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Isolation of clones of *Isochrysis galbana* rich in eicosapentaenoic acid

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ABSTRACT

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Fifty-nine clones of the microalga *Isochrysis galbana* have been isolated and cultivated. All clones were cultivated under identical conditions and were harvested in the stationary phase for fatty acid analysis. There is conspicuous variation in fatty acid profiles between clones, which is probably due to genetic variation among clones. Several clones are very rich in eicosapentaenoic acid (more than 6% of dry weight) and moderately rich in docosahexaenoic acid (more than 2% of dry weight). We suggest the commercial use of some of our clones as an unconventional source of EPA.

INTRODUCTION

In recent years, polyunsaturated fatty acids (PUFAs) have been of interest because they are essential in the nutrition of some marine organisms (Aaronson et al., 1980; Langdon and Waldock, 1981; Whyte, 1987; Fernández-Reiriz et al., 1989). More recently, they have received increased attention because of their relation to human health (Cohen, 1986; Yongmanitchai and Ward, 1989). Several PUFAs, the so-called essential fatty acids (linoleic, linolenic, arachidonic and eicosapentaenoic acids), are required in the human diet as recognized for several decades (WHO/FAO, 1977). Most recently, attention has focused on *n*-3 PUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), due to their association with the prevention and treatment of several diseases (atheroclerosis, thrombosis, arthritis, cancers, etc.) (Klausner, 1986; Yongmanitchai and Ward, 1989) through their

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metabolic conversion into eicosanoids like prostaglandins, thromboxanes and leukotrienes (Borowitzka, 1988; Kerby and Stewart, 1988; Regan, 1988; Yongmanitchai and Ward, 1989). At present, the only commercial source of 20 and 22 long chain *n*-3 PUFAs is marine fish and their oil, but world production from this source is insufficient for human requirements and there is some trouble with its flavor (Yongmanitchai and Ward, 1989).

Based on their fatty acid profile, microalgae have been suggested as another suitable source of *n*-3 PUFAs (Ben-Amotz et al., 1985; Iwamoto and Sato, 1986; Borowitzka, 1988; Yongmanitchai and Ward, 1989) and for this reason research on *Isochrysis galbana* directed toward the isolation of clones rich in *n*-3 PUFAs has been initiated.

Despite controversial data discussed below, *I. galbana* was chosen because it contains high levels of lipids (Cohen, 1986; Whyte, 1987), especially PUFAs, in comparison with other microalgae species (Helm and Laing, 1987), as corroborated by the results of this study.

The present work does not aim to optimize the culture of *I. galbana*, but to obtain clones with enhanced production of one or several PUFAs. As previously noted by Rowlands (1984) and Gudin (1989), strain improvement is an essential part of the microbial fermentation product process. The study reported here is a first step of a 'random screening' method (Rowlands, 1984) customary for other microorganisms (i.e. bacteria) but which, to our knowledge, has not been applied to microalgae. This kind of work is important both for industrial strain improvement (Gudin, 1989; Craig et al., 1988) and as a prerequisite for the future development of genetic engineering methods (Craig et al., 1988).

MATERIALS AND METHODS

The 'parent' culture used was supplied by Pescanova's Cabo de Gata factory in Almería, Spain. Cells were isolated with a micropipet under a microscope. Each isolated cell was transferred to a test tube for cultivation; 59 clones were established in this manner. All clones were cultivated under identical conditions of nutrient level, light and temperature, following standard methods (medium Algal 1, from Nutrición Avanzada, S.A., Santiago de Compostela, Spain; light intensity 55 W m^{-2}, temperature 20°C, pH 8.0 and specific air supply rate 2 volumes per min).

The seawater was sterilized in an autoclave at 120°C for 30 min and the complete culture medium was sterilized by filtration through 0.2-μm pore membranes. Cultivated clones were harvested in the stationary phase of growth for subsequent analysis. Biomass was washed with a diluted saline solution (9/1000).

