Aquacultural Engineering

Volume 6, Issue 2, 1987, Pages 141-150

# Approach to biomass production of the marine microalga *Tetraselmis suecica* (kylin) butch using common garden fertilizer and soil extract as cheap nutrient supply in batch cultures

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Fabregas J, Toribio L, Abalde J, Cabezas B, Herrero C. Approach to biomass production of the marine microalga tetraselmis suecica (kylin) butch using common garden fertilizer and soil extract as cheap nutrient supply in batch cultures. Aquac Eng 1987;6(2):141-50.

ISSN: 01448609

DOI: 10.1016/0144-8609(87)90011-2 http://dx.doi.org/10.1016/0144-8609(87)90011-2

**Abstract**

We report the possibility of an economic cultivation of the marine microalga Tetraselmis suecica, using different mixtures of a common garden fertilizer, soil extract, micronutrients and vitamins. Maximum cellular densities were obtained with a nitrogen concentration of 14 μg/ml and maximum protein concentrations were obtained with 28 μg N/ml, in all cases. The mixture of fertilizer + soil extract + micronutrients gave the best results for obtaining maximum cellular densities and protein concentrations per ml of culture. An economic evaluation showed a maximum of 2770 g of microalgal protein/dollar and $210 \times 10^{12}$ microalgal cells/dollar with the mixture of the fertilizer and soil extract.

## Introduction

A great interest in marine micro algal biomass production has been provoked by the artificial cultivation of shellfish in a hatchery environment (Ukeles, 1980). In all types of molluscan aquaculture (research investigations, pilot plants or commercial hatcheries) it is necessary to have a large microalgal biomass available as a source of food for normal development and growth of the cultured species. In fact, production of this biomass is frequently cited as critical to the success of the project and as being a serious impediment to the future development of shellfish aquaculture (De Pauw et al., 1983). The mass culture of certain species of micro algae is considered the major bottleneck in the nursery culturing of molluscs (Persoone and Claus, 1980).

Mass production of micro algae has long been successfully carried out either by using domestic or bio-industrial wastes or inorganic compounds (Shelef and Soeder, 1980; Fabregas et aI., 1985a). The culture medium should be nontoxic to larvae, so that the use in larval rearing of waste-supported micro algal biomass is often questionable. Therefore, clean nutrient sources are often used and these media are generally laboratory preparations (Stein, 1973; Soeder, 1980; Fabregas et aI., 1984a, 1985 a). Agricultural or common garden fertilizers containing nitrogen and phosphorus have been used as an alternative source of nutrients (Persoone and Claus, 1980; Ukeles, 1980; Gonzalez-Rodriguez and Maestrini, 1984). Such fertilizers are mainly composed of nitrogenous and phosphorous substances and some, if any, micronutrients such as iron, potassium or sodium (Gonzalez-Rodriguez and Maestrini, 1984), but, in general, they do not contain all the micronutrients and vitamins necessary for microalgal growth (Stein, 1973).

The marine microalga Tetraselmis suecica is at present widely used in aquaculture (WaIne, 1974; Bayne, 1976; Laing and Utting, 1980) and it has been suggested as a potential source of Single Cell Protein (SCP) (Fabregas and Herrero, 1985). This marine microalga is able to tolerate a wide range of nutrient concentration-salinity conditions (Fabregas et aI., 19 84a). The mass production of this microalga with optimum inorganic nutrient concentration has also been achieved (Fabregas et al., 1985 a, b). In this paper, we report the possibility of a more economic cultivation of this micro alga, using a common garden fertilizer, soil extract, micronutrients or vitamins, instead of inorganic nutrients of a defined medium.

## Materials and methods

The marine microalga used was Tetraselmis suecica, isolated from Ria de Arosa waters (NW Spain). It was cultured in seawater filtered through a 0-45 µm Millipore filter, autoclaved at 120°C for 20 min and enriched with the different nutrients. We used four types of nutrients: common garden fertilizer, Ferticros 7-12-7 (F); common garden fertilizer + soil extract (F + SE); common garden fertilizer + soil extract + micronutrient (F + SE + M); common garden fertilizer + micronutrient + vitamins (F + M + V). As a blank, we used a culture with the inorganic medium commonly used in our laboratory (Fabregas and Herrero, 1985; Fabregas et aI., 1984a, 1985a). All the experiments were carried out in triplicate.

