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Evaluation Of The Cellular Growth Of The Diatom *Skeletonema Costatum* (Greville) Cleve 1873 In Alternative Mediums

Evaluación del crecimiento celular del diatoma S*keletonema costatum* (Greville) Cleve 1873 en medios alternativos

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ABSTRACT

The improvement of industrial processes is still today of scientific and commercial interest, with great projection due to, among other factors, the contribution of natural marine products extracted from microalgae. In this sense, there have been numerous culture studies of autotrophic green microalgae and their adaptation to more economic forms (mixotrophic and/or heterotrophic), however, few of them are with diatoms despite them being an interesting group given that they are the most common in the natural environment and their role in the development of aquaculture is key.
Therefore, the objective of this project is the study of the adaptability of *Skeletonema costatum* (autotrophic diatom) to more economic culture mediums such as the mixotrophic and heterotrophic medium. Specifically, algae growth was compared in autotrophic conditions (control) with different mixotrophic cultures, varying the amount of organic matter dissolved and the autotrophic culture with different heterotrophic cultures in which the composition of the medium was modified, adding glucose as a carbon source and nitrogen groups such as neopeptone and yeast extract. The results obtained show that the mixotrophic culture that allows for a similar biomass to the autotrophic culture is the one which contains the highest concentration of organic matter. On the other hand, for the heterotrophic culture, although the highest biomasses are obtained by applying an organic substrate composed of glucose, the levels observed did not reach those in the autotrophic condition. These results, in the absence of further optimization of the heterotrophic culture conditions, are quite promising and demonstrate the ability of *S. costatum* to adapt to other culture media.

KEYWORDS: *Microalgae, Autotrophic Culture, Mixotrophic Culture, Heterotrophic Culture.*

RESUMEN

La mejora de los procesos industriales sigue siendo a día de hoy un tema de interés científico y comercial, con una gran proyección debido entre otros factores a la contribución de productos naturales marinos extraídos de microalgas. En este sentido, se han realizado numerosos estudios de cultivo de microalgas verdes autotróficas y su adaptación a otras formas de cultivo más económicas, sin embargo, son pocos los estudios con diatomeas, pese a ser un grupo interesante debido a que es el más abundante en el medio natural y resulta clave su papel en el desarrollo de la acuicultura.
Por ello, el objetivo de este trabajo ha sido precisamente investigar la adaptabilidad de Skeletonema costatum (diatomea autótrofa) a formas de cultivo alternativas que sean más económicas (mixotróficas y heterotróficas). En concreto, se ha comparado el crecimiento del alga en condiciones autotróficas (control) con diferentes cultivos mixotróficos, variando la concentración de materia orgánica disuelta, y el cultivo autotrófico con

diferentes cultivos heterotróficos en los que se modificó la composición del medio, adicionando glucosa como fuente de carbono y grupos nitrogenados como la neopeptona y el extracto de levadura.
Los resultados obtenidos mostraron que el cultivo mixotrófico que permite alcanzar una biomasa similar al cultivo autotrófico es el que mayor concentración de materia orgánica incorpora. En cambio, para el cultivo heterotrófico, si bien las mayores biomasas se obtienen al aplicar un sustrato orgánico compuesto de glucosa, no se lograron alcanzar las observadas en condiciones autotróficas. Estos resultados, a falta de una optimización ulterior de las condiciones de cultivo heterotróficas, son bastante prometedores y ponen de manifiesto la capacidad de adaptación de S. costatum a otras formas de cultivo.

PALABRAS CLAVE: *microalga, cultivo autotrófico, cultivo mixotrófico, cultivo heterotrófico.*

INTRODUCTION

Microalgae, especially those of marine origin, constitute a big and heterogeneous group that is almost unexploited. They represent a unique opportunity to discover new metabolites and produce other known ones at a lower cost (1). But, as many other species in nature, they have not been identified and/or physiologically characterized. Their potential awaits the exploitation in the biotechnological manufacture of biomolecules with high added value or deliberately enriched biomass (2).