Lipids were extracted by the method of Kochert (1978). Fatty acid methylation was done by direct transterification with acetyl chloride:methanol

(1:20) following the method of Lepage and Roy (1984). Analysis of methyl esters was carried out by gas chromatography using a 30-m capillary column of fused silica (SP2330, Supelco, Bellefonte, PA, USA), internal diameter of 0.25 mm, 0.20 mm standard film, split retention 100:1, and a flame-ionization detector. SIGMA Lipid Standard 189-15, Supelco Rapeseed oil mixture and Supelco PUFAs-I patterns were used for the determination.

Each clone was analysed two or more times to minimize technical errors in the determination of its fatty acid composition. Data reported here are the averages of two analyses. Pigment content was subtracted from the total lipid to calculate fatty acid content of dry weight.

RESULTS

Of the 20 fatty acids sought in 59 clones of *I. galbana*, five (16:1*n*9, 22:1*n*11, 22:1*n*9, 24:0 and 22:5*n*3) were absent in virtually all clones, seven (16:3, 18:0, 18:1*n*9, 18:2*n*6, 20:0, 20:1*n*9 and 20:4*n*6) were found in minor quantities, generally below 1% of total lipid, and the remainder (14:0,

TABLE 1

Lipid composition (percentages of total lipid) in 59 clones of *Isochrysis galbana*[a]

Fatty acid	Minimum	Maximum	Average	Standard error
14:0	4.63	16.39	11.68	0.305
16:0	7.30	27.39	15.01	0.446
16:1*n*9	0	0.43	0.02	0.010
16:1*n*7	10.15	24.28	16.16	0.310
16:3	0	2.20	1.08	0.062
18:0	0	2.31	0.90	0.065
18:1*n*9	0	1.56	0.52	0.045
18:1*n*7	0.49	7.03	3.09	0.144
18:2*n*6	0	1.70	0.44	0.049
20:1*n*9	0	3.18	1.60	0.104
20:0	0	2.74	0.15	0.054
18:4*n*3	4.54	13.88	7.68	0.239
20:4*n*6	0	2.12	0.53	0.068
20:5*n*3	13.19	31.93	23.00	0.470
22:1*n*11	0	2.06	0.03	0.035
22:1*n*9	0	0.15	0.00	0.003
22:3	0	3.31	1.27	0.079
22:5*n*3	0	0	0	0
22:6*n*3	4.25	13.36	8.05	0.208
Unsat.	52.29	73.50	63.50	0.638
PUFAs	19.29	51.24	35.98	0.715
EFAs	15.04	35.15	25.58	0.528

[a]Individual values are means of two observations. See the text for more information.

TABLE 2

Lipid composition (percentages of dry weight) in 59 clones of *Isochrysis galbana*[a]

Fatty acid	Minimum	Maximum	Average	Standard error
14:0	0.93	3.29	2.13	0.076
16:0	1.21	4.75	2.71	0.095
16:1*n*-9	0	0.10	0.01	0.002
16:1*n*-7	1.61	4.92	2.96	0.103
16:3	0	0.40	0.20	0.013
18:0	0	0.44	0.16	0.012
18:1*n*9	0	0.30	0.10	0.009
18:1*n*7	0.10	1.11	0.55	0.026
18:2*n*6	0	0.45	0.09	0.011
20:1*n*9	0	0.84	0.30	0.023
20:0	0	0.53	0.03	0.010
18:4*n*3	0.63	2.66	1.41	0.058
20:4*n*6	0	0.41	0.10	0.013
20:5*n*3	1.81	6.61	4.23	0.151
22:1*n*11	0	0.40	0.01	0.007
22:1*n*9	0	0.03	0.00	0.001
22:3	0	0.87	0.23	0.017
22:5*n*3	0	0	0	0
22:6*n*3	0.58	2.77	1.47	0.051
Unsat.	5.83	17.32	11.64	0.349
PUFAs	2.64	10.77	6.61	0.234
EFAs	2.06	7.85	4.71	0.175
Total lipid	9.00	26.30	18.30	0.504

[a]Individual values are means of two observations. See the text for more information.

