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ALGAE FOR CONTROLLED ECOLOGICAL LIFE SUPPORT SYSTEM DIET

CHARACTERIZATION OF CYANOBACTERIA "SPIRULINA" IN BATCH CULTURES.

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ABSTRACT

Spirulina sp. as a bioregenerative photosynthetic and an edible alga for space craft crew in a CELSS, was characterized for growth rate and biomass yield in batch cultures, under various environmental conditions. The cell characteristics were identified for one strain of Spirulina: S. maxima. Fast growth rate and high yield were obtained under the following conditions: temperature (30°C-35°C), light irradiance 60-100 uE m^{-2} s^{-1}, nitrate 30mM, phosphate 2mM, aeration 300 ml/min, and pH 9-10. The partitioning of the assimalatory products (proteins, carbohydrates, lipids) were manipulated by varying the environmental growth conditions. Our experiments with Spirulina have demonstrated that under "stress" conditions (i.e. high light 160 uE m^{-2} s^{-1}, temperature 38°C, nitrogen or phosphate limitation; 0.1 M sodium chloride) carbohydrate increased at the expense of protein. In other experiments, where the growth media were sufficient in nutrients and incubated under optimum growth conditions, the total proteins were increased up to almost 70% of the organic weight. In other words the nutritional quality of the alga could be manipulated by growth conditions. These results support the feasibility of considering Spirulina as a subsystem in CELSS because of the ease with which its nutrient content be manipulated.

INTRODUCTION

Pursuit of our national goals in space exploration will eventually require man's long-duration tenancy of celestial vehicles and planetary base. Requirements for life support could be met through expenditure of stored supplies and by regeneration and reuse of the waste products of human metabolism. The logistics necessary of regeneration for extended space missions are well documented (1). The primary source of all man's food and organic raw materials is solar energy. Conventional food sources consist of higher plants and animals. Unconventional food sources for human consumption are photosynthetic algae and bacteria and non-photosynthetic bacteria, yeast and fungi. Conventional food sources are highly palatable, but require a long time to produce. Algae, on the other hand, grow rapidly; their metabolism can be controlled; they produce a high ratio of edible to nonedible biomass; and their gas-exchange characteristics are compatible with human requirements. The biological components of Controlled Ecological Life Support System (CELSS) will serve as subsystems for the revitalization of air, for the long term space flight. Studies of bioregenerative life support systems for use in space indicated that they are scientifically feasible. Support of a crew in space, whether in an orbiter or on the surface of a planetary body requires that oxygen, potable water and food be supplied and that waste material be removed. Employment of photosynthetic organisms (Algae: Cyanobacteria) allows biomass production from relatively simple components which are readily recycled in a CELSS system, namely carbon dioxide, minerals (NO_3-, PO_4-3, K+, etc.) and micronutrients.

Cyanobacteria single cell protein (SCP) has been used as a food source in various parts of the world (e.g. Mexico, China and Africa) since ancient times; in fact, dried cyanobacteria and cyanobacterial tablets are now sold in health food stores in Japan, North America and Europe because they are recognized for their nutritional value. The nutritional quality of all cyanobacteria which have been tested appears

to be very high. The protein of S. maxima is easily digestible and approximately 65% of the protein is assimilatible.

The semi-microscopic blue-green algae (Cyanophyta; Cyanobacteria) occupy a taxonomic position, since they combine an autotrophic mode of growth that is common to eukaryotic plant cells with a metabolic system that is generally regarded as bacterial, rather than plant-like.

Changes in the supply and consumption of metabolites may have considerable effects on metabolic patterns. The accumulation of photosynthetic products in algae can be induced by manipulating the environmental conditions under which the algae are grown (2). The most difficult problem in using algae as food is the conversion of algal biomass into products that a space crew could actually eat over a long period of time. If algae are to be considered as a primary food source, it will be necessary to determine that they can be converted into a wide enough range of a palatable complete diet. Therefore, Spirulina, an edible alga with less nucleic acids and no cell wall , offers a good prospect for further studies by manipulating growth parameters.

In order to evaluate the potential of Spirulina for a CELSS diet, it is essential to have background information on the environmental tolerance of the species and eventually the responses of physiological characteristics. This background will be obtained from studying the species in batch and continuous cultures.

The purpose of this project was to evaluate the growth and chemical composition of Spirulina under different growth conditions in batch cultures.

MATERIALS AND METHODS

Spirulina maxima (UTEX LB 2342) was cultured in Zarrouk medium (3). The culture medium was modified for nutrient limitations. Studies for nitrogen limited cultures, sodium nitrate was replaced by potassium chloride and nitrate,

ammonia and urea were tested. For P-limited medium the P was replaced by sodium chloride and phosphoric acid was used as P-source. For salinity studies, sodium chloride was used. The pH was maintained in all cases at 9 with 2N NaoH. All experiments were incubated in 30ml medium of contimuous light, in small bottles and bubbled with air. These culture were used for evaluating the growth parameters of the alga. Cultures were placed on shelves, illuminated with cool white florescence tubes of light intensity 80 uE m^{-2} s^{-1} in culture room kept at 25°C ± 1. Light irradiation measurements were made with a Li-Cor Model Li-185 (Lambda Instruments) Meter equipped with a spherical quantum sensor.

For mass culturing, algal cells were grown in bottles. Cultures were illuminated continuously by placing them in front of a bank of two cool white fluorescent lamps (40W). Light irradiation, measured at the surface of culture bottles was 80 uE m^{-2} s^{-1}. The cultures were grown in a water bath kept at 29-30°C by the use of a heater-thermostat combination.

The cultures were aerated with air (0.03% CO_2) or air enriched with carbon dioxide. Mixtures of air (0.03%) and carbon dioxide were obtained by blending gases to a desired mixture in a two-gas proportioner. The flow rate of the mixed gas delivered to the culture was maintained at 300 ml/min.

