High yield mixotrophic cultures of the marine microalga Tetraselmis suecica (Kylin) Butcher (Prasinophyceae)

Ángeles Cid, Julio Abalde, Concepción Herrero

Laboratorio de Microbiología, Dpto. Biología Celular y Molecular, Facultad de Ciencias, Universidad de La Coruña, Campus da Zapateira s/n, 15071, La Coruña, Spain

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Abstract

The effects of three organic compounds were tested on one of the most used marine micro-algae in the aquaculture of molluscs and crustaceans, *Tetraselmis suecica*. Studies were made in axenic conditions with yeast extract, peptone and glucose added to the culture medium, each alone, in combinations of two or all together. Medium without any organic compound was used for the control. Cultures containing yeast extract grew best, reaching maximum cell density of 3.79×10^6 and 3.84×10^6 cells ml^{-1}.

The organic carbon source affected the biochemical composition. The components most affected were the carbohydrates, with values between 6.5 pg $cell^{-1}$ in control cultures and 48.5 pg $cell^{-1}$ in glucose cultures. Protein content ranged between 27.5 pg $cell^{-1}$ in control cultures and 88.6 pg $cell^{-1}$in yeast + glucose + peptone cultures. The lipid content changed little. Maximum protein yields were reached in cultures with yeast + glucose and with yeast - glucose - peptone, with values of 24.6 and 28.2 mg 1^{-1} d^{-1}, respectively. These values are 22 and 25 times those in control cultures. A maximum carbohydrate yield of 7.9 mg carbohydrate per litre per day was obtained in yeast + glucose + peptone cultures, 27 times that in the control cultures. The maximum lipid yield was obtained with yeast + glucose + peptone and yeast + glucose. Maximum energy values were 308 kcal 1^- in yeast extract - glucose - peptone cultures and 279 kcal 1^{-1} in yeast extract + glucose cultures. Gross energy values in control cultures

were 24.5 kcal 1^{-1}, but peptone cultures presented the minimum energy value, 22 kcal 1^{-1}. The yeast extract: glucose ratio in the culture medium was optimized. A ratio 2:1 produced the best yields in cells, protein, carbohydrate and gross energy.

Introduction

Interest in the mass culture of microalgae is worldwide because their cultivation is a part of the technology of growing marine molluscs, crustaceans and fishes. In many types of aquaculture systems it is necessary to have a large microalgal biomass available as source of food for normal development and growth of the cultured species (De Pauw *el al.,* 1983), Besides their use in aquaculture, microalgae also have commercial value as a source of pigments, vitamins, poly saccharides, sugars, pharmaceuticals and other biologically active compounds (Cohen, 1986; Borowitzka, 1988a; De la Noue & De Pauw, 1988; Murakami *et al.,* 1988; Richmond, 1990). Furthermore, there are other potential uses of microalgae: waste-water treatment (Oswald, 1988) fertilizers (Cohen, 1986; De la Noue & De Pauw, 1988), energy source (Borowitzka, 1988b), biocatalysts (Trevan & Mak, 1988).

Microalgae are phototrophs, but some can also grow heterotrophically (Flynn & Syrett, 1986). The carbon source is often the limiting factor in microalgal culture systems and it is therefore generally necessary to bubble CO_2-enriched air throughout the cultures. However, some freshwater microalgal species can be cultured in mixotrophic conditions and high yields at light intensities lower than those needed in autotrophic cultures have been obtained (Venkataraman *et al.,* 1980; Ogawa & Aiba, 1981 ; Richmond, 1986, Lee *el al.,* 1989). Energetic costs of aquaculture systems can be reduced using mixotrophic cultures of certain marine microalgae. Carbon can be supplied as organic solutes such as sugars, amino acids or alcohols (Ukeles & Rose, 1976; Richmond, 1986). Glucose is the most abundant sugar and the most utilized (Droop, 1974; Venkataraman *et al.,* 1980); yeast extract is a good nutrient in cultures of freshwater species of *Chlorella* (Lee *el al.,* 1989) and peptone has been utilized in mixotrophic cultures of *Nilzschia angularis* var. *affinis* (Ogawa & Aiba, 1981). Taking into account these reports, the effects of these three organic compounds were tested in axenic cultures of one of the most commonly used marine microalgae in the aquaculture of molluscs and crustaceans, *Telraselmis suecica* (Walne, 1974; Bayne, 1976; Laing& Utting, 1980; Wikfors, 1986).

