

Response of the microalga *Chlorella sorokiniana* to pH, salinity and temperature in axenic and non axenic conditions

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Abstract

Microalgal culture in non axenic conditions can be used as food source for aquaculture species, wastewater treatment and in axenic conditions for the extraction of valuable chemicals. However, the response of the microalgae in axenic conditions can be modulated with respect to non axenic conditions. The aim of this study was to test the response of the microalga *Chlorella sorokiniana* in function of pH (5, 6, 7, 8 and 9), temperature (25, 30, 37, 40 and 42°C) and salinity (15, 25 and 35ppm) in non-axenic and axenic cultures. All bioassays were carried out with photoperiod 12:12h, 98 μ mol.quanta.m⁻².s⁻¹, 28°C. Non-axenic cultures in Algal medium and by triplicate were maintained at volume of 250ml in flasks with 500ml in capacity. Whereas, axenic cultures by fire replicas were carried out in test tubes with volume of 15ml in autotrophic, mixotrophic and heterotrophic conditions with organotrophic and Algal medium. In non-axenic cultures, the microalga was more sensible to salinity, temperature and pH below 5, with optimal growth at 30°C and pH 7-8 in non saline medium. Instead, in axenic conditions, the microalga was able to grow up to 42°C, pH above 3 and salinity up to 35ppm in mixotrophy, reaching the highest growth at pH 7-8, between 0 and 25ppm and 37°C. Chlorophyll synthesis was older in non axenic cultures. These results suggest that the response of *C. sorokiniana*, to pH, temperature and salinity can be modulated by associated bacterial flora. **Key words:** *Chlorella sorokiniana*, pH, salinity, temperature, axenic, non-axenic.

Introduction

The variation of sowing conditions, such as type and concentration of nutrients, temperature, pH, luminous intensity,

photoperiod, sowing rules of discontinuous, continuous or semicontinuous crops, genetic handling, among others (15, 27, 37) have

contributed to establish systems committed to the obtaining of microalgal biomass (17, 18). The microalgae response to pH varies, since this factor determines the solubility of the carbon dioxide and of minerals in the crops and has a direct or indirect influence on its metabolism (28, 32, 38). Temperature not only affects cellular reactions but also nutritional requirements and the biomass composition, as well as gases solubility in the water (1, 14, 24, 37, 38, 40).

Massive cultures of microalgae are obtained photoautotrophically, using opened tanks and natural light or using more intensive systems of variations. However, there are production alternatives of microalgal biomass, in both mixotrophic and heterotrophic conditions, that is, when are able to assimilate a source of organic carbon exhibiting an organotrophic growth (26, 27 and 35).

Mixotrophic cultures produce an elevated quantity of biomass compare to autotrophy and heterotrophy; this might be due to the energetic effect of the light and of the organic substrate (19, 25, 28 and 36). Instead, heterotrophic cultures, seen from the engineering perspective, have advantages on the photosynthetic systems, since proteins, carbohydrates, lipids, among others, are obtained in a lower price by not using any lighting.

When microalgae are associated to bacteria on the environment (non axenic), an interaction happens, which might be good for both; in a way that the

microalga is able to assimilate products of the bacterial activity in the media. Likewise, the associated microbial flora is implied on the regulation of physiologic parameters such as pH, temperature and salinity. But, on axenic conditions microalgae do not reach a proper growth by the lack of the associated microbial flora, which would provide essential factors to stimulate growth (7, 10).

On lab conditions, strains of axenic microalgae are very important on biochemical, physiological, genetic and taxonomic studies (19). Research done with microalgae, from the physiologic point of view are very important, since these allow to clarify whether the microalga has or not physiologic mechanisms that would allow it regulate stressful environmental conditions, such as elevated salinity, pH, temperature, nutrients limitation, dehydration, among others (12, 13, 41). Therefore, it is necessary to continue researching about the reaction of a microalga towards environmental conditions, which may varies in function of the absence or presence of associated bacteria.

Among microalgae of higher importance is *Chlorella*, by its economical and nutritional value, for animals and humans. For example, *Chlorella vulgaris* has been used by its protean quality (28) and it even has properties to prevent cancer (30). Nowadays, it represents an ideal biologic system for different investigations and it also represents a high efficiency by its easy adaptation on lab conditions (4, 5, 6, 9, 19, 21, 28, 31, 32).