Currently, the cultivation of microalgae under autotrophic conditions entails a high cost because of the high energy investment involved applying light in periods of approximately 12 hours in addition as well to the costs associated with the input of nutrients. Because of this, it is crucial to study the adaptability of microalgae to more economic culture media in order to reduce costs without reducing nutritional quality. In addition, this variant offers opportunities to modify, control, and thereby increase the formation of specific compounds (3).

The few commercialized processes in which microalgae are grown under heterotrophic and mixotrophic conditions are focused on the manufacture of polyunsaturated fatty acids (PUFAs). These biotechnological processes represent a sustainable alternative to the extraction of PUFAs from fish oil (3). Being the most coveted, Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), α-linolenic acid (ALA) and docosapentaenoic acid (DPA) (4)especially n-3 fatty acids such as eicosapentaenoic acid (EPA; 20:5 n-3. Examples of commercially valuable extracellular products obtained from microalgae are L-ascorbic acid and polysaccharides (5)and balanced growth took place except for significant diel variation in chemical composition. Inorganic C and N were primarily assimilated during the photophase, and the elemental cell quotas increased accordingly. The level of storage polysaccharide, \u03b2-1,3-glucan, oscillated between 17% (end of scotophase.

One of the most important aspects to consider in the production of microalgae is the selection of appropriate (economically and chemically) medium and growth conditions. These will vary according to the different metabolisms of the species (6). For those cultures that require organic substrates to develop growth, such as the mixotrophic and heterotrophic cultures, glucose or acetate is an adequate source of energy and carbon (7). In addition, there is the possibility of low cost cultivation with molasses or carob pulp syrup, or even waste streams from the sugar or milk processing industries which have been successfully used as alternatives (8). Although microalgae grow with various carbonaceous compounds, glucose is the preferred carbon source due to its ease of handling, accessibility and safety. Acetate and ethanol are possible alternatives, but because of their respective corrosive effects or their high flammability, they are only used when an exceptional increase in productivity is desired (9). The choice of different carbon substrates is very important because they lead to different biomass yields and can also affect the formation of the target product.

The addition of nitrogenous groups such as yeast, neopeptone, nitrate, ammonia and urea are used as a nitrogen source because of their potential to improve the growth or the formation of by-products (3). Yeast extract, a complex component with a high carbon content, is not defined at the level of a single element, but is frequently used as a source of nitrogen, amino acids, vitamins and trace elements (10). On the other hand, the growth data suggests that the nitrogen source preference may vary between species.

Therefore, the objective is to study if it is possible to adapt the culture of a purely autotrophic diatom Skeletonema costatum, to mixotrophic and heterotrophic culture conditions in order to obtain an equally efficient but more economical culture, as a preliminary step to its cultivation to obtain high-value compounds for application in aquaculture.

MATERIALS AND METHODS

For the development of this project, the diatom *Skeletonema costatum* (SK3) was used, isolated in the Primary Production Laboratory of the Universidad Católica del Norte, Chile.

Collection, isolation and maintenance of the strain

The harvest of the strain, for its posterior isolation, was carried out in the Bahía de la Herradura, Guayacán (latitude 29 ° 58 '50 "S longitude 71 ° 23' 00" W), located in Coquimbo (Figure 1).

Fig. 1 Bahía Herradura, sample zone where the diatom *Skeletonema costatum* was collected. (Source: Google Earth)

For the collection of the sample, a 20 µm mesh light Juday-Bogorov type phytoplankton trawl was used. The material collected was transferred directly to the laboratory to isolate the target species.

The strain isolation method was performed by micro pipetting on a Nikon (Anti-Mold) microscope, model ALP-HAPHOT-2 YS2. Afterwards, the selected cells were deposited on sterile culture plates of 2 mL capacity per cuvette, with 1 mL of F/2 inorganic culture medium (Guillard and Ryther, 1962). Later, for maintenance, once an axenic culture is ensured, they were inoculated in flasks of 250 mL level to 150 mL with F/2 culture medium (Guillard and Ryther, 1962), and kept under controlled laboratory conditions. Finally, the obtained cultures were used as inoculum for subsequent experiments.