The composition of Ferticros 7-12-7 is: nitrogen, 7%; phosphoric anhydride, 11%; potassium oxide, 7%. This fertilizer constituted the nitrogen and phosphorus source for the microalgal cultures. It was dissoh.ed in distilled water, filtered through a paper filter and autoclaved at 120ºC for 15 min. We used six different concentrations: 0'05, 0'1, 0'2, 0’4, 0’8 and 1’6 mg/ml, giving 3’5,7'0, 14'0,28’0, 56’0 and 112'0 µg of nitrogen per ml of culture, respectively.

Soil extract was prepared by mixing 1 volume of soil with 2 volumes of distilled water, filtering through a paper filter and autoclaving at 110ºC for 20 min (Stein, 1973). This extract was added to all the cultures at a concentration of 50 ml/litre.

The micronutrients used were: $ZnCl_2$, 0’136 mg/litre; $MnCl_2$ .$4H_20$, 0'198 mg/litre; $MoO_4Na_2$ . $2H_2O$, 0’242 mg/litre; $CoCI_3$, 0’0165 mg/litre; $CuSO_4$ .$5H_2O$, 0’0249 mg/litre; ferric citrate, 6'7 mg/litre; EDTA, 9’38 mg/litre.

The vitamins used were: thiamine, 35 µg/litre; biotin, 5 µg/litre; $B_{12}$ 3 µg/litre.

The concentrations of soil extract, micronutrients and vitamins were constant in all the experiments. Cultures were carried out in Kimax screw-capped test tubes (15 x 2·5 cm) with 25 ml of medium. All cultures were maintained in a controlled environmental incubator (New Brunswick) at 15°C in 3900-lux light from fluorescent lamps (Philips TL 20W/55). A 12 h:12 h light:dark regime was maintained in order to obtain synchronous cultures. An inoculum of 1 x $10^4$ logarithmic-phase cells/ml was used.

To obtain the optical density, the transmittance T of the cultures was determined by using a Coleman II 6/20 spectrophotometer reading at 530 nm.

Cellular density of the cultures was determined by counting culture aliquots in a Thoma chamber. Protein was measured in the stationary phase by the dye-binding method (Bradford, 1976).

Chlorophylls were extracted in acetone:methanol (2: 1) at 4°C for 48 h. The extracts were filtered through a Fluoropore Millipore filter for clarification (Fabregas et aI., 1984b), and absorbances of the pigment extract at specific wavelengths were recorded. The concentration of chlorophyll a was determined by the formula of Parsons and Strickland (1965).

After the sixth day of Culturing, $CO_2$ was added to the cultures in order to maintain the pH within the optimum interval (7-4-8'5), at a rate between 02 and 2 ml per tube of culture.

The cultures were not continuously aerated, although $CO_2$ was addeddaily after the sixth day at a rate of 0·2-2 ml per culture. This $CO_2$ supply prevented the carbon source from becoming limiting and at the same time maintained the pH within the optimum range for T. suecica growth; because of this, the values obtained in the control for cell, protein and chlorophyll a concentrations are greater than those obtained in batch conditions without CO2 supply (Fabregas et aI., 1984a).

We plotted optical density, expressed as (100 - T), against time and against nitrogen concentration for each type of nutrient, obtaining threedimensional figures (Fig. 1). We also plotted cellular density against time and against nitrogen concentration for each type of nutrient, again obtaining three-dimensional figures (Fig. 2).

Maximum cellular densities were obtained with a nitrogen concentration of 14 µg/ml of culture medium, with all the different mixtures assayed. Optimum growth can be observed both by optical density measurements (Fig. 1) and by cell counting (Fig. 2). The growth (cells/ ml) was significantly greater with 14 µg/ml of nitrogen than with the other nitrogen concentrations in all cases. However, in cultures with different concentrations of the medium used as controls in these experiments, optimal growth conditions were obtained with nitrogen concentrations higher than 14 µg/rnl, not only for T suecica (Fabregas et al., 1984a) but also for other marine microalgae such as Isochrysis galbana (Fabregas et aI., 1985b).