Materials and methods

Tetraselmis suecica (Kylin) Butcher (Chlorophyta, Prasinophyceae) was isolated from the Ria de Arousa waters (NW of Spain). It was cultured in seawater filtered through a 0.45 I'm Millipore filter, autoclave at 120 °C for 60 min and enriched with $NaNO_3$ 2 mM; NaH_2PO_4 , 100 µM; $ZnCl_2$, 1 µM; $MnCl_2$; 1 µM; Na_2MoO_4 , 1 µM; $COCl_2$, 0.1 µM; $CuSO_4$, 0.1 µM; ferric citrate, 20 µM; thiamine; 35µg 1^{-1}; biotin, 5 µg 1^{-1}; B_{12}, 3 µg 1^{-1}; EDTA,26.4 mM; Tris-HCI, 15 mM; pH 7.6. Salinity of the seawater was 35‰ and the initial pH of the cultures was 7.6. Mixotrophic cultures were carried out with the addition of yeast extract, peptone and glucose to the culture medium, either singly, or in combinations of two or three compounds. Concentrations used were: peptone 1.25 g l^{-1} glucose 2.5 g l^{-1} and yeast extract 5 g l^{-1}. Cultures without any organic compound in the medium were used as control.

The cultures were grown axenically in triplicate in screw-capped Kimax tubes containing 40 ml of mediulll. All cultures were maintained at 18 ± 1ºC, and 17 µmol photon m^{-2} s^{-1} with a dark: light regime of 12: 12 h. An inoculum of 2 x 10^4 logarithmic phase cells ml^{-1}' was used. Growth was measured in a Bausch and Lomb Spectronic-20 colorimeter by recording the absorbance at 530 nm. In the stationary phase, cell density was determined by counting aliquots in a Coulter Counter model DN.

The biochemical composition was determined in the stationary phase. Protein and carbohydrates were measured in the crude extracts obtained after collecting the cells by centrifugation, resuspending them in distilled water and breaking them in an ultrasonic disintegrator. After sonication the extracts were centrifuged again, the pellets were discarded and protein and carbohydrates were measured in the supernatants. Protein was measured by the dye-binding method and carbohydrates by the phenol-suolphuric acid method as described by Kochert (1978a, b). Lipids were measured by a quantitative charring method (Marsh & Weinstein, 1966). The gross energy of cells under the different conditions was calculated in the stationary phase using the formula of the National Reserach Council (1977):

$$GE\ (kcal\ kg^{-1}) = 5.72\ (\%\ protein) + 9.50\ (\%\ lipid) + 4.03\ (\%\ carbohydrate)$$

Stationary phases, corresponding to maximum biomass production, were compared by an overall multivariate one-way analysis of variance (ANOVA) ($P \leq 0.05$).

Results

Tetraselmis suecica grew in all media tested. Microalgal growth is characterized by a sigmoid or logistic function and the growth curves and their mathematical functions are shown in Fig. I. The form of the logistic growth function was (Schanz & Zahler, 1981)

$$y(t) \sim K/1 + B \exp (- rt),$$

where $y(t)$ represents the absorbance at time I and K its ultimate limiting value ('carrying capacity'). B is a biologically unimportant constant, and its value was calcul ated by the following equation:

$(K-y_0)/y_0$. The parameter r is related with growth rate. All the parameters of the equation were fitted by non-linear regression using Marquardt's algorithm.

