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Mixotrophic Production of Marine Microalga *Phaeodactylum tricornutum* on Various Carbon Sources

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Abstract We investigated the potential use of various carbon sources (fructose, glucose, mannose, lactose, and glycerol) for culturing *Phaeodactylum tricornutum* UTEX-640 in mixotrophic and heterotrophic batch cultures. Concentrations of carbon substrates tested ranged from 0.005 M to 0.2 M. *P. tricornutum* did not grow heterotrophically on any of the C-sources used, but successive additions of organic carbon in mixotrophic growth mode substantially increased the biomass concentration and productivity relative to photoautotrophic controls. The maximum biomass productivities in mixotrophic cultures for glycerol, fructose, and glucose were 21.30 mg/l·h, 15.80 mg/l·h, and 10.20 mg/l·h, respectively. These values were respectively 10-, 8-, and 5-fold higher than those obtained in the corresponding photoautotrophic control cultures. Mannose and lactose did not significantly affect microalgal growth. The biomass lipids, eicosapentaenoic acid (EPA) and pigments contents were considerably enhanced with glycerol and fructose in relation to photoautotrophic controls. The EPA content was barely affected by the sugars, but were more than 2-fold higher in glycerol-fed cultures than in photoautotrophic controls.

Key words: Microalgae, mixotrophic growth, heterotrophic growth, sugars, *Phaeodactylum tricornutum*

The microalgae are being used as functional foods. They are a valuable source of polyunsaturated fatty acids [n3 and n6 (PUFAs n3, n6)], essential amino acids (leucine, isoleucine, valine, etc.) and pigments (lutein, β-carotene, etc.) [1]. In industrialized countries, the use of these supplements in ready-to-use dry milk products with soybean as major ingredient (baby soups, protein beverages, etc.) is increasing [2]. Some microalgal biomass has been proposed to enrich wheat flour to produce spaghetti and related products [3]. More recently, Guil *et al.* [4] reported functional properties of the biomass of three microalgal species, *Porphyridium cruentum*, *Phaeodactylum tricornutum*, and *Nannochloropsis* sp, and concluded that *P. tricornutum* biomasses have functional properties comparable to those of soybean flour and exhibit a pleasant seafood aroma. This species is rich in eicosapentaenoic fatty acid (EPA) [5–8], a PUFAs n3 that is very valuable as a nutritional supplement.

Microalgae are a potential source of eicosapentaenoic acid (EPA). Among them are *P. tricornutum*, *Porphyridium cruentum* [9], *Nannochloropsis* sp. [10], *Isochrysis galbana* [11], *Monodus subterraneus* [14], and *Skeletonema costatum* [15]. These algae are generally cultured photoautotrophically. Unfortunately, photoautotrophic growth in photobioreactors has limited productivity, because self-shading by microalgal cells in high-density cultures limits the light available for growth. Mixotrophic growth that combines photoautotrophic and heterotrophic metabolism is potentially useful for overcoming the limitations imposed by pure photoautotrophic growth. Enhancing EPA productivity requires mixotrophic and heterotrophic cultivation strategies, if feasible [16–19]. The effects of carbon sources on growth and biochemical composition have been reported for several species of microalgae [20, 16, 6, 7], and mixotrophic production of microalgal biomass, in several cases, has been demonstrated to be a viable alternative to the conventional photoautotrophic mass culture [21, 7, 19]. Mixotrophic growth requires relatively low light intensities and, consequently, can reduce energy costs [22, 19]. High productivity of mixotrophic culture is possibly due to the synergistic effect of light and the organic carbon [23, 24, 6].

An organic nutrient for potential use in commercial-scale mixotrophic cultures should be inexpensive, easy to sterilize, promote good growth, and favor the synthesis of

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the desired bioproducts. In general, sugars satisfy the above requirements. Sugars, mainly monosaccharides, have been the most used organic substrates found in the literature. The preliminary selection of the nutrients to assay has been based on the data available in bibliographies, and its facility to be assimilated because of its small molecular size and central biochemical location in the organism. Glucose has been studied at higher concentrations, and glycerol was also used to compare with new nutrients.