Axenic cultures of microalgae of great interest in biotechnology might be used for pharmacologic purposes and for human nutrition. Meanwhile, non axenic cultures where the associated bacterial flora keeps can be selected for aquaculture, animal nutrition and biofertilizers. However, the response of microalgae for each sowing condition is sensitive to be modified in function of different sowing parameters such as source of nutrients, pH, salinity and

temperature. Nevertheless, nowadays there is little physiologic information of *Chlorella* strains cultivated on axenic and non axenic conditions (32). On this matter, it is reported the effect of pH, salinity, and temperature on the growth and pigments content of the *Chlorella sorokiniana* microalga in axenic and non axenic discontinuous cultures and on mixotrophic and heterotrophic conditions.

Materials and methods

Studied Microalga. *Chlorella sorokiniana* microalga was collected coming from the Tulé dam, located on the North-Occidental Coast of Maracaibo's Lake, Mara municipality, Zulia state, and isolated using the dilution technique in series on a liquid and solid selective culture. The taxonomical located of the microalga was determined through the use of taxonomic claves and a physiologic and biologic study done to the strain (3, 19, 20, 21, 44).

Chlorella description. *Chlorella* microalga was described observing its cells on a fresh exam and on fixed trays using an optic microscope. Also, colonial morphologic characteristics of the microalga were also observed in the inorganic and organic media.

Obtaining of the axenic culture of *Chlorella* microalga. Colonies of microalga free of bacteria were isolated through unialgal cultures, sowing on inorganic solid media (figure 1). Plates were

incubated on environmental temperature, under a continuous illuminating system at a lighting intensity of 98 $\text{mmol quanta.m}^{-2}.\text{s}^{-1}$. Subsequently, colonies of isolated microalga were sowed on an organotrophic culture media (8), as a control to guarantee axenic conditions of the microalga on liquid and solid culture.

Physiologic studies. All cultures were done on a commercial media Algal with a photoperiod of 12:12 h, a lighting intensity of 98 $\mu\text{mol quanta.m}^{-2}.\text{s}^{-1}$, 28°C and an initial pH of 7. Non axenic cultures with constant ventilation initiated with an inoculum of $1 \times 10^6 \text{ cel.ml}^{-1}$ in a volume of 250 ml in matraces of 500 ml of capacity. While in axenic cultures with mechanic stirring, initiated five times with $5 \times 10^5 \text{ cel.ml}^{-1}$ in a volume of 15 ml tubes with bakelite lids.

pH. Growth and production of pigments of *C. sorokiniana* were evaluated at pH 1, 3, 5, 6, 7, 8 and 9 in axenic and non axenic cultures.

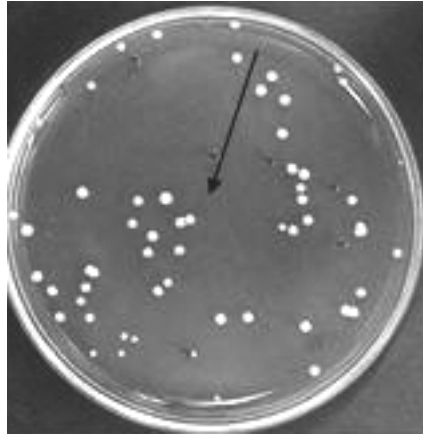
Chlorella sorokiniana

Figure 1. Colonies isolation of *Chlorella sorokiniana* microalga.

Non axenic cultures were sealed with Tris HCl at 25mM. To each treatment was adjusted pH twice a day, while the control corresponded to cultures without adjustments of pH.

pH on axenic cultures was evaluated on an organotrophic culture media with a luminous intensity of $98 \mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, using the mixotrophic condition and on heterotrophic conditions under darkness. The control corresponded to autotrophic cultures done to the inorganic media Algal.

All cultures kept for 15 days at $25 \pm 2^\circ\text{C}$, at the time where the cellular density, pigments content and final pH were determined. Axenic cultures were no sealed with the aim of avoiding the effect of these on cells free of bacteria.

Salinity. It was done in this experiment in both axenic and non axenic cultures and ocean water adjusted to salinities of 15, 25 and 35 ppm was used. The control consisted

on non saline cultures. 15 days after initiated the experiment cellular growth and pigments content were determined.