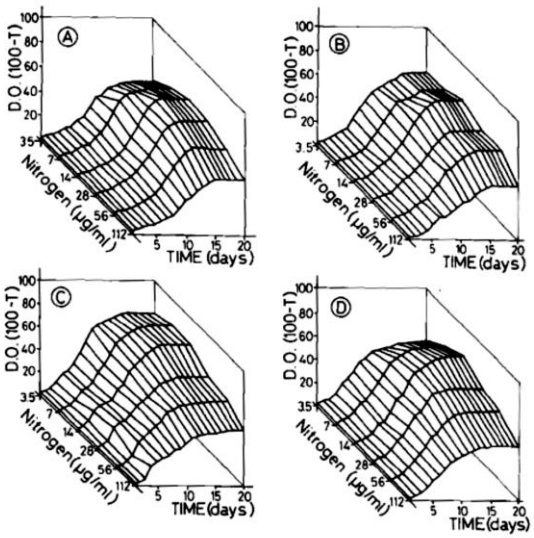


**Fig. 1.** Growth of cultures of *T. suecica* with different mixtures and concentrations of nutrients: A, fertilizer; B, fertilizer + soil extract; C, fertilizer + soil extract + micronutrients; D, fertilizer + micronutrients + vitamins.

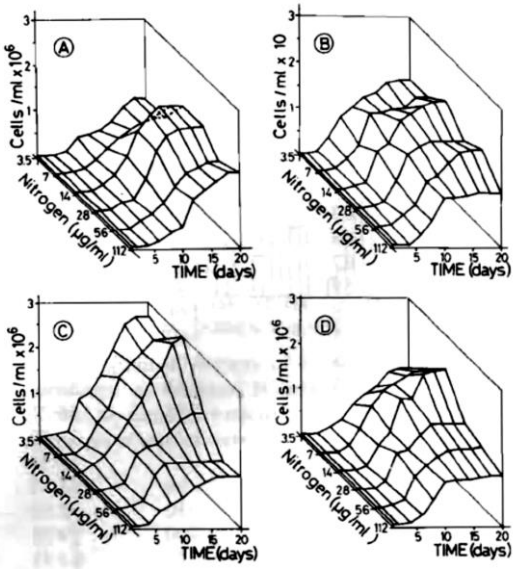


**Fig. 2.** Cellular densities of cultures of *T. suecica* with different mixtures and concentrations of nutrients: A, fertilizer; B, fertilizer + soil extract; C, fertilizer + soil extract + micronutrients; D, fertilizer + micronutrients + vitamins.

Maximum cellular density obtained at the stationary phase at this nitrogen concentration was 3 x $10^6$ cells/ml for the culture with F + SE + M (Table 1). However, this growth was 54% less than that of the control with the defined medium, which reached a final biomass at the stationary phase of 5·5 x $10^6$ cells/ml (Table 1).

For the same nitrogen concentration, the pattern of maximum cellular density was as follows:

F + SE + M > F + M + V> F + SE > F

Protein concentration is not proportional to cellular density. Maximum protein concentrations were obtained with 28 µg/ml of nitrogen in all experiments (Table 1; Fig. 3), with 64'7 µg of protein/rnl in the culture with F + SE + M. Protein concentration in the control was 147 µg/ml, 228% greater than that of each of the other nutrients.

The chlorophyll a content presented more variation depending on the different concentrations and types of nutrients. In general, maximum values were obtained with nitrogen concentrations of 14, 28 and 56 µg/ml (Table 1; Fig. 4). The maximum value of chlorophyll a was obtained in the cultures with F + SE + M, with 4-4 µg/ml. These cultures were the ones with better growth, too. However, this value is 45% less than that obtained with the control.

Excepting the control, the cultures with F + SE + M showed better growth than the other cultures, and the major protein and chlorophyll a content.