The present work was undertaken to examine the possibility of enhancing the productivity of EPA from *Phaeodactylum tricornutum* by supplementing a standard inorganic medium with various organic carbon sources (fructose, glucose, mannose, lactose, and glycerol), and the possibility of growth in heterotrophic culture by this microalga with these nutrients.

MATERIALS AND METHODS

The microalga *Phaeodactylum tricornutum* UTEX-640 was obtained from the collection of University of Texas, Austin. The inocula were grown in 100-ml conical flasks with 50 ml of culture in aseptic conditions on an orbital shaker without aeration. The culture medium of García Sánchez *et al.* [25] was used for maintenance.

Cultures were carried out in triplicate in 500-ml shake flasks containing 250 ml of culture medium. The medium was enriched seawater prepared as described elsewhere [25]. Inoculation was done using the above described inoculums in linear growth phase.

Sufficient quantity of inoculum was added to give an initial biomass concentration of 80 mg/l. Shake flasks were agitated at 150 rpm on an orbital shaker (Infors AG CH-4103 Bottmingen). Light was continuously supplied (Philips TLD 36W/54 fluorescent lamp) at an intensity of 190 μE $m^{-2}s^{-1}$ measured at the flasks' surface. The initial pH was set at 8.0. The temperature was 20±0.5°C.

The culture medium, except organic substrates, was sterilized in an autoclave at 126°C for 20 min. The organic nutrients (fructose, glucose, mannose, lactose, and glycerol) were separately sterilized by filtration through 0.2 μm membranes. Batch runs were carried out with concentrations, varying between 0.005 M and 0.2 M, of the various carbon sources. Photoautotrophic controls were included in each case. Fed-batch cultures were carried out with successive additions of C-sources. Each new addition took place, when a steady state biomass concentration had been reached. Heterotrophic cultures were carried out under the following conditions: batch cultures in the dark; batch cultures in the presence of a photosynthetic inhibitor (DCMU, 10 μM) and artificial illumination; and in the presence of DCMU in the dark. None of these conditions produced biomass growth. Consequently, *P. tricornutum UTEX-640* was concluded to be an obligate photoautotrophic species. Biomass concentration (C_b) was estimated by measuring absorbance at 625 nm [11] and periodic corroboration with dry-weight determinations. Freeze-dried biomass was used for fatty acid analysis by gas chromatography, as described by Rodriguez Ruiz *et al.* [8].