Analysis:

Growth Rate: Growth was measured by monitoring change in absorbance (O.D.) at 560 nm with spectrophotometer (Perkin Elmer Lambda I) and expressed as doublings day^{-1}. The mean daily division rate t, K, was calculated from: $\bar{K} = \frac{3.32}{t}$ $(\log_{10} OD_t - \log_{10} OD_o)$, Where, t = days since inoculation, OD_t = optical density after t days, OD_o = optical density when t = 0.

Harvesting of Cells: Cells were collected by filtration using filter paper 10um pore size (Gelman). Cells were washed with buffer solution (pH 8), diluted to known volume and processed for further analysis. Cultures were harvested at O.D. 0.1 units, to avoid light limitation.

Total Chlorophyll: An aliquot from the culture was centrifuged for 2 min at 2000g. The precipitate was suspended in methanol for 5 min in a water bath at 70°C, and therefore centrifuged. The optical density of the supernatant was determined at 655 nm.

Dry Weight Measurements (DW): A volume from the culture was filtered through a filter 10um pore size, dried in previously dried, preweighed filter paper and then weighed after cooling in a desiccator.

Ash-Free Dry Weight (AFDA): After recording the dryweight, the dried cells were ashed. The difference between dry weight and ash weight gave the organic weight of the sample.

Total Carbohydrates: The anthrone sulphuric acid method was followed.

Total lipids: Cellular lipids were solubilized by repeated extraction with methanol and methanol-chloroform (1:1), then phase separated after adjustment of the solvent rations to 10:10:9 (methanol: chloroform: water, v/v).

Total Nitrogen and Protein using Kjeldahl methods. The value of the readings was calculated in ug N, from a standard curve for nitrogen source as ammonium sulfate, which has been treated by the same method. Total protein was calculated from total N x 6.25.

Triplicate samples of the algal suspension were taken for each determination. The mean value of these triplicates was recorded.

Nutrients Requirements:

Cultures were incubated in small bottles under the same conditions as described in Section A. The original growth medium was modified by changing the concentration of one nutrient. Nitrogen, phosphorus, iron, bicarbonate and sodium chloride were studied in sufficient and limiting concentrations. The bicarbonate effect was studied together with the aeration effect.

In all experiments triplicate culture bottles were inoculated from stock cultures in the exponential phase. Growth response was measured as optical density and the growth rate was expressed as doublings per day. The yield of cultures was expressed as the total dry weight after five days of growth. The total dry weight was determined by harvesting the cells and drying it.

B. Physiological Characterization of Spirulina in Batch Cultures:

For this experiment, the alga was grown in batch cultures (Roux bottles) as mentioned in "Methods". The cultures were maintained under optimum growth conditions and monitored in the exponential phase by the absorption measurement.

O.D. of Cell Suspension versus D.W. and Chlorophyll: The species was grown in triplicate Roux bottles under the same conditions described before (see Methods). Twenty ml of culture samples were taken daily for measurements of the D.W., and chlorophyll. The experiment was continued for one week.

Under Optimum Growth Conditions: The species was grown in duplicate Roux bottles under the same conditions described before (see Methods). Cultures were analyzed for growth parameters during the eight days.

Stress Conditions:

Light and Temperature: Batch cultures were incubated in Roux bottles irradiation and others at high temperatures (38°C) in water bath.

EXPERIMENTAL DESIGN

A. Growth Parameters Characterization

Temperature, Light: The algal growth was evaluated for temperature and light tolerance on a gradient plate. Temperature could be adjusted in range from 10°C and 50°C. Illumination was provided by eight cool white fluorescent tubes (40W). The algal species was cultured in small bottles (60 ml capacity) containing 30 ml growth medium. Triplicate cultures were placed on the gradient plate, at temperatures: 20°C, 25°C, 35°C and 40°C. The cultures were exposed to two light intensities and were aerated with air (0.03% CO_2).

pH Effect:

The alga was incubated in small bottles at 35°C on a temperature gradient plate and 80 uE m^{-1} s^{-2} irradiance. The original medium was used for culturing, except the pH used for culturing was varied by using NaOH or HCl. The pH of cultures was adjusted daily to the original pH. The cultures were aerated with air (0.03% CO_2).

Aeration Rate, Carbon Dioxide Enrichment, Bicarbonate Concentration:

The alga was incubated in small bottles at 35°C on a temperature gradient plate and 80 uE m^{-1} s^{-2} irradiance. Three sets of cultures were treated differently:

a. Cultures were aerated with different flow rates (air 0.03% CO_2).
b. The flow rate which gave the best growth rate, was selected from "a". The cultures were aerated with air enriched with carbon dioxide in different concentrations 1%, 3%, 5% and 10%.
c. Cultures were treated with different bicarbonate concentrations in which one set was aerated with air (0.03% CO_2) and other set was aerated with air containing 1% CO_2. The pH of all was adjusted twice daily.

Nutrients:

Batch cultures were grown in Roux bottles in duplicate until the exponential phase was reached. One batch was analyzed and represented the culture sufficient in nutrients. Batch cultures were concentrated and diluted to the original batch volume but with a new medium modified in one element. The cultures were incubated under stressed conditions for two days and then harvested for analysis.

RESULTS AND DISCUSSION

Temperature and Light:

Figure 1 depict the growth and yield of Spirulina at two light irradiations and different temperatures ranging from 20°C to 40°C. The strain did not grow at 20°C but it started to grow at 25°C at very slow rate. Temperatures 30 and 35°C enabled the algal fastest growth rate and highest yield of cells. When the temperature was raised to 40°C, the algal cells turned yellow and gave a lower yield. The alga tolerated light irradiance 120 uE m^{-2} s^{-1}.

Aeration Rate:

The effects of air agitation rate on the growth rate and cell yield are depicted in Figure 2. The growth rate of Spirulina increased with increasing the flow rate of air in range of 150 ml/min and 500 ml/min. When the flow rate of aeration was increased to 2000 ml/min, the growth rate started to decline and cells turned yellow. On the other hand the cell yield in terms of dry weight was not affected. The pH of all cultures increased to 11. The cell yield of the strain showed parallel fluctuation to the growth rate of the alga.