16:0, 16:1*n*7, 18:1*n*7, 18:4*n*3, 20:5*n*3, 22:3 and 22:6*n*3) were present in all clones, generally in percentages much higher than 1% (Table 1).

Despite this overall picture, conspicuous variation between clones was observed. Thus, in several clones 14:0 represented over 14% of total lipid while in others it was less than 6%. As another example, the clone referred to here as AL41 was the richest in EPA with 31.93% of total lipid and, at the other end, AL26 had only 13.19% (Table 1; see also percentages of dry weight in Table 3).

Our clones of *I. galbana* were very rich in unsaturated fatty acids (Table 1) with a mean of 63.5% of total lipid. PUFAs were also found in large quantities (Table 1) averaging, 36.0%, and the essential fatty acids (EFAs) averaged 25.6% (Table 1).

Although the overall description does not change, more informative than these figures are those that represent the dry weight percentages (Table 2) where the proportion of unsaturated fatty acids reached 17.32%, PUFAs 10.77% and EFAs 7.85% of the dry weight in clone AL28 (Table 3). Clone

TABLE 3

Lipid composition (percentages of dry weight) of seven selected clones of *Isochrysis galbana*[a]

Fatty acid	Clone						
	AL26	AL28	AL34	AL36	AL38	AL41	AL58
14:0	2.11	3.14	0.93	2.45	3.20	1.05	2.78
16:0	3.75	3.25	2.70	3.71	3.29	2.12	2.59
16:1*n*9	0	0	0	0	0	0	0
16:1*n*7	3.33	4.44	2.75	3.63	3.93	2.10	3.69
16:3	0	0.30	0.16	0.26	0.33	0.22	0.38
18:0	0	0.12	0.23	0.09	0.15	0.21	0.23
18:1*n*9	0	0	0.12	0.13	0	0.21	0.06
18:1*n*7	0.96	0.43	0.52	0.53	0.46	0.10	0.53
18:2*n*6	0	0.45	0.15	0.23	0.26	0.11	0.08
20:1*n*9	0.25	0.84	0.42	0.57	0.66	0.27	0.54
20:0	0	0	0.02	0	0	0	0
18:4*n*3	0.63	1.68	1.69	1.77	1.76	2.17	2.06
20:4*n*6	0	0.32	0.18	0.17	0.15	0.29	0.15
20:5*n*3	1.81	6.25	5.75	5.91	6.08	6.61	6.12
22:1*n*11	0	0	0	0	0	0	0
22:1*n*9	0	0	0	0	0	0	0
22:3	0	0.87	0.48	0.30	0.25	0.34	0.30
22:5*n*3	0	0	0	0	0	0	0
22:6*n*3	0.58	1.75	2.48	2.07	2.06	2.77	1.92
Unsat.	7.56	17.32	14.70	15.57	15.94	15.18	15.84
PUFAs	2.64	10.77	9.62	9.50	9.79	10.61	9.49
EFAs	2.06	2.85	6.5	6.87	7.15	7.28	6.89

[a]Individual values are means of two observations. See the text for more information.

AL41 had 6.61% eicosapentaenoic acid and several other clones had over 6% (Table 3). DHA reached 2.77% in clone AL41 and several other clones had more than 2% (Table 3).

DISCUSSION

Many others have reported fatty acid variation between different taxa of microalgae (Chuecas and Riley, 1969; Shifrim and Chisholm, 1980, 1981; Pohl, 1982; Ben-Amotz et al., 1985; Borowitzka, 1988; Fernández-Reiriz et al., 1989; Yongmanitchai and Ward, 1989). Variation related to the condition or age of the culture has also been reported (Chuecas and Riley, 1969; Pohl, 1982; Whyte, 1987; Borowitzka, 1988; Fernández-Reiriz et al., 1989). Currently, the chemical composition of eukaryotic algae is generally regarded as species specific and regulated by environmental factors (Shifrim and Chisholm, 1981; Ben-Amotz et al., 1985). From these results it is evident that

fatty acid composition changes significantly among strains of a single organism. Thus, fatty acid variation is not restricted to interspecific variation but also varies from clone to clone within the same species (Shifrim and Chisholm, 1980; Whyte, 1987). Because all clones were subjected to identical culture conditions and to the same analytical techniques, the variation in fatty acid profiles among our clones of *I. galbana* is probably due to genetic variation within this species, that is genetically determined. This is not surprising, on the contrary, it is to be expected because it is commonly found in population genetics.