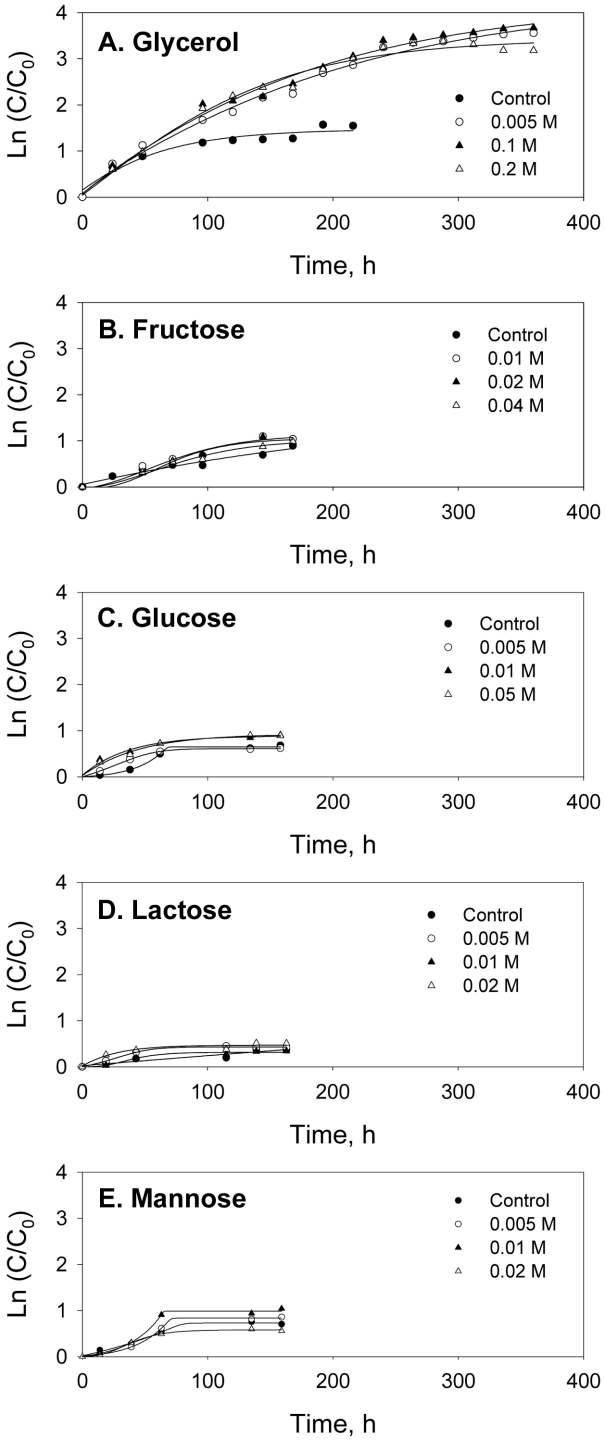

Fig. 1. Biomass growth profiles on various organic substrates.

Carotenoids were quantified by the method described by Whyte [26]. Chlorophylls were determined by Hansmann's spectrophotometric method [27] used with the equations of Parson and Strickland [28]. A sigmoidal equation was used to smooth the growth data, as previously suggested by Cerón García *et al.* [6, 7]. This procedure eliminates the influence of experimental error in calculations of growth kinetics parameters such as the instantaneous biomass productivity (P_b).

RESULTS AND DISCUSSION

Batch Cultures

Semilog plots of normalized biomass concentration for different substrates are shown in Fig. 1. The data fit the sigmoidal equation reported earlier [6, 7]. In order to more clearly reveal the effects of organic nutrient type and concentration levels on growth, a two-way variance analysis (multifactor ANOVA) was carried out, using the primary data for biomass concentration versus time. As expected in batch cultures, time factor had always a statistically significant effect on biomass concentration at the 95% confidence level (p-value <0.05), and its contribution to variance was always high.

For cultures supplemented with glycerol, growth stimulation was clearly observed at all concentrations tested compared with control (Fig. 1A). Both nutrient concentration and time influenced the biomass evolution (p-values <0.05). Relative to the lower concentrations tested, glycerol inhibited growth at concentrations >0.1 M. The other C-sources tested had barely any impact on growth relative to controls in batch culture (Fig. 1).

The p-values of the statistical analysis indicated that the biomass concentration in cultures fed with glucose and lactose was influenced only by time, but not by nutrient concentrations (Figs. 1C and 1D). Although there were no statistically significant differences, 0.05 M and 0.01 M glucose seemed to stimulate the growth, and 0.02 M lactose seemed to inhibit it in relation to photoautotrophic control (Fig. 1C). In the case of mannose (Fig 1E), the data were statistically different for four nutrient concentrations.

Table 1 shows the dimensionless maximum biomass productivity (P_d) for the various cultures. It is worth to point out the high maximum biomass productivities reached with glycerol. Fructose stimulated productivity at all concentrations tested; however, the other substrates either stimulated or suppressed productivity relative to controls depending on the concentration used.

Table 1. Dimensionless maximum biomass productivity (P_d) relative to photoautotrophic control.

Nutrient concentration	Glycerol	Fructose	Glucose	Lactose	Mannose
1*	4.78	1.95	0.63	0.96	1.29
2*	5.16	2.30	1.10	1.08	3.25
3*	4.63	1.35	0.88	0.88	0.83

*C-source concentration in increasing order for each source.

Fed-Batch Cultures

Fed-batch biomass concentration profiles for cultures fed with glycerol and fructose are shown in Fig. 2 as being representative. The most significant results for all the C-sources are shown in Table 2. The highest biomass concentration of 3.5 g/l was attained for the culture supplemented with 0.02 M fructose (Table 2). This was 6-fold higher than for photoautotrophic control. The 15.8 mg/l·h maximum biomass productivity was reached after the second addition of fructose and was 7-fold higher than the control culture (Table 2). These results were somewhat surprising, since some earlier work [29] showed that fructose did not stimulate the growth of *P. tricornutum*.

