0.32              | 29.0 ± 2.6           | 0.086                               |
| 20 glucose/40 YE            | 0                            | 7.45 ± 0.55                      | 6.15 ± 0.25              | 24.7 ± 1.2           | 0.113                               |
| 20 glucose/20 YE            | 0                            | 5.22 ± 0.27                      | 7.00 ± 0.20              | 26.0 ± 1.1           | 0.095                               |

content was highest at 35.8% in cultures grown with 5 g l<sup>-1</sup> glucose, the highest yield of DHA (0.575 g l<sup>-1</sup>) was achieved by cultures grown in 30 g l<sup>-1</sup> glucose.

In an attempt to investigate the factor limiting biomass production, further steps to optimise biomass and lipid production involved increasing the yeast extract and peptone content of the medium and therefore reducing the C:N ratio. The data from this experiment are shown in Table 5. For all culture regimes, the concentration of

yeast extract was the determining factor for increasing biomass production. Although the medium containing 20 g l<sup>-1</sup> glucose, 40 g l<sup>-1</sup> yeast extract and 20 g l<sup>-1</sup> peptone supported high biomass and produced the highest lipid in the biomass (11.2%), only a relatively low proportion of the lipid (18.7%) was present as DHA. The highest percentage of DHA (38.5%) was found in the lipid from cultures grown in 40 g l<sup>-1</sup> glucose, 20 g l<sup>-1</sup> yeast extract and 20 g l<sup>-1</sup> peptone, but the lipid content in biomass from this medium

was the lowest achieved (3.9%, w/w) in all of the media combinations tested. The values for DHA production in Tables 4 and 5 indicate the significance of C:N ratio in stimulating fatty acid production by thraustochytrids. This observation concurs with the work of Yokochi et al. (1998), who recorded an increase in total fatty acid content of the cells as the concentration of the nitrogen source introduced into the medium was reduced.

