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Preservation of the marine microalga, *Isochrysis galbana*: influence on the fatty acid profile

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Abstract

Preservation of marine microalgae is of interest for storage in culture collections as well as a method of having living biomass available for aquaculture feed. The present paper reports the effects of lyophilization, freezing and maintenance of refrigerated cell concentrates on the viability and fatty acid profile of *Isochrysis galbana*. Lyophilization led to a viability loss of about 98%, and the initial fatty acid profile remained unchanged throughout the experiment (1 month). Survival rate after freezing was enhanced by a 10% v/v glycerol addition and remained around 20% at the end of the experiment. No effect was observed on the fatty acid profile. A concentrate of 9 $g \cdot l^{-1}$ maintained at 4°C for 30 days retained up to 85% viability. There was a significant decrease in saturated and mono-unsaturated fatty acids, while polyunsaturated content remained constant.

1. Introduction

Today most mass cultures of microalgae are used in aquaculture as food for marine animals, in particular larval and juvenile molluscs, crustaceans and fish. Live food is necessary for raising fish through the early critical larval stages. Thus, in hatcheries, living microalgae biomass becomes the bottleneck of production in a short period of time. During this stage, a microalgae culture failure, due to adverse environmental conditions or contamination by other organisms, can interrupt the food chain from larvae to commercial-sized individuals. Aquaculture

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production could be improved by using microalgae biomass stored (Sommer et al., 1990, Nell and O'Connor, 1991), which could be supplied under quantitative and qualitative control.

To reach this goal, preservation techniques for the storage of microalgae must be developed, bearing in mind that a good survival rate must be accompanied by adequate biochemical composition. Information in the literature regarding the preservation of marine microalgae is rather limited. For long-term maintenance of a wide variety of cell types, cryopreservation methods including lyophilization, deep freezing, storage in liquid nitrogen or even simply refrigerated algal concentrates have been described (Cordero Esquivel et al., 1993).

In this study, preservation experiments carried out with the marine microalga *Isochrysis galbana* are described. The preservation methods tested were lyophilization, freezing with and without cryoprotectant and storage of concentrated cultures at 4°C.

As polyunsaturated fatty acid (PUFA) content of microalgae plays an important role in cell quality as food in aquaculture, and the biochemical composition of microalgae might be altered by the preservation technique, the effect on the fatty acid content of *I. galbana* was also determined.

2. Materials and methods

The microalga used was an isolate selected from among 42 isolates of a single strain of *Isochrysis galbana* in a phenotypic selection programme carried out in our laboratory to select an EPA-rich strain of microalgae (López Alonso et al., 1992a,b). This isolate (labeled AL II4) is lodged with the CCAP. Algae were grown in a 10-liter stirred tank reactor at 20°C, 1% CO_2-enriched air was bubbled in and continuous light (2×10^{16} quanta·cm^{-2}·s^{-1}) was provided. Culture medium was as described by Molina Grima et al. (1992). Stationary phase culture (0.939 mg·l^{-1}) was harvested by centrifuging at 2500 rpm ($686\times g$) for 10 min. Microscopic examination revealed no apparent cellular damage. The concentrate was divided into equal volumes for preservation: by lyophilization in a Modulyo Edwards freeze-dryer after pre-freezing at −20°C, by freezing at −20°C, −2°C·min^{-1}, by freezing with the addition of 10% v/v of glycerol at −20°C, −2°C·min^{-1} and by storing at 4°C with constant illumination of a 9 g·l^{-1} concentrate.

Survival rate measurements were carried out by staining with Evan's blue and counting live cells (non-stained) under the light microscope in a Neubauer hemocytometer (Harrison, 1988). Staining was carried out by addition of 0.1 ml of 1% (w/v) stock solution of Evan's blue (Sigma) to a 2 ml culture sample yielding a final concentration of 5×10^{-7} g stain per ml of culture. Samples were incubated for 20 min at room temperature. Evan's blue is a dead-cell stain that turns the organic matter of dead cells deep blue. It is repelled by living cells, with a functional cell membrane. Counts were accomplished with appropriate serially diluted samples.