Temperature. Cultures were let to grow at 25, 30 and 37°C in non axenic conditions and at 25, 37, 40 and 42°C in axenic conditions. The organotrophic culture media was used in mixotrophic and heterotrophic cultures, while the control was kept with inorganic media Algal and corresponded to autotrophic cultures.

Culture media: a) Inorganic. Sterile distilled water was used as autoclave at 121°C and 15 psi for 20 minutes. Subsequently, water was enriched with culture media Algal at a concentration equivalent to 6.0 and 8.0 mM of NaNO_3 (14). Solid media was done with Agar-Agar 1.5% supplemented with Algal nutrients. B) Organic. Liquid media was prepared in tubes with bakelite lid of 20 ml capacity and solid media on Petri plates. After sterilized the

organotrophic media formed by yeast extract, peptone and glucose at 5.0; 1.5 and 2.5 g.L⁻¹ respectively, Algal was added at a concentration equivalent to 8.0 mM de NaNO₃ (8).

Biomass evaluation:

A) Cellular Count. Growth was calculated counting cells with a binocular optic microscope on an improved Neubauer chamber of 0.1 mm of depth (15).

B) Determination of pigments.

Chlorophyll a, b and total carotenoids were determined through a metabolic extraction. The concentration expressed in pg.cel⁻¹ was determined through the equation proposed by Wellburn (42).

Statistical analysis. Growth and production of pigments of the microalga in non axenic and axenic cultures were analyzed using the Scheffé test using the statistical design Stat Most for Window, 3.0.

Results and discussion

Morphological characteristic of Chlorella. It is an unsteady unicellular microalga, without any constriction on the medium area of the cell, with spherical shape, smooth cellular wall and with chloroplast with crown shape (4, 5, 22). Colonies of chlorella are sometimes associated to the mucilage and the modality of the cell adding is very characteristic of this specie, which facilitates its identification. 90% approximately are apart of the plankton and benthos of fresh water (16).

pH. Microalga growth (cellular density) in axenic and non axenic cultures incremented with pH. For non axenic cultures, the highest values were obtained at pH 8 and 9 with 344.68±33.45 and 352.34±51.47x10⁶ cel. ml⁻¹, respectively (table 1). However, pH 1 and 3 was lethal for microalga, on the contrary when pH incremented at 4 the growth of the microalga began, but still with an inhibition of 81.79% in relation to control.

On axenic conditions, the

microalga also incremented its grow in function of pH, which indicates that its cellular density optimizes on the alkaline rank, but different to the non axenic cultures showed tolerance to pH 1 and 3, even though growth kept inhibited at pH 1, the microalga was able to grow through pH 3, in autotrophic, heterotrophic and mixotrophic cultures with an increment of 4; 77.5 and 169 times respectively, compare to the cellular growth (0.5x10⁶ cel.ml⁻¹) to what initiated the culture (figure 2).

Mixotrophic and heterotrophic cultures with a final pH of 7.8 produced the highest values of cellular density with 122.64±1.33 and 109.43±1.48x10⁶ cel. ml⁻¹. Different to the lather, the highest value on the autotrophic cultures of cellular growth was found at final pH 8.2 and 9.6 with 15.29±0.03 and 16.87±0.04x10⁶ cel. ml⁻¹.

Likewise, the growth of the microalga was stimulated in function of the presence of organic substrates on the culture media

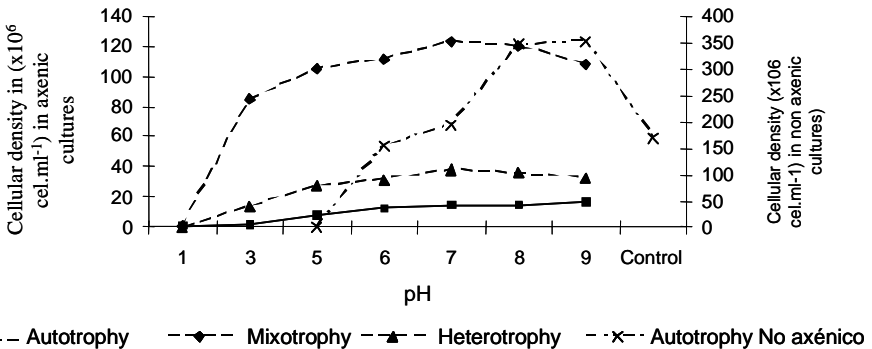
Table 1. Cellular density ($\times 10^6$ cel.ml⁻¹) and pigments' content (pg.cel⁻¹) of the microalga *Chlorella sorokiniana* in function of pH in non axenic conditions.

