# Eicosapentaenoic acid production by *Phaeodactylum tricornutum* under different culture condition

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***Summary:*** *Phaeodactylum tricornutum* UTEX 640 strain of microalgae was screened under different culture conditions for their capacity to produce eicosapentaenoic acid (EPA) the most abundant polyunsaturated fatty acid (PUFA). In our experiments, the effect of sodium chlorid, nitrogen source, phosphate, initial pH, as well as the $CO_2$ content of the medium on production of the eicosapentaenoic acid (EPA) by P. tricornutum were investigated. The EPA content of biomass was enhanced by the low pH of the medium, with increased concentrations of $B_{12}$ vitamin and nitrate, and also with decreasing concentrations of sodium chlorid. The EPA is most likely associated with polar (membran) lipids and the role of EPA appears to be involved with membran permeability in microalge. The synthesis of phospholipids, enhances the EPA content of the cells, as expected. The maximum EPA yields were observed under optimum culture condition 43 – 48 mg/g of dry cell weight.

***Key words:*** *P. tricornutum*, EPA, polar lipids, vitamins $B_{12}$, initial pH

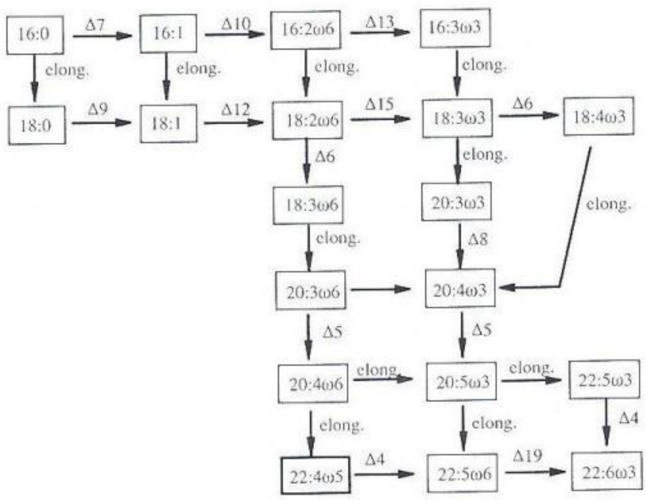
## Introduction

The importance of the ω-3 fatty acid, eicospentaenoic-acid (EPA) has been established in the prevention and treatment of various human heart and circulatory diseases. Cold watered marine fishes are rich in EPA. It is unclear whether the fishes were capable of synthesizing any of the polyunsaturated fatty acids (PUFA's) (*Norday* 1991, *Yangmanitchai & Ward,* 1989, *Kyle & Glaude,* 1992). In the food chain, microalgae (diatoms, dinoflagellates, unicellular red and green algae) are well known as primary producers of ω-3 fatty acids and potential sources of them (*Vonshak,* 1990, *Yangmanitchai & Ward,* 1991). The biomass of microalgae can be enriched with ω-3 fatty acids by low temperatures, and nitrogen starvation, as well as with controlled illumination. The incubation of the cells with sodium-chloride and silica compounds cause an increased the EPA production (*Velos* et. al. 1991, *Kyle and Glaude,* 1992, Pulz, 1995). The pathways of polyunsaturated fatty acid synthesis in microorganism are shown in *Table 1*.

The EPA is most likely associated with polar (membran) lipids in EPA producing microorganisms (*Kyle & Rutten,* 1989). Unsaturated fatty acids are generally considered as modulators of membrane fluidity and permeability. Thus we suppose that the role of EPA appears to be involved with membran permeability in microalgae.

The eicosapentaenoic acid synthesis in a strain of a *Phaeodactylum tricornutum* (UTEX 640, Algal collection of University of Texas) has been studied at the Institute for Drug Research. The various shaking flask cultures were

***Table 1*** ω-6 and ω-3 pathways of PUFA biosynthesis and the possible branching points for the interconversion of the pathways. Elongation steps (elong) and desaturation steps (Δn, where n indicates the position of the double bond) are indicated. (Kyle and Glaude 1992).




analysed by gas chromatography after performing the appropriate lipid isolation and trans-esterification methods (*Bligh & Dyer,* 1959, *Kyle & Rutten,* 1989). The EPA production increased by media of different kind. The concertation of nitrate, phosphate and vitamin $B_{12}$ are important factors in multiplication of algae. The sodium-chloride concentration is a limiting factor for the existance of seawater strains (*Borowitzka & Borowitzka* 1988, *Lee* et. al, 1989, *Al –Hasan* et al., 1990).