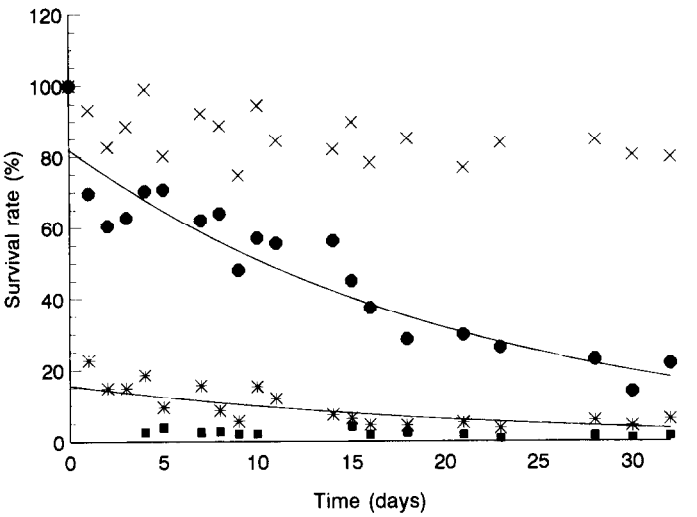

Fig. 1. Influence of storage time and preservation method on survival rate of *Isochrysis galbana*. ×, Concentrate at 4°C; ●, Frozen biomass with 10% (v/v) glycerol; ⋆, Frozen biomass without cryoprotectant addition; ■, Lyophilized biomass; solid lines represent theoretical values.

A control sample, obtained after centrifuging the culture and possessing the same concentration as the stored biomass ($9\ g \cdot l^{-1}$), was used to obtain the relative survival rate measurement. Live cells (non-stained) from 16 squares of the hemocytometer were counted 3 times for each preservation method and the control sample every time. The survival rate estimate was the average of the 3 totals compared to the control sample, expressed as a percentage. The control sample was the 100% survival rate.

The margin of error of the Evan's blue method in survival rate measurements was determined by mixing known amounts of living and dead microalgae, staining the samples and counting the living cells. The average error thus found was about 1.27% (data not shown).

For testing, two samples at a time were taken from each type of stored biomass, and subjected to fatty acid analysis. Fatty acid methylation was done by direct transesterification with acetyl chloride/methanol (1:20) following the method of Lepage and Roy (1984). The 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 μm standard film, split ratio 100:1, and a flame ionization detector. Sigma Lipid Standard 189-15, Supelco rapeseed oil mixture and Supelco PUFA-1 patterns were used for the determination. Nonadecanoic acid was used as an internal standard to quantify fatty acid content in dry weight biomass.

3. Results

The survival rate found in lyophilized biomass of *I. galbana* was very low and remained approximately constant at around 2% although a slight decrease was

Table 1
Variation of fatty acid composition of preserved biomass over time (% of dry weight)