pH	Cellular density	Chlorophyll	Carotenoids	Chlo/caro
Control	168.27± 1.43	1.58±0.04	0.37±0.04	4.17
5.0	0.22± 0.01	ND	ND	ND
6.0	154.52± 1.11	0.33±0.01	0.06±0.01	5.35
7.0	192.46± 6.46	1.59±0.01	0.27±0.01	5.88
8.0	344.68±33.45	1.60±0.01	0.17±0.01	9.41
9.0	352.34±51.47	1.40±0.05	0.19±0.02	7.37

Cultures done in axenic conditions. Control: non sealed and non adjusted with final pH of 9. Chlo/Caro: chlorophyll/carotenoids relation. Cultures at pH 5.0 with an inhibition percentage of 81.79% in relation to the control culture. ND: non determined by low cellular density.

(autotrophy<heterotrophy<mixotrophy) (figure 2). That is, the organotrophic culture media with lighting was the one which induced a higher grow of *Chlorella* microalga. Different reports indicate that the mixotrophic condition of grow contributes to a higher production of biomass in different microalgae, in relation to heterotrophy and autotrophy (10, 11, 23, 33, 38).

These results also indicated that the culture media resides pH, influenced on grow. On the inorganic media, the highest cellular density was reached at pH 9.6 while on the orgnotrophy media with lighting and darkness, a final pH of 7.8 produced (figure 2). It is possible that the net charge of some organic substrate on the culture media varies in function of pH which might influence on its growth.



*Controlled crop with final pH of 8.9

Figure 2. Influence of pH on the growth ($\times 10^6$ cel.ml⁻¹) of *Chlorella sorokiniana* microalga in axenic and non axenic cultures.

On the other hand, the fact that this strain of *Chlorella* was able to tolerate a culture media on axenic conditions resulted of physiologic interest. However, on its natural habitat characterized by its association to bacteria, the microalga did not grow and lost viability at pH inferior to 6. This means, that on axenic conditions it presented physiological mechanisms exclusively inherent to the microalga, able to regulate very acid environmental conditions, which lots of photosynthetic microorganisms were not able to tolerate, though when are free of bacteria. On this matter, it has been reported that species *C. ellipsoidea* and *C. saccharophila* grow satisfactorily at pH between 2 and 3 and this property also constitutes a useful characteristic to separate species inside the genre (21).

Chlorophyll conditions in non axenic conditions was superior on the control and cultures at pH 7 and 8,

with a significant difference ($P < 0.05$). Instead, on axenic conditions, pH did not have any influence on the content of chlorophyll, because between pH 3 and 9 similar values were found between 0.37 and 0.45 $\text{pg}\cdot\text{cel}^{-1}$ (figure 3). However, this pigment produced even more in non axenic conditions with a significant difference ($P < 0.05$) with values of 3.5; 5.3 and 5.7 times more superior to the obtained in axenic, mixotrophic, heterotrophic and autotrophic cultures respectively. These results are likely reflected by the elevated relation chlorophyll/ carotenoids registered in non axenic cultures. Likewise, this relation tended to increase with pH, in both axenic and non axenic cultures. That is, the synthesis of chlorophyll was stimulated at the time the increased the pH, while the concentration of carotenoids tends to reduce (table 1 and 2).