Air Enrichment with Carbon Dioxide:

Figure 3 shows the effect of air enriched with different concentrations of carbon dioxide on the growth rate of Spirulina:

. Cultures aerated with 10% CO_2 - in air, did not grow and turned yellow. The pH of the cultures were maintained at 9.4 by the addition of sodium hydroxide. How-

ever, the pH of cultures aerated with 1% CO_2 - enriched air was maintained stable. . Cultures aerated with air (0.03% CO_2) grew at more or less the same growth rate of those aerated with 1% CO_2 - enriched air. The yield of cultures treated with different CO_2 concentrations, in terms of dry weight, was equivalent to the growth rate.

The results of this experiment are in agreement with those of Faucher and Coupal (4). They reported that Sparging 1% CO_2 - air in Spirulina cultures could maintain a constant pH of the culture medium, and at the same time generate HCO_3 ions which were used as carbon source for S. maxima. In a similar study with green algae, Golden and Graham (5), reported that in batch cultures, maximum growth rates were achieved at the CO_2 levels present in atmospheric air and at HCO_3^- concentrations of 3 mM.

pH Effect:

The growth rate of Spirulina strain was clearly affected by the pH of the growth medium as is shown in Figure 4. The alga exhibited higher growth rate in media of pH range of 9 to 10. The growth rate decreased with increasing pH above 10 and the cells turned yellow. The cell concentration increased when increasing the pH of the medium from 8 to 10 and then decreased above pH 10.

Nutrient Requirements:

Nitrogen: Nitrogen sources in the form on nitrate and urea were tested in different concentrations in order to determine their effectiveness as N-sources. The results of nitrate-N and urea-N are represented (Figure 5). The growth rate of Spirulina was enhanced with increasing the concentration of urea-N and nitrate-N. The urea-N at 20mM concentration enhanced the growth rate, while further increase in its concentration limited the growth of the strain.

On the other hand, nitrate-N at concentration 30 mM, enabled the strain to reach fast growth rate and high yield in terms of dry weight. The experiment demonstrated that the least amount of nitrate-N necessary to maintain the growth of Spirulina in culture was 10 mM. Microscopically, the trichomes became shorter with average 6 turns/trichome, in media limited in nitrogen concentration. In agreement with our results, Faucher (4) reported that urea-N in low concentration could support the growth of S. maxima, at high concentration of nitrate-N.

Phosphate: Increasing the phosphate-P concentration in the culturing media to 1 mM to 5 mM, enhanced the growth rate of the strain (Figure 6). But as the concentration increased to 10 mM, the growth rate declined. The mass yield showed similar responses coinciding with the growth rate. Microscopically, the trichomes became shorter in media of phosphate-P concentration below 1 mM and with few number of turns in case of S. maxima (5 turns/trichome). Generally, cyanobacteria require small concentrations of phosphate-P for growth. They grow in phosphorus-limited media (6).

Sodium Chloride: Spirulina grew in media lacking sodium chloride (Figure 7). The growth rate increased as the sodium chloride concentration increased to 0.01M Further increase in sodium chloride concentration (0.1M) affected the growth rate and resulted in lower yield of cells. In addition, microscopic examination of the strain indicated that in media treated with a high concentration of sodium chloride 0.1M, the trichomes were short and with less turns, the average turns per trichome was 6. The results of this experiment, indicate that Spirulina tolerate increases in sodium chloride concentration up to 100 mM. Spirulina tolerance to salt had been previously reported (4).

Iron: Iron concentrations ($FeSO_4$) influenced the growth and yield of the culture (Figure 8). Concentration of 0.05 mM was sufficient for the growth, although media deficient in iron did not show any growth response. Increasing the concentration of iron beyond 0.1 mM lowered the yield of alga and cells turned yellow.

Bicarbonate Concentration: Figure 9 shows that S. maxima grows in the medium even without bicarbonate salt, providing that the culture was aerated with air (0.03% CO_2). As the bicarbonate concentration increased, the growth rate as well as productivity increased. Further increase in bicarbonate concentration above 16g/L (190 mM) did not affect the growth rate. When the carbon dioxide concentration in the air increased from 0.03% to 1%, as shown in Figure 9, the growth rate increased remarkably by decreasing the bicarbonate concentration in the medium as low as 4g/L i.e. one quarter of the concentration in the Zarrouk medium (see Methods). The results of this experiment indicate that Spirulina can utilize atmospheric carbon dioxide when the media bicarbonate concentration is minimum in the culture medium. The pH of all cultures was adjusted daily to 9.4.

Physiological Characterization of Spirulina in Batch Cultures:

Batch Cultures:

Optical Density (O.D.) of Cell Suspension versus Dry Weight (D.W.) and Chlorophyll: Results are presented in Figure 10. For all samples within the first three days of cultivation, which contain relatively small concentrations of biomass (400 mg DW/L or less), readings fell within the accurate range of the O.D. scale and they could be read directly from the spectrophotometer without dilution. However, for all samples during the later cultivation periods which contained high concentration of biomass (500 mg DW/L), dilution of the samples with distilled water was necessary prior to OD readings. The graph show linearity between OD and dry weight.

Each OD unit is equivalent to a concentration of 700 mg/L in the case of S. maxima. It is obvious from this experiment that other reliable indicators of estimating algal productivity can be computed from OD measurements. Therefore, OD measurements can be translated into biomass yield in terms of dry weight or chlorophyll.

Physiological Characteristics of Culture, under Optimum Growth Conditions: The cultures were assayed for growth parameters during the eight days. (Fig. 11) Increments of carbohydrates, proteins, dry weight and chlorophyll are expressed as ug/ml culture. The results show that increases in the synthesis of chlorophyll, protein and yield of the culture are correlated. Growth parameters of cultures analyzed after eight days started to level off, due the nutrient exhaustion and light limitation caused by increasing cell concentration.