Fatty acid	Days of storage												
	0	1	2	4	5	8	10	14	18	21	24	28	30
Lyophilized biomass													
14:0	0.86			0.76	0.83	0.93	0.82	0.78	0.74	0.95	0.78	0.73	0.65
16:0	1.98			1.78	1.83	1.92	1.89	1.78	1.71	2.17	1.82	1.70	1.52
16:1	1.95			1.76	1.85	2.00	1.85	1.73	1.66	2.13	1.78	1.66	1.49
18:4	0.51			0.45	0.49	0.56	0.49	0.45	0.46	0.56	0.42	0.39	0.39
20:5	2.32			2.47	2.22	1.97	2.03	2.51	1.90	2.51	1.90	1.74	1.70
22:6	0.85			0.93	0.85	0.76	0.76	0.96	0.71	0.96	0.71	0.65	0.67
Frozen biomass													
14:0	0.86	0.76	0.77	0.47	0.76	0.65	0.67	0.64	0.71	0.72	0.71	0.69	0.68
16:0	1.98	1.83	1.79	1.39	1.58	1.47	1.69	1.43	1.68	1.76	1.60	1.63	1.77
16:1	1.95	1.81	1.78	1.41	1.64	1.29	1.68	1.33	1.66	1.74	1.60	1.61	1.75
18:4	0.51	0.42	0.36	0.38	0.39	0.34	0.39	0.32	0.33	0.37	0.34	0.31	0.47
20:5	2.32	1.98	1.73	1.91	1.92	1.62	1.83	1.49	1.54	1.65	1.63	1.47	1.65
22:6	0.85	0.67	0.61	0.77	0.67	0.52	0.66	0.49	0.53	0.57	0.58	0.50	0.62
Frozen biomass with 10% v/v glycerol													
14:0	0.86	0.83	0.79	0.75	0.79	0.66	0.74	0.62	0.68	0.75	0.81	0.68	0.67
16:0	1.98	1.88	1.81	1.74	1.71	1.77	1.72	1.51	1.59	1.74	1.76	1.58	1.56
16:1	1.95	1.86	1.81	1.73	1.78	1.71	1.66	1.33	1.56	1.68	1.78	1.56	1.52
18:4	0.51	0.47	0.44	0.43	0.46	0.45	0.42	0.35	0.34	0.36	0.41	0.33	0.34
20:5	2.32	2.14	2.02	2.01	2.17	2.13	1.90	1.76	1.61	1.64	2.01	1.52	1.56
22:6	0.85	0.72	0.68	0.67	0.74	0.72	0.64	0.56	0.55	0.57	0.65	0.52	0.54
Concentrated culture at 4°C													
14:0	0.86	0.86	0.76	0.74	0.73	0.70	0.74	0.79	0.75	0.84	0.84	0.67	0.67
16:0	1.98	1.76	1.61	1.47	1.36	1.22	1.23	1.06	0.94	1.06	0.93	0.81	0.84
16:1	1.95	1.80	1.65	1.56	1.45	1.24	1.38	1.33	1.19	1.37	1.29	0.93	0.99
18:4	0.51	0.51	0.46	0.47	0.49	0.49	0.51	0.56	0.60	0.56	0.60	0.49	0.47
20:5	2.32	2.24	2.11	2.17	2.28	2.20	2.30	2.40	2.23	2.36	2.44	1.96	1.94
22:6	0.85	0.78	0.77	0.77	0.82	0.77	0.78	0.83	0.77	0.80	0.84	0.65	0.64

observed over time (Fig. 1). Its fatty acid profile was not affected by time during storage (as tested by analysis of variance, data not shown), 14:0, 16:0, 16:1*n*-7, 18:4*n*-3,EPA(20:5*n*-3) and DHA (22:6*n*-3) being the main fatty acids found in the saponificable lipid fraction (Table 1).

Freezing ($-20°C$, $-2°C \cdot min^{-1}$) led to 20% viability after 1 day of storage, which rose to 70% with the addition of 10% v/v of glycerol. During the following days, viability of the frozen biomass was observed to decrease to 5% without cryoprotectant and 20% with it (Fig. 1). Analogous to lyophilized biomass, the fatty acid profile remained unchanged during storage (Table 1) regardless of the use of glycerol.

A concentrate of 9 $g \cdot l^{-1}$ was kept at 4°C under constant light. Up to 85% via-

bility was retained for at least 30 days (end of the experiment) (Fig. 1). The influence of storage time on the fatty acid profile of the biomass was observed. There was a significant decrease in saturated and mono-unsaturated fatty acids although polyunsaturated fatty acids remained approximately constant (Table 1).

4. Discussion

Lyophilization of micro-organisms has been proposed for long-term preservation of culture collections, in medicine and industrial biological processes (Chang and Elander, 1986). A loss of viability over time which may indicate some residual metabolic activity in lyophilized cells (Holm-Hansen, 1967) was found. The percentages of viability reported in the present study are in agreement with those reported for *Chlorella* sp. after 1 day of storage (Holm-Hansen, 1967).

The nutritional value of microalgal species is mainly related to the content of certain PUFAs, in particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There is a plethora of papers dealing with the importance of these fatty acids in the growth of marine species (De Pauw et al., 1984). It is therefore important that preservation methods keep the fatty acid content of the algae unchanged, more so when growth conditions are controlled to attain a planned chemical composition of the biomass.