Wide discrepancies appear in different studies on *Isochrysis galbana* (Pohl, 1982; Ben-Amotz et al., 1985; Helm and Laing, 1987; Fernández-Reiriz et al., 1989; Yongmanitchai and Ward, 1989; present work). For example, of the total fatty acids, Yongmanitchai and Ward (1989) reported 0% EPA, Helm and Laing (1987) 3.6% and our own data showed a total clone average of 23% (Table 1). Some DHA results reported are: 0.26% (Fernández-Reiriz et al., 1989), 18.9% (Helm and Laing, 1987) and 8.05% (present study; Table 1).

Differences in culture conditions, the analytical methods and the growth phase sampled can explain these discrepancies. As noted by several authors, different culture techniques lead to important differences in the final composition of a culture (Materassi et al., 1980; Shifrim and Chisholm, 1981; Pohl, 1982; Borowitzka, 1988; Fernández-Reiriz et al., 1989; Yongmanitchai and Ward, 1989). At the same time, genetic variation between the samples cultivated by different workers can account for a large amount of the variation observed among reports. As shown, wide genetic variation is present in our sample of *I. galbana* and discrepancies among different studies suggests that 'geographical' variation in this species is likely to be high. It is therefore temerarious to generalize from one sample studied to the whole species, as is currently done.

This discussion poses another question worthy of remark: comparisons of figures referring to percentages of total fatty acids or total lipids are very inadequate, because lipid content varies greatly from species to species (Langdon and Waldock, 1981; Shifrim and Chisholm, 1981; Pohl, 1982; Borowitzka, 1988) and from sample to sample within the same species (Shifrim and Chisholm, 1980), as discussed above. In this sense, from a biological as well as commercial point of view, the critical data are for 'real' production, and results should be reported as percentages of dry weight for suitable evaluation.

On the other hand, suggestions on the use of microalgae for the commercial production of PUFAs (Ben-Amotz et al., 1985; Borowitzka, 1986; Yongmanitchai and Ward, 1989) are supported by our results. Many clones of *I. galbana* contained large quantities of PUFAs, with a maximum of 10.77% and a mean of 6.61%, expressed as percentages of dry weight (Table 2). Some clones were rich in EPA and DHA, two fatty acids of great commercial interest.

Clones AL28, AL38, AL41 and AL58 were the highest producers of EPA (Table 3). Clones AL34, AL36, AL38 and AL41 were the richest in DHA (Table 3). However, our clones were poor in linoleic acid and arachidonic acid (AA). This may be because in *I. galbana* EPA and DHA are produced by an alternative metabolic pathway to that of AA, as has been suggested for microalgae (Yongmanitchai and Ward, 1989), and which is in agreement with reports on *Porphyridium cruentum* (Cohen et al., 1988).

At present, a limited number of unconventional sources of EPA and DHA have been analysed. The fungus *Morteriella alpina*, which has 15% EPA of total fatty acids, has been suggested as a source of EPA (cited in Yongmanitchai and Ward, 1989). EPA average production of our clones was 23% of total lipid and many of them were near 30%. The microalgae *Porphyridium cruentum* (Vonshak et al., 1985) and *Chlorella minutissima* (Borowitzka, 1988) yielded 1.5% and 2.6% EPA of dry weight, respectively. Average EPA production of *I. galbana* clones was 4.23% and several of the clones reported above had over 6% of dry weight. At the same time, our clones averaged 1.47% DHA and several clones produced nearly 3%.