For carotenoids, the most elevated values at pH 7 were reached

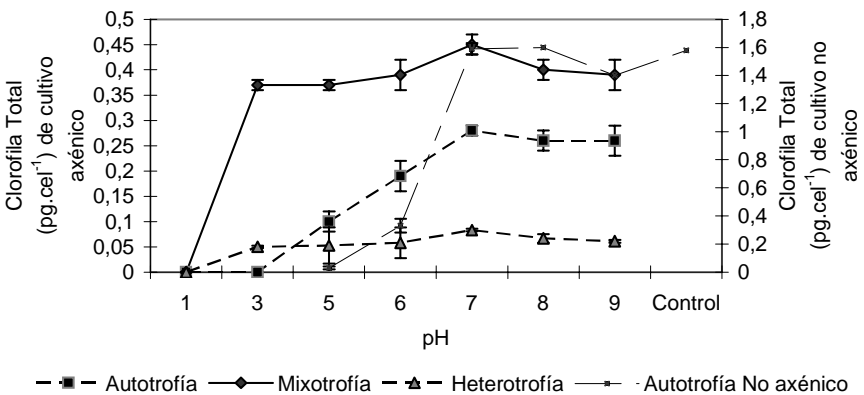


Figura 3. Influencia del pH sobre el contenido de clorofila ($\text{pg}\cdot\text{cel}^{-1}$) de la microalga *Chlorella sorokiniana* en cultivo axénicos y no axénicos.

Table 2. Chlorophyll/carotenoids relation in mixotrophic and heterotrophic cultures of *Chlorella sorokiniana* microalga in function of pH, in axenic conditions.

pH		Chlorophyll/carotenoids relation		
Initial	Final	Autotrophy	Mixotrophy	Heterotrophy
1	1.1	ND	ND	ND
3	3.4	ND	1.61	1.05
5	5.8	1.25	1.85	1.06
6	6.4	1.36	1.95	1.10
7	7.8	1.47	1.96	1.11
8	8.2	1.53	2.05	1.41
9	9.6	1.53	1.95	1.46

ND: non determined by its low cellular density.

independent of the axenic condition; though without a significant difference between both types of cultures (table 1 and 2).

Results indicate that the highest production of chlorophyll in non axenic cultures might had been related to the provision of nutrients rich on nitrogen sources and minerals that might had been provided by bacteria associated to the microalga, though the organotrophic media where the microalga free of bacteria grew, was very enriched with different nitrogen sources.

Salinity. Cellular densities in the most elevated stationary phase were reached in a non saline media with $59.18 \pm 3.63 \times 10^6$ cel.ml⁻¹ for non axenic cultures and 109.11 ± 1.07 and $89.70 \pm 1.0 \times 10^6$ cel.ml⁻¹ for mixotrophic and heterotrophic cultures with significant differences ($P < 0.05$) (figure 4). However, the increment of salinity produced an inhibitory effect on the growth of the microalga. At 25

and 35 ppm the cellular density reduced in 97.9% and in 98.5% respectively, in non axenic cultures. In mixotrophic cultures, the microalga grew in all salinities, with a reduction of even 61.8% when was cultivated at 40 ppm. In heterotrophic cultures also grew at all salinities, until it reached 1.3% in relation to non saline cultures.

These results indicate that the effect of salinity was more drastic in axenic, autotrophic and heterotrophic cultures than in non axenic. However, when the microalga grew in mixotrophic cultures was able to keep elevated cellular densities until salinities of 25 ppm. Instead, at 35 ppm reaches 62.3% of the grow obtained in absence of salinity (figure 4). It is important to mention that the organotrophic culture media used favored the grow of the microalga and it seems that in presence of light the microalga tolerated more salinity. On this matter, salinity had a more

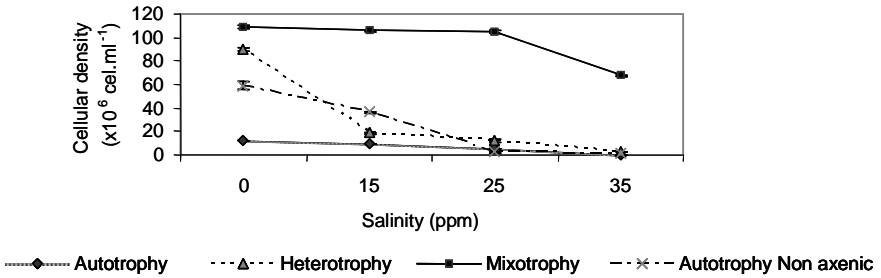


Figure 4. Influence of salinity (ppm) on the growth ($\times 10^6$ cel.ml⁻¹) of *Chlorella sorokiniana* microalga in axenic and non axenic cultures.

drastic effect under darkness, being the grow relation mixotrophy/heterotrophy 30 times higher. That is, in mixotrophic cultures was produced an increase of the inhibition of 38.3% while in heterotrophic cultures reached 98%.