Acclimatization of the strain for posterior organic cultures

The strain was previously acclimatized for 2 weeks. The culture medium used for the acclimatization of S. costatum consisted of natural seawater (34 UPS) previously sterilized with UV and filtered through 0.45 µm membrane filters, enriched with Guillard F/8 solution and 1.06 x 10-4 M Na2SiO3 · 9H2O (silicate). The solution was distributed in 250 mL flasks, with 100 mL of the solution. The flasks were then placed in an All-American Electric 14.5 Quart 1050 Watts/8.75 amps autoclave where they were subjected to 121° C temperature and 1 atm pressure for 15 min, in order to sterilize the culture media. Finally, they were allowed to cool for the subsequent cellular inoculation. For this purpose, 50 mL of the flasks kept in the laboratory were used for inoculation. The quality of the inoculation is very important, since it will condition the future culture. It has to be made from cell cultures that are in their exponential phase.

Mixotrophic Culture (Organic)

The mixotrophic culture medium was prepared using a solution of dissolved organic matter from fish meal, at a concentration of 166.66 g·L-1. This was provided by the company BioMar (Chile), whose biochemical composition according to a proximal analysis, contains 53.89% crude protein; 18.16% total lipids; 19.57% free nitrogen; 8.37% ash.

The culture media consisted of natural sea water with 34 practical salinity units (PSU), sterilized with UV, microfiltered to 0.45 µm, sodium silicate in a concentration of 1.06 x 10-4 M, with different concentrations of MOD: T1 (1.3 mL); T2 (2.6 mL); And T3 (3.2 mL).

They were kept in the Primary Production Laboratory at a temperature of 20° C with a variation of ±2° C and with a light cycle: light:darkness of 12:12h and with an irradiance of 17.35 µmol·m-2s-1, which was determined using a Li-CoR® model cuantometer.

Heterotrophic Culture (Organic)

For the cultivation under heterotrophic conditions glucose was added as a carbon source, and yeast and neopeptone as a contribution of nitrogen groups. Three different treatments were used; TG (glucose 0.5 g·L-1), TG + L (glucose 0.5 g·L-1+ yeast extract 0.05 g·L-1) and TG + N (glucose 0.5 g·L-1+ neopeptone 0.5 g·L-1).

Due to the associated problem of contamination in the heterotrophic culture, antibiotic was added. A solution of Florfenicol (Sigma-aldrich) in 15 µg·mL-1 concentration was prepared by dissolving the antibiotic in ethanol:water (50:50).

The culture media consisted of natural sea water with 34 practical salinity units (PSU), sterilized with UV, microfiltered to 0.45 µm, the addition of 1.06 x 10-4M sodium silicate and Florfenicol (15 g·mL-1).

All were kept in the Primary Production Laboratory incubator at 140 r.p.m., using a Green SSeriker II shaking at a temperature of 14° C and total darkness.

***S. costatum* Cell Count**

The cultures of diatom S. costatum under different growth conditions (autotrophy, mixotrophy and heterotrophy) were maintained for 14 days. During this time period, the daily cell count was performed with a hematocytometer to determine the density (cells·mL-1) in order to characterize the growth curve.

First, the culture medium was homogenized by shaking. The sample was then extracted using a previously autoclaved glass Pasteur pipette. Afterwards, one drop of the sample was transferred to a 0.1 mm deep hematocytometer or Neubahuer® chamber. The camera was then placed on the Nikon (Anti-Mold) microscope, model ALPHA-PHOT-2 YS2, and the count was performed and the daily cell density was determined using the following formula:

Density = N° Total Cells· 10.000 = cells·mL-1 (1)

RESULTS

Effect of organic matter on the growth of *S. costatum* in mixotrophic conditions