Maximum cell densities reached in the stationary phase are shown in Table I. Optimal combinations for obtaining better growth and maximum cell densities in the stationary phase were all those including yeast extract (Fig. I): i.e. alone, with glucose or peptone, or with both. Maximum cell densities were 3.84 x 10^6 cells ml $^{-1}$ in cultures with yeast extract + glucose + peptone and 3.79 x 10^6 cells ml $^{-1}$ in cultures with yeast extract + glucose; there was no significant difference between them ($P \leq 0.05$). The yield in peptone cultures and in glucose cultures was less than that in the control cultures. However, the addition of peptone + glucose improved the yield (Fig. I; Table 1). The organic carbon source also affected the cellular composition (Table I). The cellular component most affected was the carbohydtates, with contents of between 6.5 pg cell^{-1} in control cultures and 48.5 pg cell^{-1} in glucose cultures. Protein content ranged between 27.5 pg cell^{-1} in control cultures and 88.6 pg cell^{-1} in yeast + glucose + peptone cultures (Table 1). The lipid content did not change significantly along the cultures with the different organic carbon sources, ranging from 20.6 to 28.6 pg cell^{-1}

Differences in cell densities and in cell contents resulted in large differences in the yields of protein, lipids and carbohydrates (Fig. 2). Yields of protein (mg 1- 1 d - I) in mixotrophic cultures were significantly higher than those in control cultures ($P \leq 0.05$) (Fig. 2). Maximum protein yields were reached with yeast + glucose and with yeast + glucose + peptone, with values of 24.6 and 28.2 mg l^{-1} d $^{-1}$, respectively; there was no significant difference between these. These values are 22 and 25 times those in control cultures (1.1 mg l^{-1} d $^{-1}$). Maximum carbohydrate yields were obtained in yeast + glucose + peptone cultures, with 7.9 mg l^{-1} d $^{-1}$, 27 times those reached in control cultures. Regarding lipids, we observed three groups among the cultures: (I) control,

peptone, glucose, and peptone + glucose cultures, with lipids yields between 0.9 and 1.8 mg l^{-1} d^{-1}; (2) yeast and yeast + peptone cultures, with 3.2 and 3.7 mg l^{-1} d^{-1}, respectively; and (3) yeast + glucose + peptone and yeast + glucose cultures, with 6.6 and 7.2 mg l^{-1} d^{-1}. There are no significant differences within a group, but there are significant differences among the groups ($P \leq 0.05$). The first group includes the cultures with lower growth, and the third group includes those with higher growth.

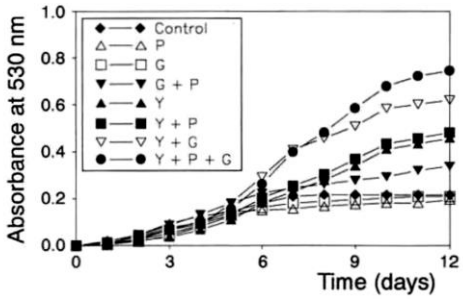

Fig. 1. Growth curves of *Tetraselmis suecica* in mixotrophic cultures with different organic compounds and autotrophic cultures (Control). Growth yield was measured by absorbance at 530 nm. (P = peptone; G = glucose; Y = yeast extract).

$y(t)_O = 0.219/[1 + 23.333 \exp(-0.787t)]$ $(r = 0.996)$
$y(t)_P = 0.176/[1 + 13.667 \exp(-0.847t)]$ $(r = 0.990)$
$y(t)_G = 0.202/[1 + 63.333 \exp(-0.914t)]$ $(r = 0.999)$
$y(t)_{G+P} = 0.307/[1 + 22.615 \exp(-0.679t)]$ $(r = 0.984)$
$y(t)_Y = 0.476/[1 + 58.5 \exp(-0.583t)]$ $(r = 0.998)$
$y(t)_{Y+P} = 0.523/[1 + 36.357 \exp(-0.507t)]$ $(r = 0.999)$
$y(t)_{Y+G} = 0.603/[1 + 119.6 \exp(-0.795t)]$ $(r = 0.999)$
$y(t)_{Y+G+P} = 0.786/[1 + 86.33 \exp(-0.645t)]$ $(r = 0.999)$

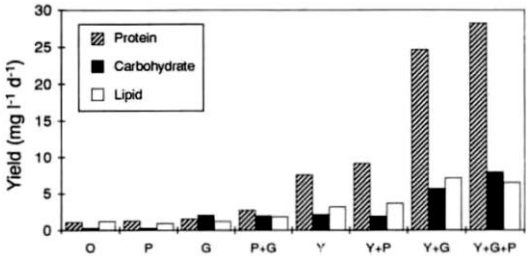

Fig. 2. Yields in different biochemical components (mg l^{-1} d^{-1}) in mixotrophic cultures of *Tetraselmis suecica* with different organic substrates (O = control; P = peptone; G = glucose; Y = yeast extract).