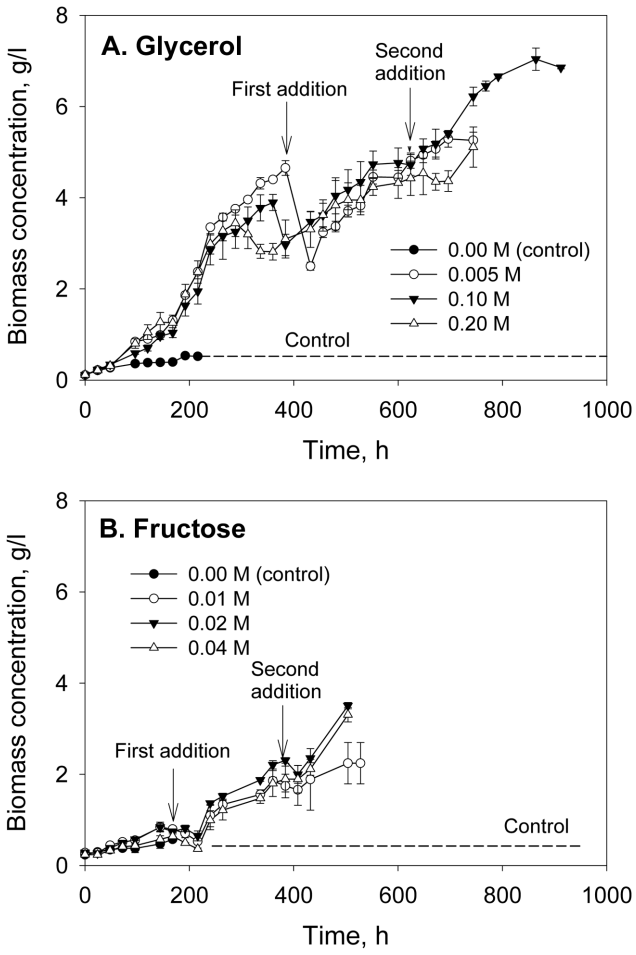

Fig. 2. Mixotrophic growth of *P. tricornutum* in fed-batch mode for different substrate concentrations: **A**. glycerol; **B**. fructose. Arrows indicate nutrient addition.

Table 2. Influence of nutrients on maximum biomass productivity (P_{max}) and concentration (C_{max}) in fed-batch cultures.

	Control	Glycerol (0.1 M)	Control	Fructose (0.02 M)	Control	Glucose (0.05 M)	Control	Mannose (0.01 M)	Control	Lactose (0.005 M)
P_{max}	2.20	21.30 (A)	2.00	15.80 (C)	2.20	10.20 (C)	2.40	7.80 (A)	2.40	2.60 (A)
C_{max}	0.53	7.04 (C)	0.57	3.50 (C)	0.88	2.20 (C)	0.80	1.05 (C)	0.78	0.77 (A)

(A) Initial culture phase; (B) after first nutrient addition; (C) after second nutrient addition.

Glycerol clearly supported the microalgal growth (Fig 2A), and this stimulatory effect was observed at all glycerol concentrations tested. Growth was enhanced with supplementation levels up to 0.2 M (Fig. 2A). The highest biomass concentration of 7.04 g/l was reached with 0.1 M glycerol concentration. These results agree with other similar observations about *P. tricornutum* [29, 30]. Furthermore, glycerol has been shown to be a better C-source than glucose for algal species such as *Nannochloropsis*, *Rhodomonas*, and *Cyclotella* [31]. At all glycerol concentrations tested, the highest biomass productivities were reached at the beginning of culture. The maximum productivity of 21.3 mg/l/h was obtained at 0.1 M glycerol. This corresponded to nearly 10-fold productivity of the control culture (Table 2). In heterotrophic growth on glycerol (0.1 M), Ukeles and Rose [30] reported a productivity of 1.9 mg/l/h or about a tenth of that obtained in mixotrophic culture.