Physiological Characterization of Cultures, under Stress Conditions:
The results of analysis were expressed on the basis of organic weight (Ash Free Dry Weight: AFDW) and represented in Table 1.
Light Irradiance and Temperature: Increasing the light irradiance to 120 uE m^{-2} s^{-1}, led to an increase in the total carbohydrate content and a decrease in protein content: S. maxima 19.58%, 29.06%. Increasing the temperature of culture incubation to 38°C, influenced the composition of the strain, in a similar manner to the light irradiation experiment: S. maxima, 45.28%, 18.75%, for protein and carbohydrates, respectively. The culture produced a low percentage of lipids, when grown in high temperature experiments. Cells turned yellow green in color. Studies with light-limited cyanobacteria showed a high level of polysaccharide formation when they exposed to high light intensities (8).

Nutrient Limitation: Media limited in nitrate-N and phosphate-P, favored the accumulation of carbohydrate rather than protein. Nitrate and phosphate

limited cultures: S. maxima had 37.52%, 35.21% carbohydrate and 21.56%, 41.25% protein. When the cultures were transferred to media limited in nitrogen and phosphate, cultures changed in color from blue to yellow-green. N-limited cultures of Anacystis nidulans (7), and P-limited cultures of Oscillatoria agardhii (8), showed elevated levles of polysaccharide storage.

Sodium Chloride: As Zarrouk (3) media were enriched with 0.1M and 0.5M NaCl, the carbohydrate content of the cells increased, when compared to that of the control (Zarrouk: 0.01M NaCl), to 26.25%, 36.73% in S. maxima. On the other hand, the total protein decreased respectively to: 52:62%, 45.64% in S. maxima. The lipid percentages showed little increase when compared to those of the complete media (control). Many cyanobacteria are capable of adapting to a range of salinity in the environment by synthesizing internal osmotic support in the form of carbonhydrates.

Bicarbonate: When bicarbonate concentration of Zarrouk media was reduced to one quarter (4.g/L), the culture showed much difference in the chemcial composition as compared with the control media except their yield was somewhat below the control. The carbohydrate increased to 38.53% when 0.03% CO_2 in air was used for aeration and to 40.23% when 1% CO_2 air was used for aeration.

Conclusions of this study are summarized as following:

- The lipid percentage, in particular, did not show much increase in different culture treatments. But, increasing the temperature of culturing to 38°C or light irradiance to 120 uE m^{-2} s^{-1}, reduced the total lipids drastically. However, increasing sodium chloride to 0.1M in the culturing media, the lipids increased somewhat higher than in the control media.
- The ability of the alga to utlize macroelements and microelements, and to convert it into biomass.

- A slight inverse relationship was observed between the protein content and carbohydrates which means that one increased at the expenses of the other. This suggests that quality of biomass may be manipulated for dietary purposes. An adequate supply of nutrients is therefore a pre-requisite for producing a uniform quality of biomass, which in turn could then be used in the formation of diets. (see Sufficient Nutrients). The possibility of manipulating the quality of the biomass could have potential for the NASA/CELSS Program, when specific diet formulation is needed (e.g. low protein content).
- Overall algal productivity and quality could be manipulated by means of varying nutrient concentrations or temperature and light irradiance.

It can be concluded that through manipulating environmental conditions of the algal growth, one can modify the photosynthetic products. Thus, Spirulina can be, through manipulating growth factors, used as palatable diet comparable to higher plants.

Further work is needed to characterize the efficiency of the algal cells under such environmental conditions in terms of gas exchange and energy loss or gain in steady state.

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Table 1. Molecular Compositon of Spirulina maxima

Growth Conditions	% Organic Wt. (AFDW)		
	Protein	Carbohydrate	Lipids
*Sufficient Nutrients	69.75	11.5	4.68
High Light ($160uEm^{-2}s^{-1}$)	29.06	19.58	3.56
High Temperature (38°C)	45.28	18.75	3.75
N-limited	21.56	37.52	4.68
P-limited	41.25	35.21	5.20
Sodium Chloride 0.1M	52.62	26.25	4.68
0.1M	45.64	36.73	7.52
Bicarbonate (4.4g/L) (0.03% CO_2)	45.67	38.53	6.22
1% CO_2)	43.52	40.23	6.53

* Experimental conditions were:
temperature 30°C; light irradiance $80uEm^{-2}s^{-1}$; air flow rate 300 ml/min;
The values shown are averages of four independent determinations.