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## Material and method

**Organism**. P. *tricornutum* UTEX 640 was obtained from the Culture Collection of Algae at the Universitry of Texas at Austin. The culture was maintained by subculturing on slant agar containing the Mann and Myers medium and kept at 18 °C.

**Culture condition**. Shaking flask cultures were grown autotrophically at 20 °C (*P. tricornutum*) using the Mann and Meyers medium (Mann & Meyers, 1968) in 500 ml Erlenmeyer flasks with a working volume of 100 ml. The sterilized medium was inoculated with a 14-day-old agar slant culture. The culture flasks were incubated in an orbital shaker (Braun, Certomat S-II) at 150 rpm and 20 °C for 7 and 10 days. Light adjusted to 300 μE $m^{-2}$ $s^{-1}$ with continous illumination provided by cool-white fluorescent tube (Tungsram). Optimization experiments o fair addition were carried out in 8 liter air lift (Felföldi, 1964) photobioreactor with illumination provided by double cool-white light at the light intensity of 300 μE E $m^{-2}$ $s^{-1}$. Air, supplemented with 1.5% $CO_2$, was bubbled at the rate of 0.5 $m^3$/hour (approximately 1 v v m).

**Extraction and analysis.** The determination of total EPA (as methyl ester derivative) content was performed by a rapid microanalytical procedure using 5–50 mg of dried algal cells (*Kyle & Rutten*, 1989). An aliquot of cells was centrifugated and washed with isotonic solution before lyophilization. 100 μl of internal standard (methyl pentadecanoate 10 mg $ml^{-1}$) was added to each lyophilized algal sample (25 mg), as well as 2 ml of methanolic base reagent (Supelco Inc., Bellefonte). The tubes were then flushed with nitrogen, sealed and heated up to 70 °C for 15 min. for simultaneous extraction from the cell biomass and transesterification. After cooling 2.0 ml of water was added to the samples. The fatty acid methyl esters were extracted with 2 ml hexane. The hexane extracts were injected directly into gas chromatograph equipped with an SP 2330 packed column (flow rate of the 30 ml/min, column temperature 210 °C).

## Results

The effects of nitrate, phosphate, sodium chlorid and vitamin $B_{12}$ concentration on the EPA synthesis by *P. tricornutum* are presented in Table 2.

Highest algal growth and EPA production has been observed at nitrate concentrations of 0.5 and 0.25%, respectively, with latter value resulting in highest EPA production. In the cultures containing 0.25% of potassium-nitrate, an average of 37.5 mg $g^{-1}$ of cellular EPA content were measured.

The phosphate concentration, in the range of – 0.05–0.1% –, caused an inhibitory effect on EPA production. The cultures containing $K_2HPO_4$ more than 0.05% were unable to grow. The optimal EPA levels were observed at a phosphate level of 0.01% of the medium.

The EPA production of the cells was increased when the level of sodium chlorid concentration was decreased from 0.5% to 0.1 and 0%.

Studies were carried out on the influence of vitamin $B_{12}$ on EPA production. The $B_{12}$ supply at concentration of 10 mg/liter, resulted a 45% increase of the EPA production compared to the control.

The effect of initial pH in modified Mann & Myers medium on culture growth and EPA production were investigated. The results are presented in *Table 2*., and *Fig. 1 and 2*. An effective growth was observed in cultures having initial pH values between 6.0 and 7.0, and the EPA content of the cultures ranged from 42.5 to 48.0 mg $g^{-1}$ dry weight. Optimal EPA production per unit volume of cell culture occurred when the initial pH was 7.0.

The effect of carbon dioxid was investigated by supplementation of air with $CO_2$ of 1.5%. The results are presented in *Fig. 2*. Maximum biomass production per unit of culture was observed at initial pH of 6.5, when the air was not supplemented with $CO_2$. However the maximum EPA production was observed when the culture pH was decreased and the air was supplemented with 1.5% of $CO_2$. The EPA content of the cells cultivated in an airated cultures ranged from 35.5 to 40.0 mg. $g^{-1}$. This value was ranged from 40.0 to 46.0 mg $g^{-1}$ dry weight when the airation was combined with $CO_2$.