As might be expected, fatty acid composition did not vary during storage. Analogous results were obtained by Cordero Esquivel et al. (1993), with freeze-dried biomass of the diatom *Chaetoceros* sp. after 2 months of storage. Nevertheless, if high viability is required, this preservation method may be rejected for food in aquaculture. On the other hand, it may be the best for biochemical profile preservation.

Freeze stress is related to the formation of ice inside the cells (intracellular ice) or around the cells (extracellular ice). The former is generally lethal and the latter produces extracellular hypertonicity due to the ionic-concentration effect in the liquid phase surrounding the solid phase during the ice formation process. This hypertonicity must be balanced by osmotic movement of water from the cell to the extracellular solution. The cooling rate is determined by the effect predominating during the freezing process. At slow cooling rates, cells may be exposed to the damaging effects of hypertonic stress. As the cooling rate is increased, the probability of solute damage decreases, but the probability of intracellular ice formation increases, as less time is available for water to leave the cell osmotically (McLellan, 1989).

As can be seen in Fig. 1, the addition of glycerol enhanced the survival rate of *I. galbana*, although it was unable to avoid the decrease in viability over time. This influence of storage time on viability regardless of the preservation method is of relevant importance, as by modelling this variation the viability at any given time may be found. Other papers dealing with the effects of freezing on microalga viability refer only to time of storage, and do not describe its variation during this

Table 2
Two-way ANOVA of the survival rate of preserved biomass

Source of variation	DF	Sum of squares	F-ratio
A: Treatment	3	3524.61	297.45***
B: Time	15	147.91	2.50**
C: Sample	2	0.12	0.36ns
Interaction AB	45	177.74	23.97***
Residual	126	20.76	

*$0.01 < P < 0.05$; **$P < 0.05$; ***$P < 0.001$; ns = not significant.

period. In this sense, the significance of these data has been validated by a two-way ANOVA treatment, in which sources of variation included were preservation method, storage time and sample examined (Table 2). The effects of preservation method and time on survival rate variation were statistically significant ($P < 0.001$), and the type of sample exerted no influence on it. With regard to interaction among sources of variation, method and time were found to be significant ($P < 0.001$), but no significant interaction appeared between them and the sample (Table 2). This confirms that survival rate is a function of time, as affected by treatment.

Two stages can be distinguished in the survival rate of frozen biomass over time (Fig. 1). The first step in which viability suffers a dramatic decrease could represent the change from liquid to solid state, i.e. the ice formation period. Beyond this time, viability decreased slowly and death kinetics were exponential:

$$-\frac{dS}{dt} = K_d \cdot S \tag{1}$$

$$\int_{S_0}^{S} \frac{dS}{S} = -K_d \int_0^t dt \tag{2}$$

$$S = S_0 \cdot e^{-Kd \cdot t} \tag{3}$$

where S is the viability at time t, K_d is a mortality constant which could depend on the preservation method and storage temperature, and S_0 is the viability just after the ice formation process.

Fitting the experimental data obtained without cryoprotectant to Eqn. (3), the following expression was obtained:

$$S = 15.0 \cdot e^{-0.046 \cdot t} \quad r^2 = 0.617 \tag{4}$$

Theoretical and experimental data are shown in Fig. 1. When the same fit was applied to viability data from frozen biomass with glycerol, the expression obtained was:

$$S = 78.6 \cdot e^{-0.047 \cdot t} \quad r^2 = 0.895 \tag{5}$$

Eqns. (4) and (5) show how, contrary to what might be expected, K_d is not dependent on the addition of cryoprotectant. Thus, the difference between viability

observed in frozen biomass with and without glycerol originates during the freezing period and the function of cryoprotectants is related to a decrease in risk of physical and chemical damage during the ice formation process.

On the other hand, lipid accumulation has been reported to protect cells from injury during freezing. Morris and Clarke (1978) suggested that the lipid bodies found in *Chlorella* cells reduce the size of the vacuole, and that this is associated with improved survival following freezing. In this sense, McLellan (1989) observed that intracellular ice formation may be prevented if cells accumulate lipid droplets, which coalesce during cooling and fill the cell. During thawing, droplets again become discrete. Thus, the high lipid content of *I. galbana* (Molina Grima et al., 1992, 1993) could enhance the ability of this species to survive under freezing.