Energy measurements of organic compounds were carried out knowing the composition in protein, carbohydrate and lipid. If we take into account that these microorganisms are used in feeding molluscs, larvae, etc, suspended in liquid medium at a known density, we can calculate the energy of microalgae per litre of culture. Maximum energy values were 308 kcal l^{-1} in, yeast extract + glucose + peptone cultures and 279 kcal l^{-1} in yeast extract + glucose cultures. Gross energy values found in control cultures were 24.5 kcal l^{-1} but peptone cultures presented the minimum energy value, 22.3 kcal l^{-1}, (Fig. 3).

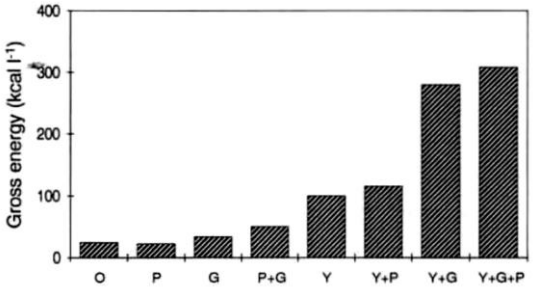

Fig. 3. Gross energy (kcal l^{-1}) of *Tetraselmis suecica* in mixotrophic cultures with different organic substrates (O = control; P = peptone; G = glucose; Y = yeast extract).

Table 1. Cell density and biochemical composition per cell in the stationary phase in mixotrophic cultures of *Tetraselmis suecica* grown with different organic compounds (O = control; P = peptone; G = glucose; Y = yeast extract).

Culture	Cell density (10^6 cell ml^{-1})	Proteins (pg $cell^{-1}$)	Carbohydrates (pg $cell^{-1}$)	Lipids (pg $cell^{-1}$)	RNA (pg $cell^{-1}$)
O	0.56	27.45	6.50	26.68	11.96
P	0.43	39.23	8.59	27.18	16.42
G	0.52	39.73	48.48	28.60	14.17
P + G	0.75	46.20	31.79	28.20	19.62
Y	1.76	52.79	14.87	20.55	12.90
Y + P	1.59	70.48	14.65	28.12	12.70
Y + G	3.79	78.42	18.02	22.90	13.86
Y + G + P	3.84	88.61	25.38	20.64	20.44

The highest results were obtained in cultures with yeast + glucose + peptone and those with yeast + glucose, without significant differences between them. Therefore, taking the medium with yeast extract + glucose as reference, the improvement in microalgal yields by optimizing the ratio between yeast extract and glucose was studied. For this optimization, and after a previous screening, a minimum yeast extract concentration of 1.25 g l^{-1}, was used, and four glucose concentrations between 0.08 and 0.625 g l^{-1} resulting in yeast extract: glucose ratios (w/w) of 16: I, 8: I, 4: I and 2: 1. Cultures without glucose were used as control. Changes in the yeast extract: glucose ratio in the medium affected the growth of the marine microalgae *Tetraselmis suecica* (Fig. 4). Significant differences in the cell densities reached at stationary phase were found *(P*≤0.05) (Table 2). Cell density reached in the stationary phase increased as the yeast: glucose ratio decreased. At constant yeast concentration, a correlation between the glucose concentration in the medium and number of cells reached can be established and this is represented by the equation $y = 5.52x + 0.98$ *(r*= 0.99), where *x* is the glucose concentration in the medium and *y* the cell density in 10^6 cells ml^{-1}. The optimum ratio for obtaining a maximum cell density was 2: 1, with 4.34 x 106 cells ml-'; this value is significantly higher *(P*≤0.05) than values obtained in the other cultures, with higher yeast: glucose ratios (Table 2). The minimum cell density of 0.93 x 10^6 cells ml^{-1}, was obtained in control cultures without glucose. Cultures with a yeast: glucose ratio 16: 1, i.e. a glucose concentration of 0.08 g l^{-1} had a cell density of 1.35 x 10^6 cells ml^{-1} significantly higher than control. Therefore, the addition of this minimum quantity of sugar produced cell densities 50% higher than densities reached in the cultures without glucose (Table 2).