***Table 2*** Effect of sodium-chlorid on EPA production by *P. tricornutum*

| | EPA content mg/g (dry weight) | |
|---|---|---|
| | 7 days | 10 days |
| 0,5% NaCl, Controll | 26.0 ± 2.82 | 32 ± 1.41 |
| 0,10% NaCl | 29.5 ± 0.70 | 23.0 ± 0.84 |
| 0% NaCl | 36.5 ± 2.12 | * 41.7 ± 7.07 |

## Discussion

The nitrogen stress has an effect on the proportion of unsaturated fatty acids in marine microalgae (*Borowitzka & Kaixian*, 1993). In contrast, the proportion of polyunsaturated fatty acids in freshwater algae *Scenedesmus* and *Chlorella* incraesed at high nitrogen concentrations (*Piorreck* et al., 1984), which is consistent with our studies. It was noted that the lipid content of *P. tricornutum* incraesed at low nitrogen levels (*Lewin* et al., 1958, *Holdswortrh & Colbech*, 1976, *Thomas* et al., 1984 ). *Kyle* et. al (1989) found the nitrogen–rich cultures of the *P. tricornutum* had a high percentage of EPA in the nitrogen-deficient cultures the oil level was elevated, but at a depressed EPA level. EPA may associate preferentially with membrane lipids in this species, and when triglyceride production is stimulated by nitrogen deficiency, the EPA is diluted by the accumulation of triglyceride-associated fatty acids.

As regard the phosphate concentrations CHU (1943) found that the growth of the diatoms was decreased at about

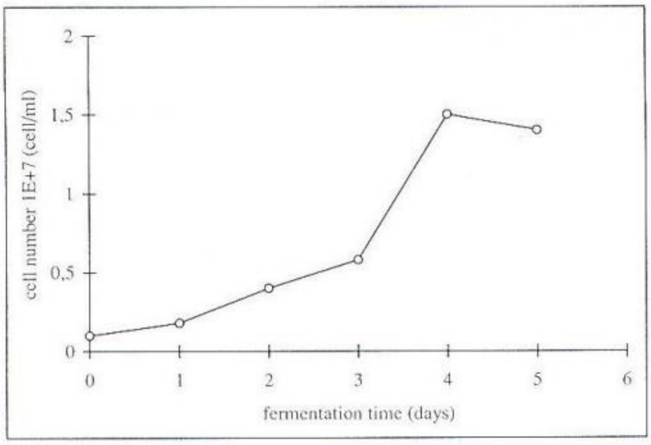


*Figure 1* The growth curve of *P. tricornutum* culture conditions modified Mann & Myers medium (initial pH = 7.0) in shake flasks

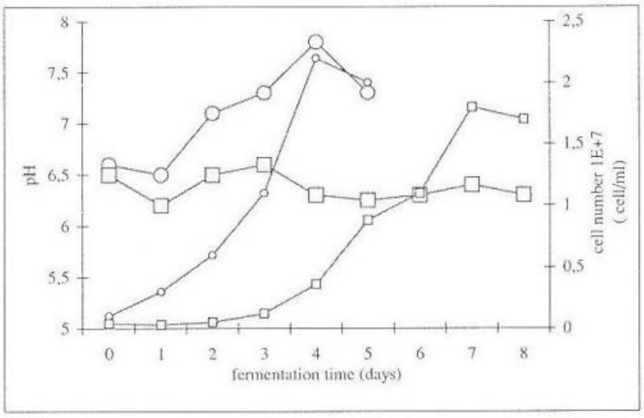


*Figure 2* *P. tricornutum* strain growth and pH curve in 8 litres volume (modified Mann & Myers medium). O-O: pH with 1 litre/minute air addition, o-o: cell number with 1 litre/minute air addition, □: pH with 1 litre/minute air addition with 1.5 % $CO_2$ and □: cell number with 1 litre/minute air addition with 1.5% $CO_2$

0.02% of phosphate, and was optimal at 0.001–0.01%.

Composition of fatty acid in *P. tricornutum* was affected by decreased NaCl concentrations significantly. However, the *Chlorella minutissima*, a marine alga, had maximum cell growth in media containing 0.2% NaCl and a higher percentage of total fatty acids (*Seto* et al., 1984). The decrease of NaCl concentration (from 0 to 0.1%), causes a higher ratio of EPA in the cells.