Likewise, salinity produced an increment of the cellular content of chlorophyll and total carotenoids, in non axenic conditions. The highest values were reached at 25 and 35 ppm in relation to non saline cultures (figure 5). The highest cellular size result on a low growth velocity of the microalga which salinity might had

been related to the accumulation of metabolites, therefore the cellular content of chlorophyll and carotenoids was superior to those found in cells without any exposure to the saline media. The microalga accumulated 20 and 24 times more chlorophyll and carotenoids in sweet aquicolous media, in relation to the cultivated at 35 ppm in mixotrophy. However, when it grew in darkness only produced 1.8 and 1.6 times more chlorophyll and carotenoids respectively, compare to those kept at 35 ppm (figure 5).

The statistical analysis showed that there was a significant difference

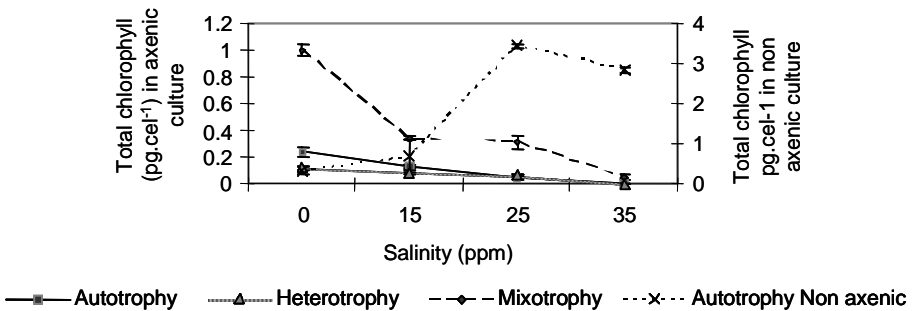


Figure 5. Influence of salinity (ppm) on the chlorophyll production (pg.cel⁻¹) in axenic and non axenic cultures of *Chlorella sorokiniana* microalga.

($P < 0.05$) compare to chlorophyll, between cultures at 35 ppm and controls (table 4). For carotenoids there significant differences were also obtained ($P < 0.05$) between the control and cultures at 15 and 25 ppm. This might had been due to the presence of salts of Mg, Ca, SO_4 and Fe in ocean water, to which a higher availability of nutrients was obtained to improve the production of biomass and chlorophyll in microalgae as *Chlorella*.

In both culture conditions, the chlorophyll/carotenoids relation reduced to salinity (table 3). The increment of salinity stimulated the synthesis of carotenoids by effect of saline stress, in a way that increased the molecules proportion of these pigments in relation to chlorophyll.

Even though these results suggested that the microalga was sensitive to salinity on its natural habitat, in axenic conditions was able to keep an elevated grow on an enriched culture media and until salinities of 35 ppm. It is unknown the

reason that cells tolerated more salinity under lighting than in darkness when grew on organotrophic media.

Temperature. In non axenic cultures the microalga grew in all the studied temperatures, with an optimum of 30°C. However, at 37°C growth reduced 25.4% in relation to the optimum temperature (figure 6). The elevated cellular densities produced between 25 and 35°C, indicated the adaptation of this microalga in tropical water. Therefore, this *Chlorella* strain might develop efficiently in opened cultures in our tropical areas (26).

In axenic cultures, *Chlorella* showed an excellent growth between 25 and 42°C with an optimum of 37°C. In mixotrophic conditions the highest growth at 37°C was obtained, which was 2 and 15 times superior to the obtained in heterotrophic and autotrophic cultures respectively. At the same time, at 40°C and 42°C the microalga exhibited an elevated growth with 72.62 ± 0.75 and

Table 3. Chlorophyll/carotenoids relation in axenic and non axenic cultures of microalga *Chlorella sorokiniana* in function of salinity.

Total Chlorophyll /Carotenoids Relation				
Salinity (ppm)	Autotrophy	Mixotrophy	Heterotrophy	Non axenic autotrophy
0	1.60	1.41	1.44	4.71
15	1.30	1.43	1.40	5.30
25	1.25	1.11	1.29	3.05
35	NC	1.00	NC	1.04

NC: there was not any growth.