The cell composition was less affected by differences in yeast: glucose ratio (Fig. 5). The cell constituents more affected were proteins. A maximum protein content of 56.2 pg $cell^{-1}$ was reached in cultures with a yeast: glucose ratio 4: 1. Yields in protein increased with the decrease of yeast: glucose ratio, and were highest at the ratio 2: 1 with 19.2 mg l^{-1} d^{-1}. This was significantly higher than values obtained in the remaining

cultures (Fig. 6). Yields of carbohydrates also increased when the yeast extract: glucose ratio decreased. The maximum value was 5.3 mg l^{-1} d^{-1} in cultures with 2: I yeast: glucose ratio. This yield is about 4 times higher than that in the control cultures. Lipid yields varied along the same pattern as carbohydrates, with a maximum value of 9.3 mg l^{-1} d^{-1}, (Fig. 6). Energy increased proportionally to the glucose concentration, i.e. gross energy increased when yeast: glucose ratio decreased. Values were between 243 kcal l^{-1} in yeast: glucose ratio 2:1 , and 50.7 in cultures without glucose (Table 2).

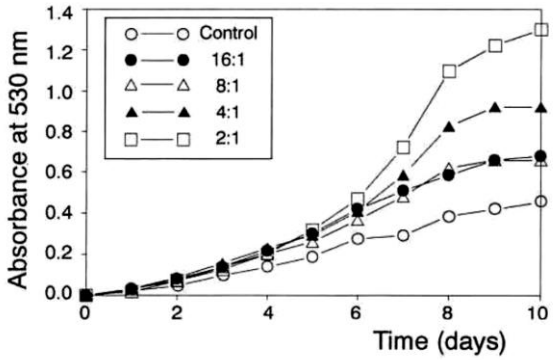

Fig. 4. Growth curves of *Tetraselmis suecica* in mixotrophic cultures with different yeast extract: glucose ratios. Growth yield was measured by absorbance of 530 nm.

$$y(t)_O = 0.463/[1 + 26.235 \exp(-0.590)] \quad (r = 0.997)$$
$$y(t)_{16:1} = 0.716/[1 + 30.109 \exp(-0.640)] \quad (r = 0.999)$$
$$y(t)_{8:1} = 0.768/[1 + 29.72 \exp(-0.580)] \quad (r = 0.997)$$
$$y(t)_{4:1} = 1.308/[1 + 42.6 \exp(-0.548)] \quad (r = 0.996)$$
$$y(t)_{2:1} = 1.485/[1 + 116.96 \exp(-0.706)] \quad (r = 0.997)$$

Table 2. Influence of yeast extract: glucose ratios on cellular density in the stationary phase and gross energy in mixotrophic cultures of *Tetraselmis suecica*.

Culture	Cellular density (10^6 cell ml^{-1})	Gross energy (kcal l^{-1})
Control	0.93	50.68
16:1	1.35	75.57
8:1	1.79	117.36
4:1	2.97	221.86
2:1	4.34	243.76

Discussion

Organic carbon nutrition has been studied extensively in a number of freshwater microalgal species, especially *Chlorella* and *Scenedesmus,* which are of interest with respect to biomass production (Ukeles & Rose, 1976; Richmond, 1986). However this kind of nutrition has less studied in marine species. The type or organic solutes and the light intensity had marked effects on the cultured micro algae. Mixotrophic culture does not stimulate growth at high light intensities (Ukeles & Rose, 1976). It was for this that experiments were carried out at 17 µmol photon m^{-2} s^{-1} light intensity, lower than the saturation level reported for *T. suecica* (Fabregas *Cl a/.,* 1985). In the same way, glucose added to C. *pyrenoidosa* and C. *vulgaris* cultures had a growth stimulating effect only at light intensities less than saturation (Ogawa & Aiba, 1981).