Specific vitamin requirements for vitamin $B_{12}$ are common among diatoms (*Guillard*, 1968), as it was observed with *P. tricornutum* UTEX 640.

The effect of initial pH was very interesting. The pH of the medium may alter the rate of phosphate uptake by a direct effect on the permeability of the cell membran (Epstein, 1956). The water permeability of the algal protoplasm reaches the maximum at about pH 7 (*Seemann*, 1950). Phosphorus plays an important role in photosynthesis and phospholipid synthesis, an influence of light on phosphate uptake was expected. *Ketchum* (1939) found that in the case

*Table 3* Effect of potassium-nitrate on EPA production by *P. tricornutum*

| | EPA content mg/g (dry weight) | |
|---|---|---|
| | 7 days | 10 days |
| 0.1% KNO3, Controll | 26.0 ± 2.82 | 32 ± 1.41 |
| 0,50% KNO3 | 27.5 ± 1.69 | 24.0 ± 11.31 |
| 0,25% KNO3 | 37.5 ± 0.98 | 37.0 ± 2.8 |

*Table 4* EPA content of *P. tricornutum* on different phosphate and nitrate concentration

| | EPA content mg/g (dry weight) | |
|---|---|---|
| | 7 days | 10 days |
| 0,01% $K_2HPO_4$ with 0.1% $KNO_3$, Controll | 26.0 ± 2.82 | 32 ± 1.41 |
| 0,05% $K_2HPO_4$ with 0,25% $KNO_3$ | 13.3 ± 1.27 | 24.5 ± 0.70 |
| 0,1% $K_2HPO_4$ with 0,25% $KNO_3$ | 25.3 ± 1.62 | no measure |

*Table 5* Effect of $B_{12}$-vitamin on EPA production by *P. tricornutum*

| | EPA content mg/g (dry weight) | |
|---|---|---|
| | 7 days | 10 days |
| 0 mg/l $B_{12}$-vitamin, Controll | 26.0 ± 2.82 | 32 ± 1.41 |
| 10 mg/l $B_{12}$-vitamin | 46.7 ± 4.66 | 24.0 ± 1.97 |
| 20 mg/l $B_{12}$-vitamin | 43.0 ± 1.83 | 35.0 ± 0.50 |

*Table 6* EPA content of P. tricornutum strain in different culture conditions

| | EPA content mg/g (dry weight) | |
|---|---|---|
| | 7 days | 10 days |
| initial pH = 8.0 with 0,5% NaCl, 0 mg/l $B_{12}$-vitamin 0.1%, $KNO_3$ with 0,01% $K_2HPO_4$, Controll | 26.0 ± 2.82 | 32 ± 1.41 |
| initial pH = 7.0 with 0% NaCl, 0,25% $KNO_3$ and 10 mg/l $B_{12}$-vitamin **(modified Mann & Myers)** | 43.0 ± 7.49 | 48.0 ± 0.56 |
| initial pH = 6.0 with 0% Na Cl, 0,25% $KNO_3$ and 10 mg/l $B_{12}$-vitamin | 40.3 ± 0.28 | 42.5 ± 1.69 |

of *Phaeodactylum tricornutum* the absorption of phosphate per cell in the light was dependent on its concentration in the medium. The nitrate concentration of the medium also influences the rate of phosphate uptake (*Ketchum*, 1939). At the same time the nitrate uptake is attended by a higher pH. The permeability of cell membran increases with the concentration of EPA. Unsaturated fatty acids in polar (membran) lipids (phospholipids, galactolipids) increase if we lowered the initial pH and moderately increased the concentration of nitrate. Aside from triglycerids, the major algal lipids are phosholipids and glycolipids, and these oils may comprise 10–47% in total lipids of bacillariophyceae

(*Borowitzka*, 1988).

An increased EPA content of *P. tricornutum* cells was observe when the $CO_2$concentration of the air supply rose as it was noted in autotrophically grown cells of *Chlorella fusca* (Dickson et al., 1969, *Chen & Jones*, 1991).

The capacity of *P. tricornutum* to produce maximum EPA percentage of dry weight with relatively low optimum pH (pH=6.5), 0% NaCl, 0.25% $KNO_3$ concentration and with 10 mg / l $B_{12}$ vitamin in the culture medium and when the air supply was substituted by 1.5% carbon-dioxide.

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