There was no effect of freezing on the fatty acid profile. Analogous to survival measurements, ANOVA treatment of each fatty acid took into account preservation process, time and sample as sources of variation on fatty acid content. As a result, it may be said that fatty acid variation is dependent on treatment ($P<0.001$) and time ($P<0.001$), and that the interaction between them is statistically significant (Table 3). Therefore, the effect of time on the variation of fatty acid content may not be understood without considering the effect of the treatment.

Long-term preservation of microalgal cultures at low temperatures (4°C) has been used previously to induce freezing tolerance (Benz-Amotz and Gilboa, 1980; Benz-Amotz and Rosenthal, 1981). Using this method, several marine diatoms could be maintained without subculture for many months at 5°C, provided the cells were exposed to light for short periods several times per day (Umebeyashi, 1972).

I. galbana cultures were illuminated constantly and a slow decrease of viability over time then was measured (Fig. 1). As cell metabolism was still active, the fatty acid profile underwent continuous change. Initially, palmitic and palmitoleic acids constituted 2% of dry weight, while EPA, the major PUFA, represented 2.3% of dry weight. During the following 5 days, the content of 16:0 and 16:1 decreased rapidly. Beyond this time, the decrease continued at a slower rate. However, EPA content remained constant as did 18:4*n*-3 and 22:6*n*-3.

An explanation for this fact comes from the function of these fatty acids in cellular metabolism. In a previous study, lipids were fractionated and fatty acid composition of the neutral lipid, glycolipid and phospholipid fractions was determined (Molina Grima et al., 1994). The main fatty acids found in neutral lipids, which are related to a cellular storage function, were 16:0 and 16:1. Polyunsaturated acids were found mainly in the glycolipids, which are related to membrane organelles. Thus, during the maintenance period, cells take energy from their storage lipids, causing a decrease in the fatty acids contained in this fraction.

Table 3
Two-way ANOVA of the fatty acid content of the preserved biomass

Fatty acid	Source of variation	DF	Sum of squares	F-ratio
14:0	A: Treatment	3	22.31	71.22***
	B: Time	13	18.73	13.80***
	C: Sample	1	0.07	0.71ns
	Interaction AB	39	23.14	5.68***
	Interaction AC	3	0.08	0.25ns
	Interaction BC	13	2.18	1.61ns
	Residual	39	4.07	
16:0	A: Treatment	3	910.26	571.12***
	B: Time	13	73.16	10.59***
	C: Sample	1	1.18	2.21ns
	Interaction AB	39	165.98	8.01***
	Interaction AC	3	1.08	0.68ns
	Interaction BC	13	9.10	1.32ns
	Residual	39	20.72	
16:1*n*-7	A: Treatment	3	468.68	223.79***
	B: Time	13	90.40	9,96***
	C: Sample	1	0.46	0.66ns
	Interaction AB	39	176.14	6.47***
	Interaction AC	3	1.70	0.81ns
	Interaction BC	13	9.74	1.07ns
	Residual	39	27.22	
18:4*n*-3	A: Treatment	3	60.16	232.29***
	B: Time	13	8.02	7.14***
	C: Sample	1	0.00	0.00ns
	Interaction AB	39	17.97	5.34***
	Interaction AC	3	0.24	0.91ns
	Interaction BC	13	0.70	0.62ns
	Residual	39	3.37	
20:5*n*-3	A: Treatment	3	668.76	114.32***
	B: Time	13	217.47	8.58***
	C: Sample	1	0.33	0.17ns
	Interaction AB	39	257.12	3.38***
	Interaction AC	3	3.30	0.56ns
	Interaction BC	13	16.33	0.64ns
	Residual	39	76.05	
22:6*n*-3	A: Treatment	3	123.12	93.97***
	B: Time	13	31.54	5.56***
	C: Sample	1	0.18	0.41ns
	Interaction AB	39	41.76	2.45***
	Interaction AC	3	1.22	0.93ns
	Interaction BC	13	3.30	0.58ns
	Residual	39	17.03	

*$0.01 < P < 0.05$; **$0.001 < P < 0.05$; ***$P < 0.001$; ns = not significant.

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