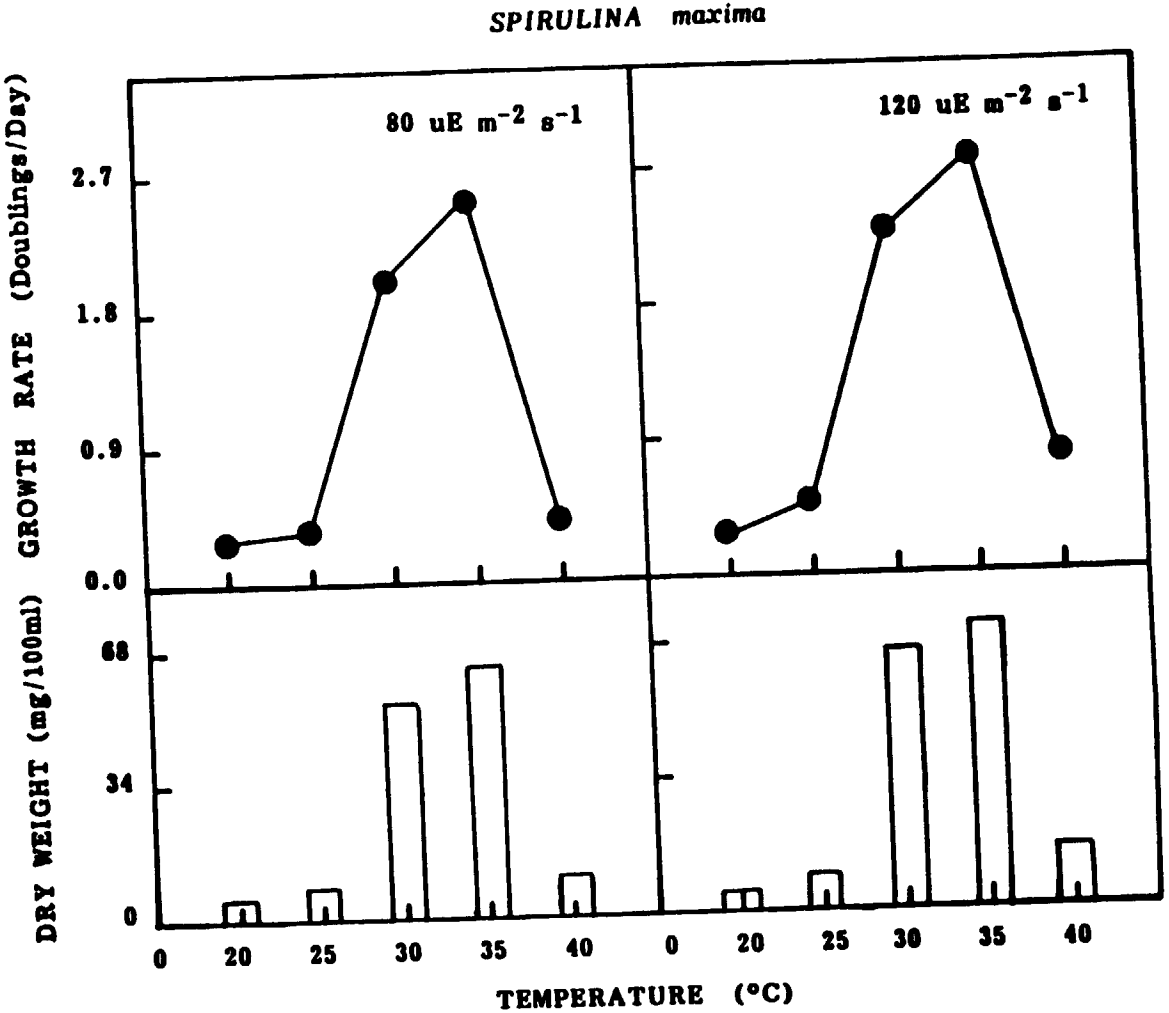

Figure 1. Growth Rate and Yield of S. maxima as Function of Temperature and Light Irradiance