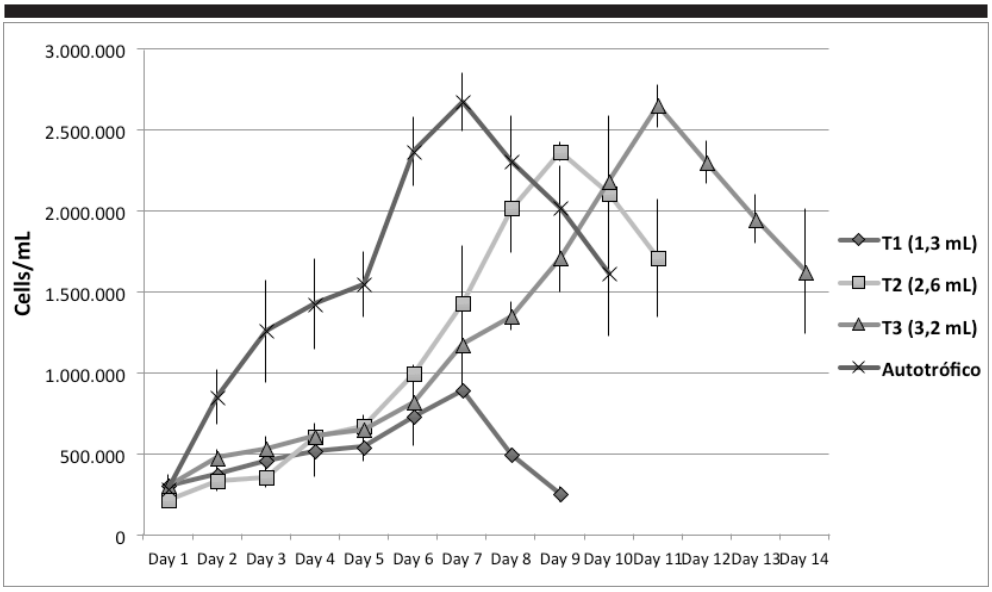

Fig. 2 Growth curves of *Skeletonema costatum* (cells·mL-1) in mixotrophic and autotrophic conditions.

The mixotrophic culture reaches a higher cell concentration the higher the concentration of the dissolved organic matter (figure 2).

In T3 treatment, the concentration reached is similar to that obtained in autotrophic conditions with a value higher than 2,500 cells·mL-1. However, the autotrophic culture reached this concentration in a shorter time. This difference could be due to the fact that the strain used for the culture should have been better adapted or because an increase of organic matter in the culture medium is required. Nevertheless, as it reached the same growth than the autotrophic culture, we tend to think that the mixotrophic culture can be a more economical alternative than the autotrophic culture.

Effect of the composition of the organic substrate in the growth of *S. costatum* grown in heterotrophic conditions

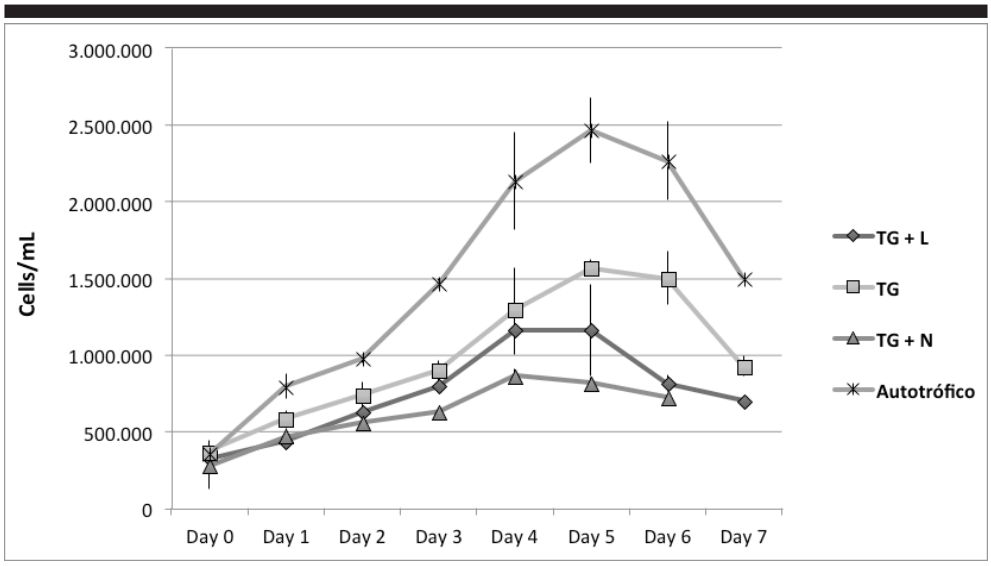

Fig. 3 Growth curves of *Skeletonema costatum* (cells·mL-1) in autotrophic and heterotrophic conditions.