58.79±0.04 x10⁶ cel.ml⁻¹, while in autotrophic cultures the microalga only tolerated temperatures until 37°C, though the growth was always lower in relation to mixotrophy and heterotrophy (figure 6). This was due to microalgae in non axenic cultures required additional nutrients derived from the associated bacterial flora. Therefore, its growth was not stimulated when was free of bacteria.

These results found in relation to the temperature agree to those reported in axenic cultures of the microalga *Tetraselmis suecica*, where a low grow in autotrophy conditions was also produced, in absence of organic substances (18).

Results indicated that this strain of *Chlorella* was able to grow at elevated temperatures between 37 and 42°C. This characteristic, plus the tolerance at acid pH and elevated salinity, also allowed locating this strain in the *C. Sorokiniana* specie (17, 19, 38 and 39). In non axenic cultures of *Chlorella vulgaris* an

optimum temperature has been reported for its growth of 32.4°C (20 and 30). However, the behavior of microalgae in axenic and unialgae conditions in relation to temperature might vary, since in non axenic cultures of the studied *Chlorella* specie, an optimum temperature of 30°C (non reported results) was obtained.

It is important to highlight that the elevated productivity of some microalgae in mixotrophic conditions is related to the simultaneous and efficient use of organic compounds and CO₂ as carbon source, and light as energy source in a way that the biomass production incremented respect to heterotrophy or autotrophy cultures (6 and 34).

Axenic cultures of microalgae are strictly studied since these provide direct results product of different environmental factors on its growth, physiology or biochemical. These cultures are necessary for further taxonomic research (38),

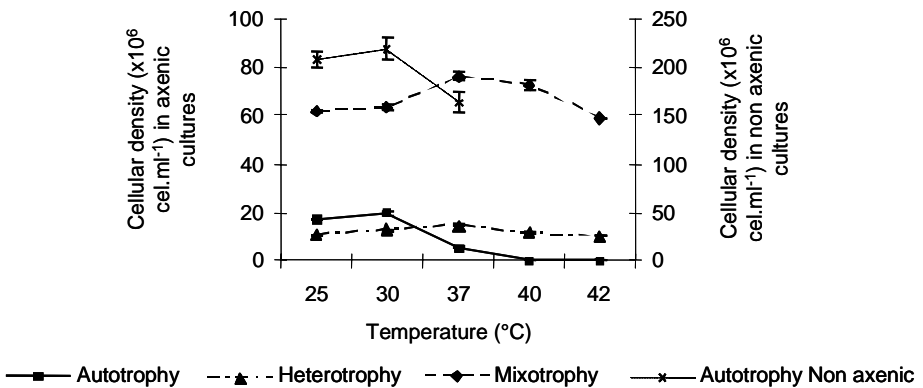


Figure 6. Temperature influence on the growth (x10⁶ cel.ml⁻¹) of *Chlorella sorokiniana* microalga in axenic and non axenic conditions.

metals removal (2), and biotransformation (29). On this matter, *Chlorella sorokiniana* in axenic conditions presented

differential characteristics in relation to its behavior in presence of its associated bacterial flora.

Conclusions

The response of the microalga *Chlorella sorokiniana* at pH, salinity and temperature varied in function of the presence or absence of the bacterial flora. In non axenic conditions it only was able to grow satisfactorily at pH between 6 and 9, but also to optimize its cellular density at 30°C and to grow in absence of salinity.

In axenic conditions its tolerance response changed to acid pH; at temperatures of even 42°C and salinities of 25 ppm.

This behavior suggests that the physiological response of the microalga on the natural environment was modified when exempted the relation to its associated bacteria.

Also, the mixotrophy showed to be the most adequate condition that favored the growth of the microalga towards environmental changes.

Therefore, this autochthonous microalga under axenic conditions has a great biotechnological potential by its growth capacity in mixotrophic and heterotrophic cultures in presence of organic nutrients and on a wide pH interval. This might serve as an alternative to the autotrophy condition of growth for the production of biomass at elevated temperatures, therefore the production of enzymes, proteins or compounds might be induced with biological activity with pharmacologic purposes.

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