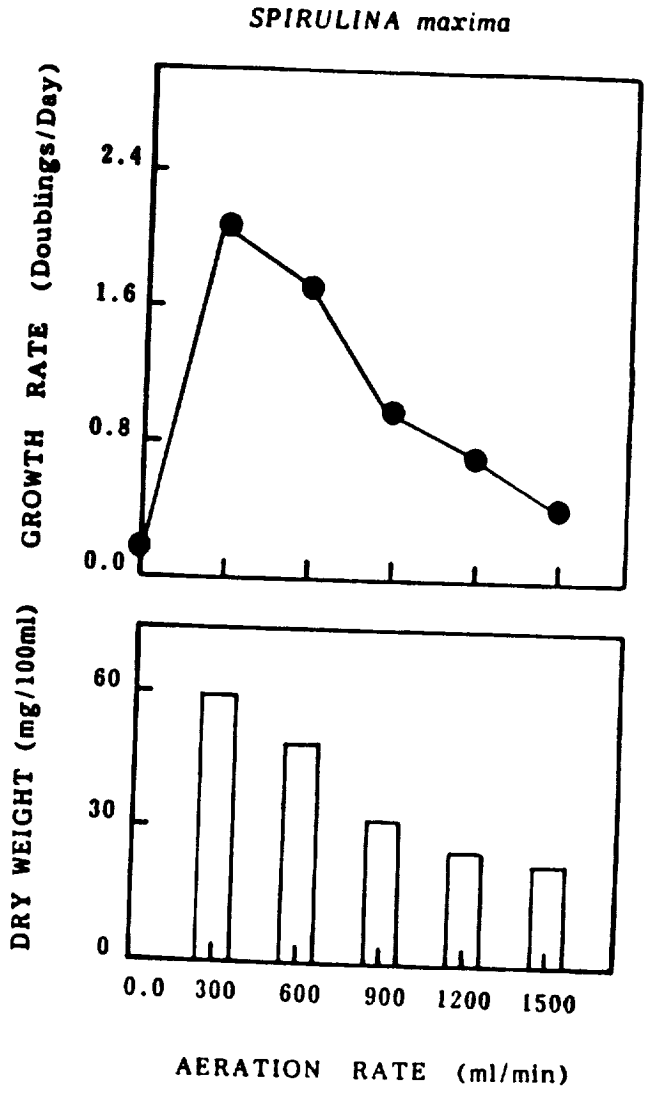

Figure 2. Growth Rate and Yield of S. maxima as a Function of Aeration Rate

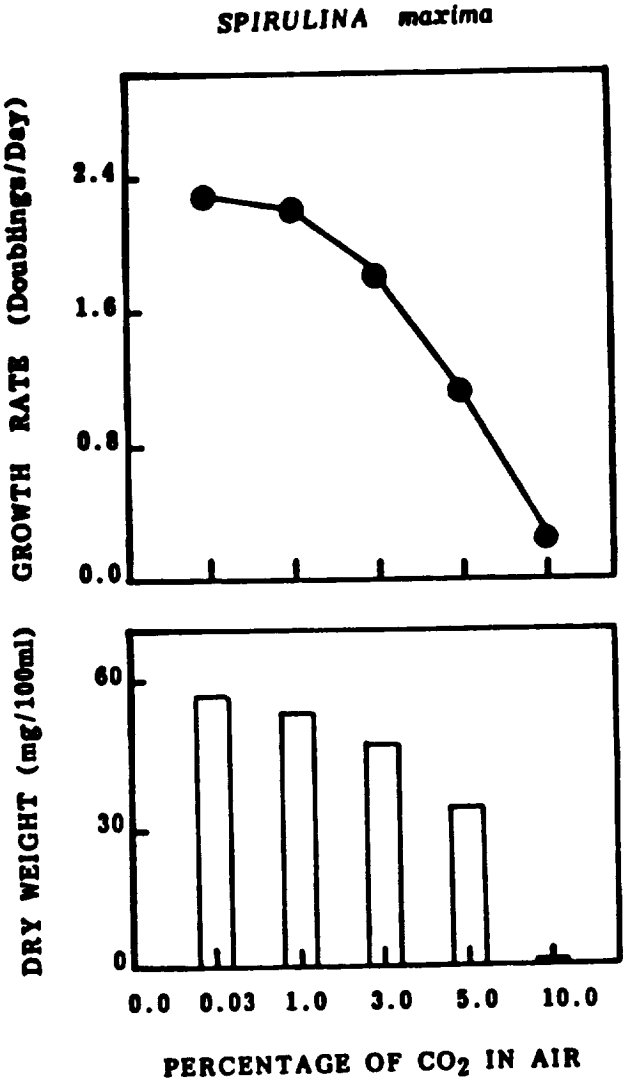

Figure 3. Growth Rate and Yield of S. maxima as a Function of Carbon Dioxide Concentration in Air

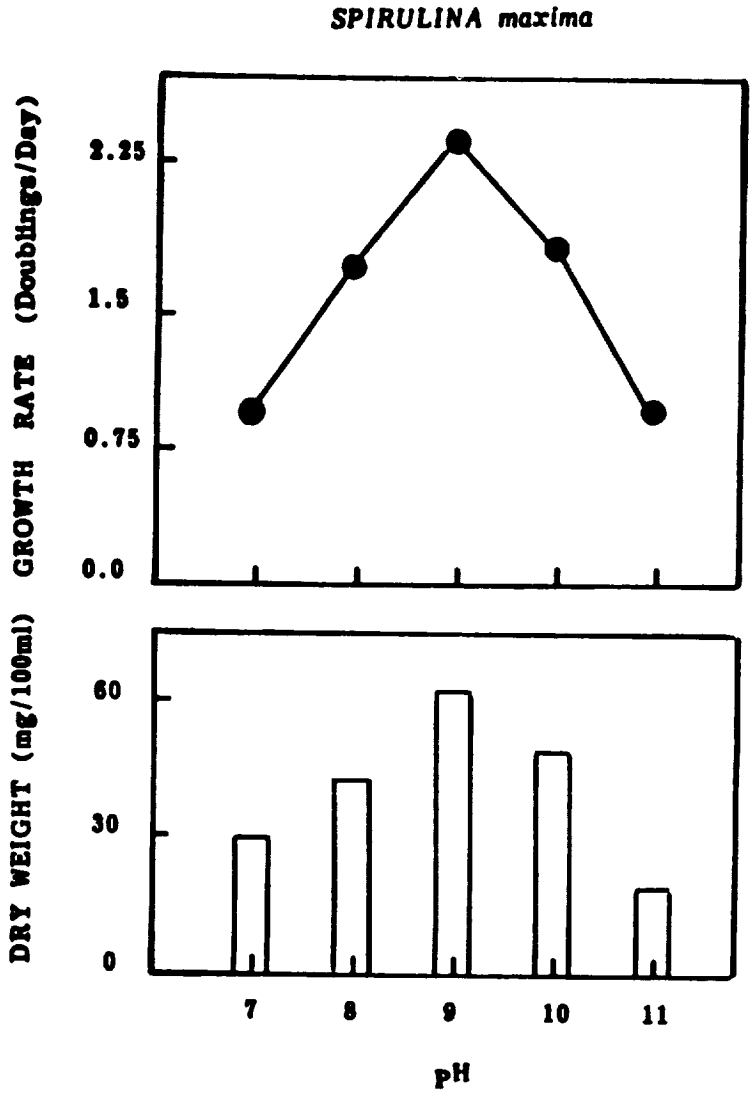

Figure 4. Growth Rate and Yield of S. maxima as a Function of pH.