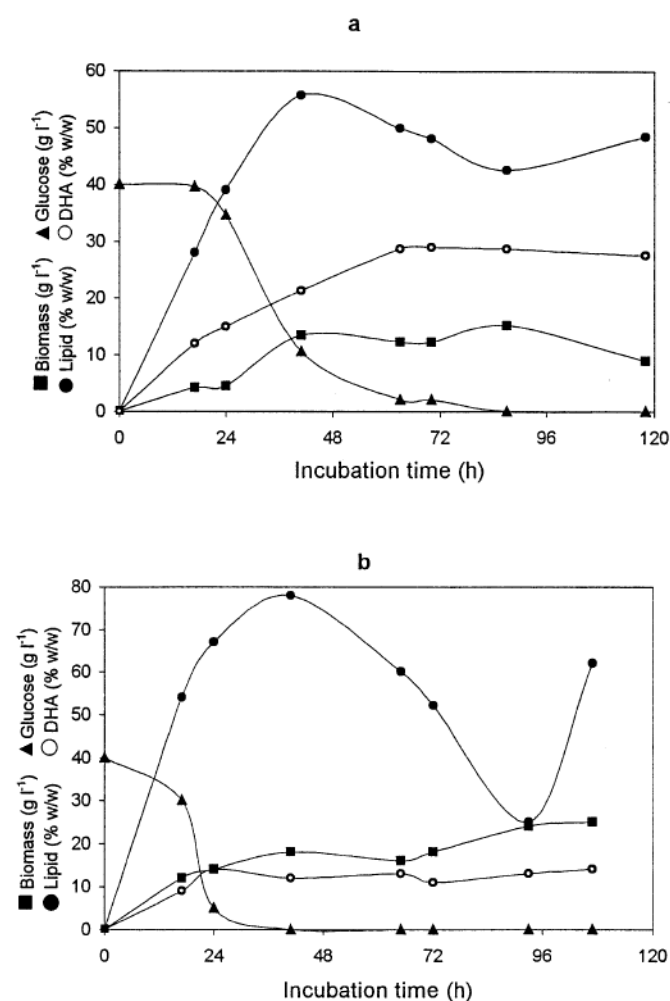


Fig. 3. Biomass, lipid and DHA production and glucose utilisation by thraustochytrid strain G13 maintained in a 2-l bioreactor: (a) for 118 h with medium composed of 40 g l<sup>-1</sup> glucose, 20 g l<sup>-1</sup> yeast extract and 27 g l<sup>-1</sup> ASW; (b) for 107 h with medium composed of 40 g l<sup>-1</sup> glucose, 5 g l<sup>-1</sup> yeast extract and 20 g l<sup>-1</sup> sodium sulphate. A total of 30 ml Mazu added over 96 h; aeration, 300 ml min<sup>-1</sup>; agitation, 750 rpm; temperature, 24°C. pH values ranged between (a) 6.1 and 7.6 and (b) 5.8 and 6.4.

### 3.2.2. Cultivation studies

The biomass, lipid and DHA produced by strain G13 over a 120-h period of growth in a bioreactor are presented in Fig. 3a. The growth curve depicted is typical of several achieved under the conditions documented, i.e. 40 g l<sup>-1</sup> glucose, 20 g l<sup>-1</sup> yeast extract, 27 g l<sup>-1</sup> ASW, 30 ml Mazu antifoam added over 4 days, 300 ml min<sup>-1</sup> aeration and 750 rpm agitation. Maximum biomass production (ca 14 g l<sup>-1</sup>) was reached at 41 h, close to the point of glucose exhaustion from the medium. This was also the point at which the lipid content of the biomass reached a maximum of 56% (w/w). The DHA content of the lipid remained stable at around 28% (w/w) from 63 to 120 h cultivation. There was a slight but insignificant rise from pH 6.5 throughout the growth period.

The medium used in the cultivation study was modified further by substituting the ASW with 20 g l<sup>-1</sup> sodium sulphate as a source of sodium, and the concentration of yeast extract was reduced from 20 to 5 g l<sup>-1</sup>, thus establishing a high carbon:nitrogen regime.

A representative result from a bioreactor cultivation of strain G13 in this medium is shown in Fig. 3b. The culture produced a maximum biomass of 14 g l<sup>-1</sup> at 24 h around the point of glucose exhaustion from the medium. At 41 h the lipid content of the cells was maximal at 78% (w/w) of which the DHA content was 18% of the lipid. Thereafter, there was some increase in the DHA content of the lipid and a maximum DHA yield of 2.17 g l<sup>-1</sup> was achieved at 107 h. Recent investigations to optimise DHA production have recorded yields of 4 g l<sup>-1</sup> (Yokochi et al., 1998) and 13.3 g l<sup>-1</sup> (Yaguchi et al., 1997) from a strain of *Schizochytrium* sp. This clearly indicates the potential for further work with the thraustochytrid group in order to maximise even greater yields.

The findings from the investigations described here are, firstly, that although thraustochytrids contain high levels of PUFA, there is wide diversity with regard to the synthesis of individual fatty acids. The possibility for 'tailor made' single cell oil (SCO) may exist. Thraustochytrids from warmer climates such as sub-tropical mangroves

produced more biomass (at 25°C), but this was not generally associated with high levels of DHA in the fatty acids present in the cells. In contrast, the cold water isolates were low biomass producers under the same culture conditions, but produced consistently higher levels of DHA in the total fatty acids.

To ensure an optimum fatty acid profile, emphasis should be placed on strain selection when considering the use of thraustochytrids for SCO production for a specific application. Results indicate that the highest DHA production occurred at the time of glucose exhaustion and that high levels might be enhanced, or prolonged, by fed-batch cultivation. Thraustochytrid oil was found to be a free-flowing triacylglycerol with an acceptable peroxide value of less than 5 meq of peroxide oxygen kg<sup>-1</sup> of oil, both features of advantage for commercial uses.

Ratledge (1998) considered that, despite improvements in the efficiency of photobioreactors, it is doubtful whether the growth of microalgae in bioreactors could be scaled up to satisfy even a modest demand for SCO rich in *n* – 3 PUFA, and suggested that heterotrophic microbes might be a more productive source. Our findings and those of other researchers highlight one group of marine microbes with a commercial future.

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