The conventional source of EPA and DHA is marine fish oil (Yongmanitchai and Ward, 1989). Three species of oily fishes — sardine, anchovy and jurel — have been analysed for comparison. All three have a similar fatty acid profile (Fig. 1). Jurel was the richest in EPA with 1.68% of dry weight, significantly smaller than the *I. galbana* clone average of 4.23% (Table 2; see also Fig. 1). On the other hand, the three fish species were very rich in DHA:

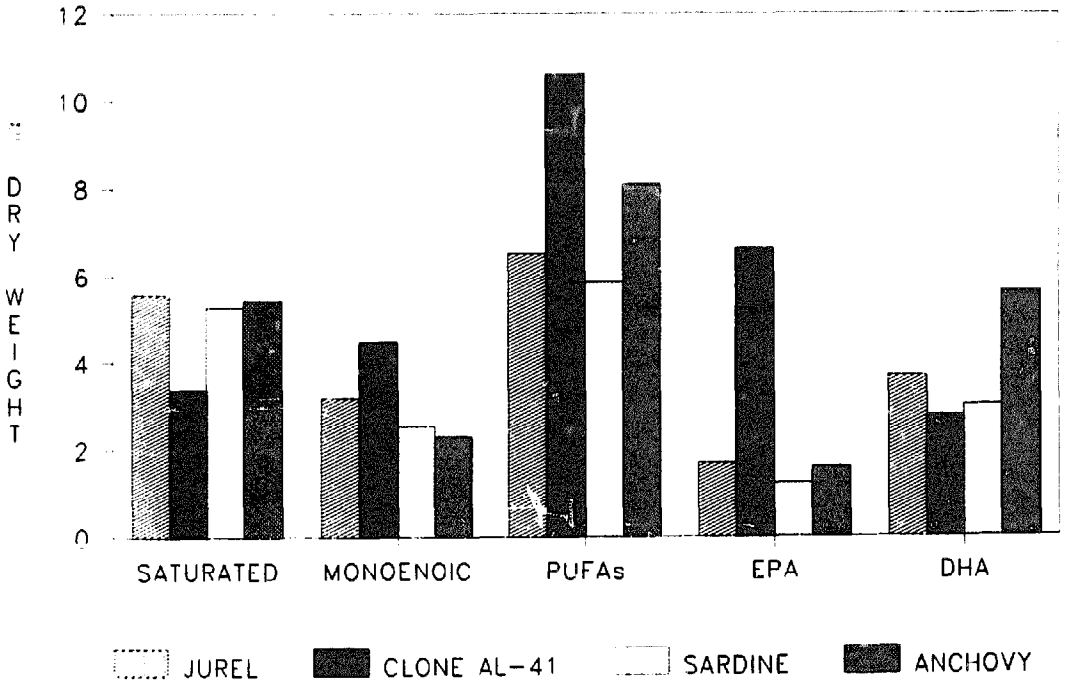

Fig. 1. Lipid composition (percentages of dry weight) in three fish oils and the AL41 clone.

3.02%, 5.62% and 3.66% for sardine, anchovy and jurel, respectively, compared with our *I. galbana* clone maximum of 2.77% (Table 2; Fig. 1).

Overall comparison suggests the use of some of our *I. galbana* clones for the commercial production of EPA, obtaining at the same time some DHA. The marketing of selected clones of *I. galbana* requires economic evaluation of mass culture, harvesting, extracting/processing and marketing (Borowitzka, 1986; Gudin and Thepenier, 1986), but with market prices reported by Borowitzka (1986, 1988), this seems promising. The present study is only a first step in the development of a systematic attainment of EPA from *I. galbana*. Additional random screening steps are necessary and processes conditions of the new clones will probably need improvement to show its maximum advantage (Rowlands, 1984). This is currently being looked at.

Finally, attention is called to the application of random screening methods to other microalgae. Improvement requires not only the development of optimum culture parameters but also the selection of the most adequate species and genome for the metabolic target (Gudin and Ferreira dos Santos, 1990).

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