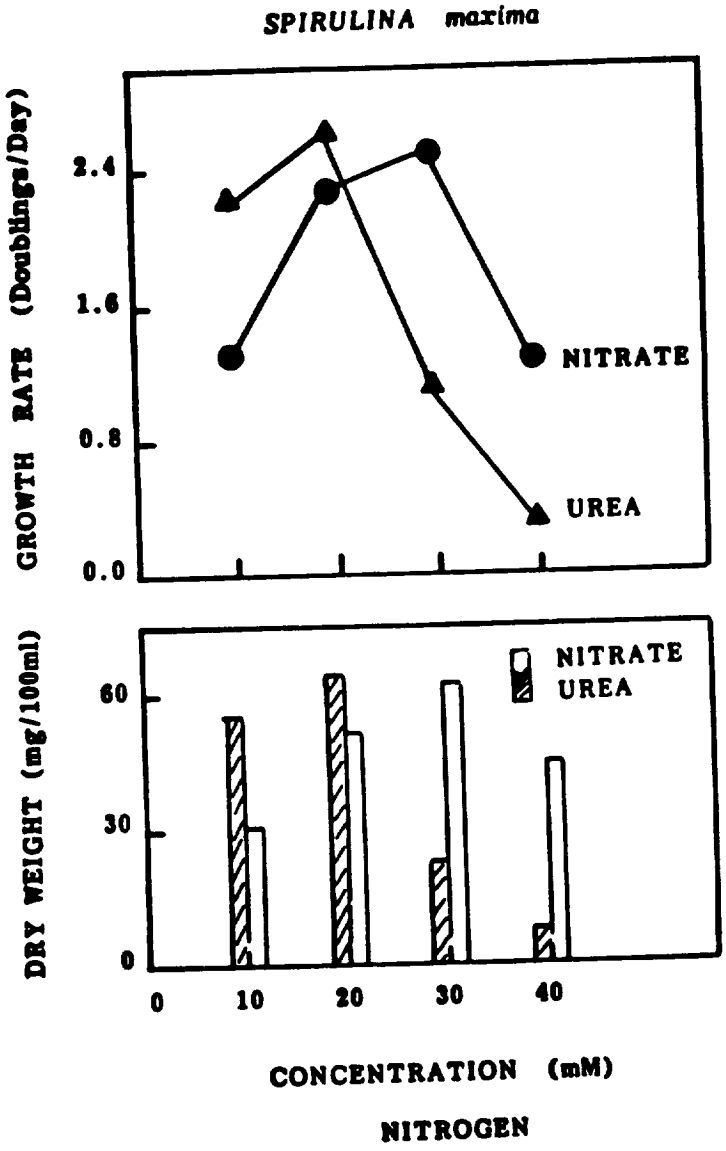

Figure 5. Growth Rate and Yield of S. maxima as a Function of Nitrogen Concentration

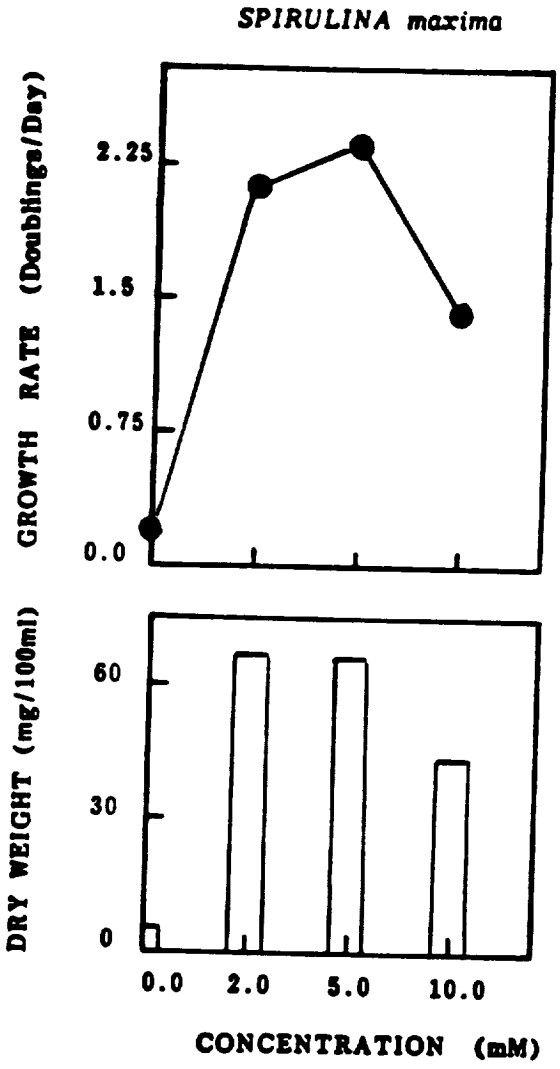

Figure 6. Growth Rate and Yield of S. maxima as a Function of Phosphate Concentration

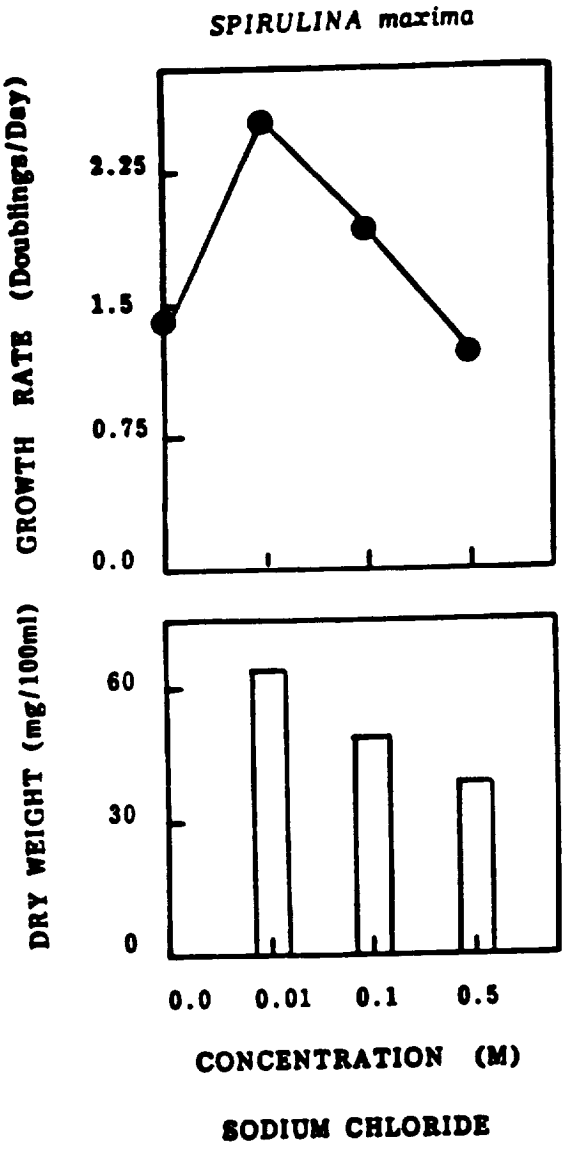

Figure 7. Growth Rate and Yield of S. maxima as a Function of Sodium Chloride Concentration