In the heterophic culture growth, the treatment with glucose (TG) gave the best results, obtaining a maximum value of 1.566.667 cells·mL-1 (Figure 3). But failed to reach the maximum values obtained in the autotrophic medium: 2.466.666 cells·mL-1. This may be due to the simplicity of the glucose molecule, as it allows for rapid metabolization and assimilation of carbon. Unlike the inclusion in the medium of nitrogenous groups such as neopeptons (TG + N) or even more complex groups, such as the case of yeast derivatives (TG + L).

Preliminary results suggest that the mixotrophic culture may be a better alternative due to energy being obtained from light and nutrients. Accordingly, organic matter could be obtained from other sources such as wastewater, waste streams from industries and waste from animals. This type of culture should continue to be optimized to achieve a yield as competitive as that obtained under autotrophic conditions.

DISCUSSION

There is a large number of projects that show the viability of microalgae culture in culture media alternative to the "natural media". Generally, these studies were applied to autotrophic microalgae and their adaptability to mixotrophic and heterotrophic culture conditions.

For this, a preliminary study of the organic substrates as possible sources of carbon to be supplied in the mixotrophic and heterotrophic medium is usually carried out. This is because the variability of the organic substrates to be chosen provides different yields of biomass (as has been seen as well in this study) and directly affect the formation of the target product (3).

Below is a summary table (Table 1) which shows the values of maximum growth rate obtained in different tests. Our data is incorporated in bold letters.

The results suggest that there is a proportional relationship between the addition of nitrogen groups to the organic substrate and the growth rate.

Studied Species	Culture	μmax	Sources of carbon and nitrogen groups	Products	References
Skeletonema Costatum	M	0.299*	Fish meal	Not analyzed	
Skeletonema Costatum	H	0.292*	Glucose	Not analyzed	
Skeletonema Costatum	H	0.321*	Glucose and Neopeptone	Not analyzed	
Skeletonema Costatum	H	0.318*	Glucose and Yeast extract	Not analyzed	
Chlorella protothecoides	M	0.09	Glucose, acetate glycerol	Lipids, biodiesel	O'Grady and Morgan (11)
Chlorella Pyrenoidosa	M	0.201	Glucose, acetate, lactate	Ascorbic acid	Running *et al.* (12)
Chlorella Regularis	M	< 0.24	Glucose, acetate, ethanol	Biomass	Sansawa and Endo (13)
Haematococcus pulvialis	M	0.009	Acetate, glucose	Astanthaxin Canthaxanthin	Hata *et al.* (14)L-1
Crypthecodinium cohnii	H	0.089	Glucose, acetate Acetate, glucose,	DHA	Jiang and Chen (15)
Nitzschia alba	H	0.106	Glutamate	Biomass, EPA	Lewin and Lewin (16)
Nitzschia laevis	H	0.017	Acetate, glucose	EPA	Wen *et al.* (17)

Table 1.Data of alternative cultures studied in different species.
(M: mixotrophic cultivation; H: heterotrophic cultivation; *: average growth rate).

CONCLUSIONS

From the results obtained in the present study we can conclude that:

- The cultivation of *Skeletonema costatum* under mixotrophic conditions gives better results in terms of biomass, the greater the content of dissolved organic matter, allowing to reach a biomass similar to that offered by the culture under autotrophic conditions.
- Also, from the study under heterotrophic conditions, it has been observed that the substrate that provides the best results is the one composed only of glucose, although it would be desirable to continue to optimize the conditions of this culture to achieve a performance similar to that obtained in autotrophic conditions.
- Finally, the results show that the highest average growth rate is obtained when nitrogen groups such as neopepton and yeast extract are applied.

It is therefore possible to conclude that the diatom *Skeletonema costatum* (Greville) Cleve 1873 allows the adaptation of its cultivation under different conditions: Autotrophic, Mixotrophic and Heterotrophic, although it would be desirable to optimize the conditions of cultivation under heterotrophic conditions to improve this yield. And therefore, these are very promising results that would allow for more economic forms of cultivation that could be applied in different sectors.

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