Experiments to establish the influence of an organic source of carbon must be carried out in axenic conditions (Neilson *et al.,* 1973). On the other hand, bacteria-micro algae

interactions may provide better yield than that reached in axenic photoautotrophic cultures (Atlas & Bartha, 1987). However, the maximum cell densities obtained in these axenic mixotrophic cultures at µmol photon m^{-2} s^{-1} light intensity are three times those obtained in non-axenic autotrophic cultures of *T. suecica* at 215 µmol photon m^{-2} s^{-1} light intensity (Fabregas *cl al.,* 1984). This fact means the use of a light intensity twelve times lower, with the consequent reduction in the energetic costs for the production of the micro algal biomass.

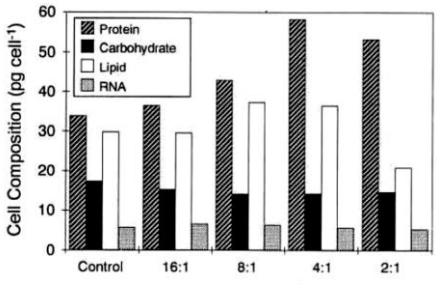

Fig. 5. Biochemical composition (pg $cell^{-1}$) in the stationary phase of mixotrophic cultures of *Tetraselmis suecica* using different yeast extract:glucose ratios.

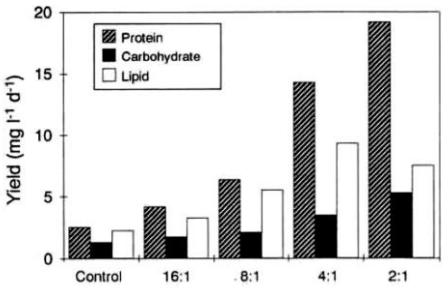

Fig. 6. Yields of cell components (mg l^{-1} d^{-1}) in mixotrophic cultures of *Tetraselmis suecica* using different yeast extract:glucose ratios.

Among the different compounds assayed the best results were obtained in all the combinations which included yeast extract. Yeast extract has already been described as a good nutrient in cultures of freshwater species *Chlorella* (Lee et al*.,* 1989), but its use has not been reported for marine species. Growth in peptone cultures and in glucose cultures was less than that in the control cultures. However, the addition of peptone + glucose improved the growth. This synergistic effect was also been observed for other organic compounds in freshwater micro algal mixotrophic cultures (Ogawa & Aiba, 1980; Lee et al., 1989).

The best results were obtained in cultures with yeast extract + glucose and yeast extract + glucose + peptone with maximum cell densities of 3.79 and 3.84 x 10^6 cells ml^{-1}. These results are three times higher than maximum cell density reached in non-axenic autotrophic cultures of this micro alga, with optimal conditions of salinity Ifnd nutrient concentration and a light intensity of215 µmol photon m^{-2} s^{-1} (Fabregas et al*.,* 1984). Biomass yields increased when the yeast extract: glucose ratio was optimized. Biochemical cell composition was less affected by changes in the yields extract: glucose ratio in the medium than the yeast in biomass. The only affected cell fraction was the protein, whereas carbohydrates, lipids and RNA did not change. Similar results have been reported previously (Becker & Venkataranlan, 1982), showing a decrease in the protein content of microalgal cells in response to a decrease in the carbon

concentration in the culture medium. However, the protein fraction was more affected by the carbon compound used than by the yeast: glucose ratio in the medium. Growth and biochemical variability of the marine micro alga *Tetraselmis suecica* grown with different sources and concentrations of organic carbon compounds can change its nutritive value, with a potential effect on its value as single-cell protein (Fabregas & Herrero, 1985), or as feed mariculture (Wikfors *cl al. ,* 1984; Fabregas & Herrero, 1986).

These results all show that mixotrophic cultures results in a higher biomass yield compared with other processes, possible due to the energetic effect of light and organic substrate. Such mixotrophic cultures with high yields and minimum energetic cost can be used to supply the microalgal biomass needed in certain aquaculture systems.

Acknowledgements

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