**TABLE 1**

Growth and Protein and Chlorophyll *a* Concentrations of Cultures of *T. suecica* with Different Mixtures and Concentrations of Nutrients

| *Nutrient mixture*[a] | *Nitrogen (µg/ml)* | *Optical density, 100 − T* | *$10^6$ × cells/ml* | *Protein (µg/ml)* | *Chlorophyll a (µg/ml)* |
|---|---|---|---|---|---|
| F | 3·5 | 52 | 1·34 | 9·58 | 0·09 |
| | 7 | 63 | 1·26 | 17·77 | 0·44 |
| | 14 | 68 | 1·90 | 37·82 | 2·03 |
| | 28 | 67 | 1·85 | 55·73 | 1·52 |
| | 56 | 59 | 1·42 | 37·65 | 2·43 |
| | 112 | 48 | 1·60 | 26·99 | 1·20 |
| F+SE | 3·5 | 65 | 1·67 | 22·04 | 1·25 |
| | 7 | 65 | 1·69 | 36·30 | 2·48 |
| | 14 | 73 | 1·99 | 38·49 | 2·64 |
| | 28 | 68 | 1·81 | 56·31 | 3·20 |
| | 56 | 63 | 1·74 | 38·60 | 3·27 |
| | 112 | 48 | 1·16 | 39·34 | 2·05 |
| F+SE+M | 3·5 | 71 | 2·66 | 31·73 | 1·11 |
| | 7 | 75 | 2·50 | 33·50 | 1·48 |
| | 14 | 75 | 3·00 | 59·25 | 3·30 |
| | 28 | 62 | 1·72 | 64·71 | 4·40 |
| | 56 | 57 | 1·10 | 29·40 | 3·95 |
| | 112 | 48 | 1·17 | 39·91 | 2·58 |
| F+M+V | 3·5 | 56 | 1·45 | 10·17 | 0·56 |
| | 7 | 69 | 1·82 | 15·48 | 0·84 |
| | 14 | 78 | 2·25 | 32·77 | 2·11 |
| | 28 | 66 | 1·42 | 61·68 | 2·79 |
| | 56 | 57 | 1·26 | 44·15 | 2·53 |
| | 112 | 49 | 1·11 | 28·49 | 2·35 |
| Control | 28 | 96 | 5·58 | 147·46 | 9·88 |

[a] Abbreviations: F, fertilizer; SE, soil extract; M, micronutrients; V, vitamins.

The fertilizer Ferticros does not contain all the elements necessary for maximum microalgal growth, although it can be used as a source of nitrogen and phosphorus.

The use of soil extract for marine microalgal growth in seawater is very common (Prakash and Rashid, 1968; Prakash et al., 1973). This soil extract can supply vitamins, micronutrients and, perhaps, certain other growth factors, acting besides as a chelating agent (Hoeffner and Manahan, 1980) because humic substances chelate ions of Fe, Ca, Al, Cu and Co, so that these ions are prevented from precipitating essential microalgal nutrients such as phosphate (Prakash et al., 1973); on the other hand, the involvement of humic substances in cellular metabolic processes is also a possibility (Prakash and Rashid, 1968).

We established the efficiency as the ratio between the nitrogen added to the culture medium and the protein nitrogen produced per culture. Maximum efficiency was obtained in the cultures with the mixture of F + SE (Table 2).

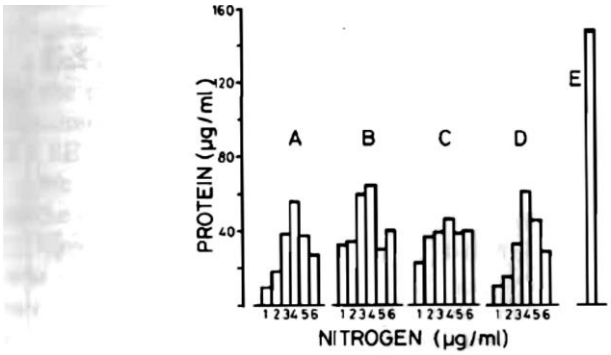


**Fig. 3.** Protein concentrations in cultures of *T. suecica* with different mixtures and concentrations of nutrients: A, fertilizer; B, fertilizer + soil extract; C, fertilizer + soil extract + micronutrients; D, fertilizer + micronutrients + vitamins; E, control. Nitrogen contents (µg) per ml of culture were: 1, 3·5; 2, 7; 3, 14; 4, 28; 5, 56; 6, 112.