In glucose-fed cultures, the concentration of glucose had a lesser effect on growth than did the concentration of fructose in fructose-fed cultures. A maximum biomass concentration of 2.2 g/l was attained in the culture supplemented with 0.05 M glucose (Table 2). Higher concentrations of glucose did not inhibit the growth.

Feeding with lactose and mannose had no significant effect on growth, comparised with the batch and control cultures: Other observations [29, 30] showed that lactose and mannose are poor substrates for *P. tricornutum* Bohlin. In fed-batch cultures, glycerol at 0.1 M provided the highest biomass concentration and productivity, followed by fructose (Table 2). The same relative effectiveness for these substrates was seen in batch cultures.

Pigments and Fatty Acids Profiles

The pigments and fatty acids profiles of the biomass were determined at the end of batch and fed-batch cultures, and the results are shown in Table 3 for fed-batch cultures grown on various carbon sources. Mixotrophic cultures on glycerol were richer in chlorophylls and carotenoids than the control cultures. The carotenoids content generally increased with increasing concentration of organic carbon source. The highest total pigment content (3.5% of biomass dry weight) was obtained with 0.1 M glycerol.

The literature on the influence of C-source on the pigment content is scarce. Most available reports suggest that mixotrophic cultures have a higher growth rate and chlorophyll content compared with photoautotrophic controls [32, 33]. Márquez Sasaki *et al.* [34] concluded that *Spirulina platensis* produced 1.5 times more biomass and twice as much photopigments in mixotrophic growth compared with photoautotrophic growth. For *Chlorella*, Shi and Chen [35] observed that pigments content in photoautotrophic cultures was merely 1% of those in mixotrophic cells.

The contents of individual fatty acids in the biomass did not follow a pattern similar to the concentration of C-source, but were influenced by the type of nutrient. Marked differences were observed in fatty acid profiles of biomass cultured on glycerol and fructose: the saponifiable lipids content for biomass grown on glycerol were almost twice as high as for biomass grown on glucose. The main fatty acids of *P. tricornutum* were myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n7), and EPA (20:5n3). Mixotrophic growth on glycerol clearly enhanced the content of these fatty acids in relation to the control (Table 3).

The influence of C-substrate on fatty acid profiles of microalgal biomass is known to depend on numerous factors, including light-dark cycling, nitrogen level and the species being grown [20]. As shown by Wood [31], the degree of unsaturation of the fatty acids is also influenced by the C-source used in photoheterotrophic growth. A high concentration of sugars appears to favor the synthesis of increasingly saturated fatty acids.

The microalga *P. tricornutum* is incapable of heterotrophic growth on glycerol, glucose, fructose, mannose, and lactose. Of the C-substrates tested, glycerol was the most effective in substantially enhancing the mixotrophic production of

Table 3. Fatty acids and pigment contents (% on biomass dry weight) in *P. tricornutum* grown in fed-batch mode with glycerol and fructose. The results are averaged values based on all nutrient concentrations tested.

Nutrient	Chlorophylls	Carotenoids	14:00	16:00	16:1*n*7	20:5*n*3
Control	2.02±0.16	0.15±0.00	0.47±0.03	0.88±0.07	1.32±0.87	1.60±0.01
Fructose	2.15±0.56	0.50±0.13	0.61±0.02	0.90±0.09	2.28±0.01	1.59±0.02
Glycerol	2.62±0.33	0.45±0.20	1.50±0.57	2.00 ±0.18	4.78±0.32	2.40±0.50

the biomass, relative to photoautotrophic controls. However, glycerol concentrations of higher than 0.2 M were inhibitory. In fed-batch cultures, relative to photoautotrophic controls, all C-sources concentration-dependently enhanced the maximum biomass concentration until inhibitory levels of substrates were reached. Glycerol at 0.1 M was the best substrate, increasing the final biomass level by ~7-fold relative to control cultures. Mixotrophic cultures had generally elevated levels of chlorophylls, carotenoids, and the major fatty acids, relative to controls. Compared with other substrates, glycerol had the most stimulatory effect on the production of these metabolites.

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