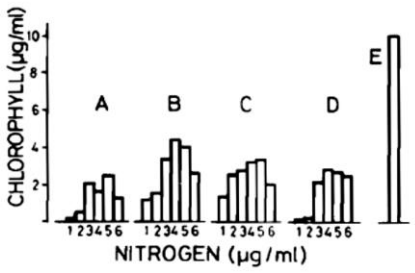


**Fig. 4.** Chlorophyll *a* concentrations in cultures of *T. suecica* with different mixtures and concentrations of nutrients: A, fertilizer; B, fertilizer + soil extract; C, fertilizer + soil extract + micronutrients; D, fertilizer + micronutrients + vitamins; E, control. Nitrogen contents (µg) per ml of culture were: 1, 3·5; 2, 7; 3, 14; 4, 28; 5, 56; 6, 112.

**TABLE 2**
Economic Evaluation of *T. suecica* Culture Media with Different Mixtures and Concentrations of Nutrients

| *Nutrient mixture*[a] | *Nitrogen (µg/ml)* | *$10^3$ × cost ($/100 litre)* | *Protein/100 litre (g)* | *Protein/$* (g)* | *Efficiency (%)* | *$10^9$ × cells/ 100 litre* | *$10^{12}$ × cells/$* |
|---|---|---|---|---|---|---|---|
| F | 3·5 | 0·73 | 1·0 | 1 360 | 45 | 134 | 182 |
| | 7 | 1·47 | 1·8 | 1 224 | 41 | 126 | 85 |
| | 14 | 2·94 | 3·8 | 1 292 | 43 | 190 | 64 |
| | 28 | 5·88 | 5·5 | 935 | 31 | 185 | 31·5 |
| | 56 | 11·76 | 3·7 | 314 | 10 | 142 | 12 |
| | 112 | 23·52 | 2·7 | 102 | 3·8 | 160 | 6·8 |
| F+SE | 3·5 | 0·79 | 2·2 | 2 770 | 99 | 167 | 210 |
| | 7 | 1·53 | 3·6 | 2 373 | 82 | 169 | 110 |
| | 14 | 3·00 | 3·8 | 1 283 | 44 | 199 | 66 |
| | 28 | 6·47 | 5·6 | 947 | 32 | 181 | 30·5 |
| | 56 | 11·82 | 3·8 | 326 | 11 | 174 | 14·7 |
| | 112 | 23·58 | 3·9 | 166 | 5·6 | 116 | 5 |
| F+SE+M | 3·5 | 10·91 | 2·2 | 198 | 99 | 266 | 24 |
| | 7 | 11·65 | 3·3 | 287 | 76 | 250 | 21 |
| | 14 | 13·12 | 5·9 | 451 | 67 | 300 | 22 |
| | 28 | 16·06 | 6·4 | 402 | 37 | 172 | 10 |
| | 56 | 21·94 | 2·9 | 134 | 4·8 | 110 | 5 |
| | 112 | 27·82 | 3·9 | 143 | 5·7 | 117 | 4·2 |
| F+M+V | 3·5 | 46·41 | 1·0 | 21 | 45 | 145 | 3 |
| | 7 | 47·17 | 1·5 | 33 | 35 | 182 | 4 |
| | 14 | 48·65 | 3·3 | 67 | 37 | 222 | 4 |
| | 28 | 51·59 | 6·1 | 119 | 35 | 142 | 3 |
| | 56 | 57·47 | 4·4 | 77 | 12 | 126 | 2 |
| | 112 | 68·82 | 2·8 | 41 | 4 | 111 | 1·6 |
| Control | 28 | 2 450·00 | 14·7 | 7 | 84 | 558 | 0·26 |

[a] Abbreviations as in Table 1.

We carried out an economic evaluation, but only referred to the cost of the different chemical compounds of the different mixtures.

Maximum concentration of microalgal protein, expressed as g/dollar, was obtained with F + SE, with 2770 g of protein/dollar, at a nitrogen concentration of 3'5 µg/ml. Maximum cellular density of 210 x $10^{12}$ cells/dollar was obtained with the same mixture of F + SE and at the same nitrogen concentration (Table 2). Comparing these data with the control, we obtained better economic results with F + SE than with the control, the differences being up to 404-fold in terms of protein/dollar and 810-fold in terms of cells/dollar.

**Acknowledgements**

We thank Professor Dr B. Regueiro Varela for his interesting comments and Cros SA for their cooperation. This work was supported by a grant from Direccion General de Ordenacion Pes que ra, Ministerio de Agricultura, Pesca y Alimentacion, Madrid, Spain.

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