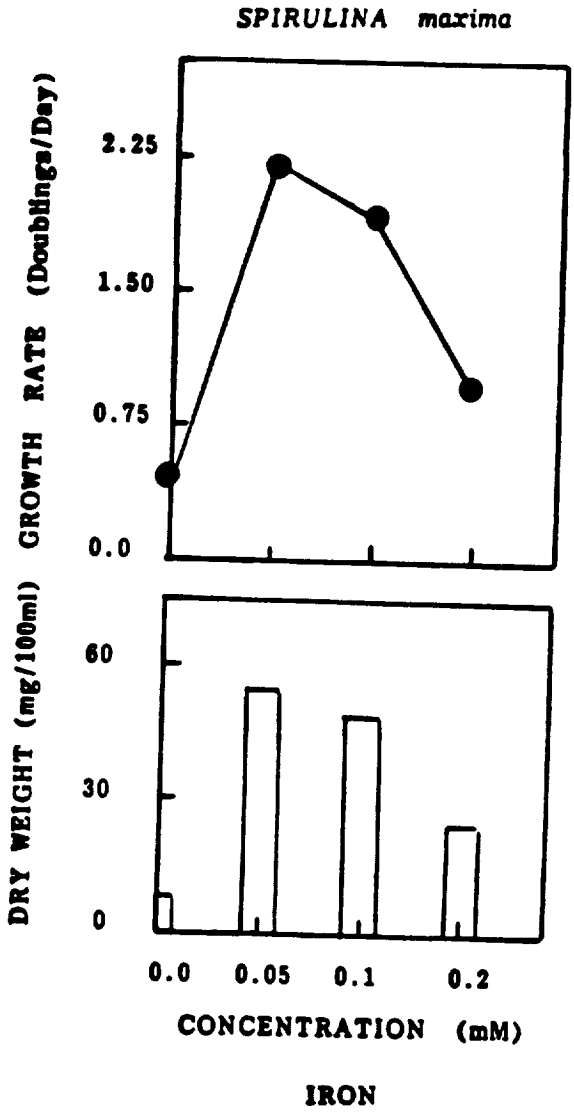

Figure 8. Growth Rate and Yield of S. maxima as a Function of Iron Concentrations

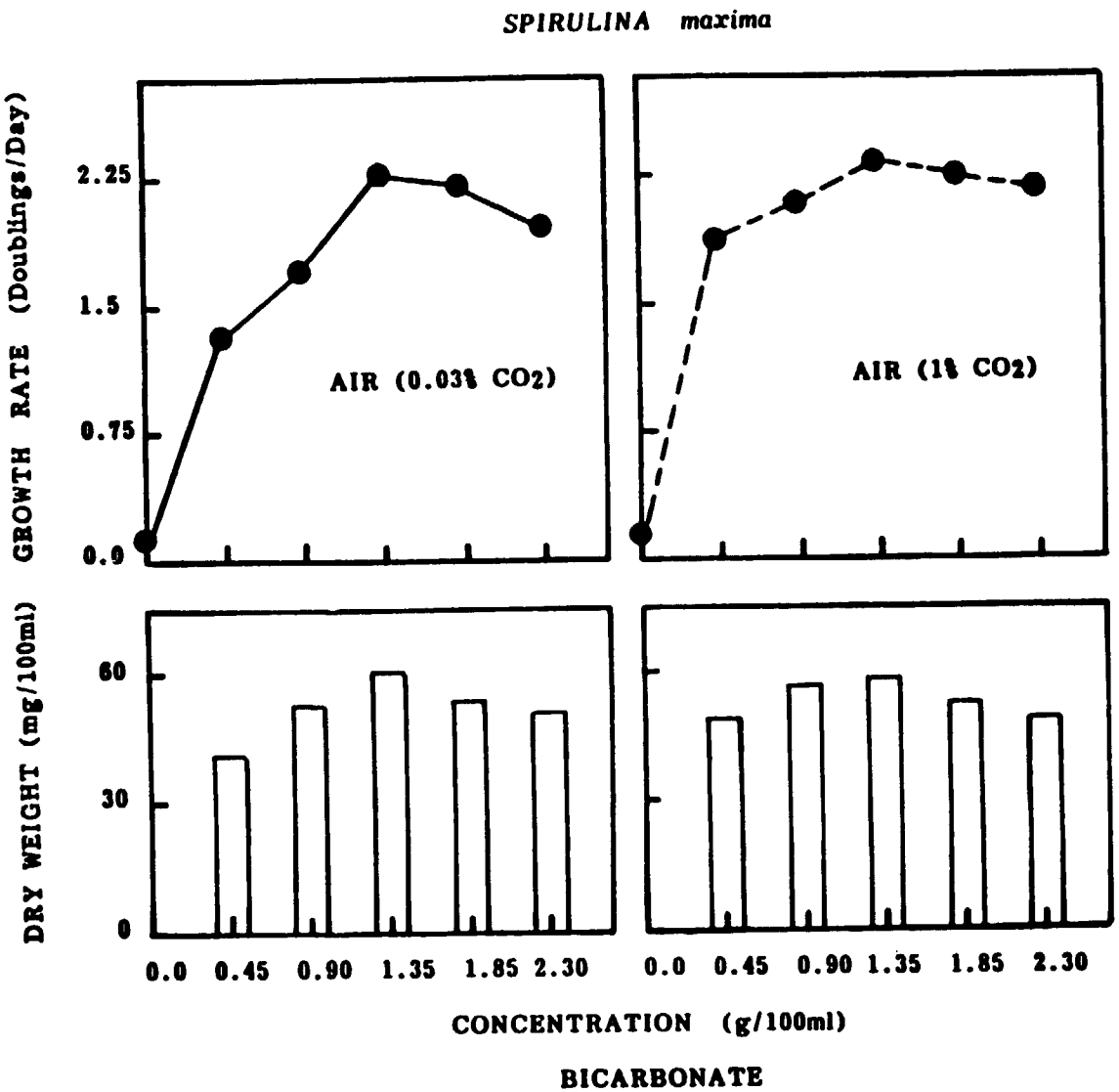

Figure 9. Growth Rate and Yield of S. maxima as a Function of Bicarbonate Concentration

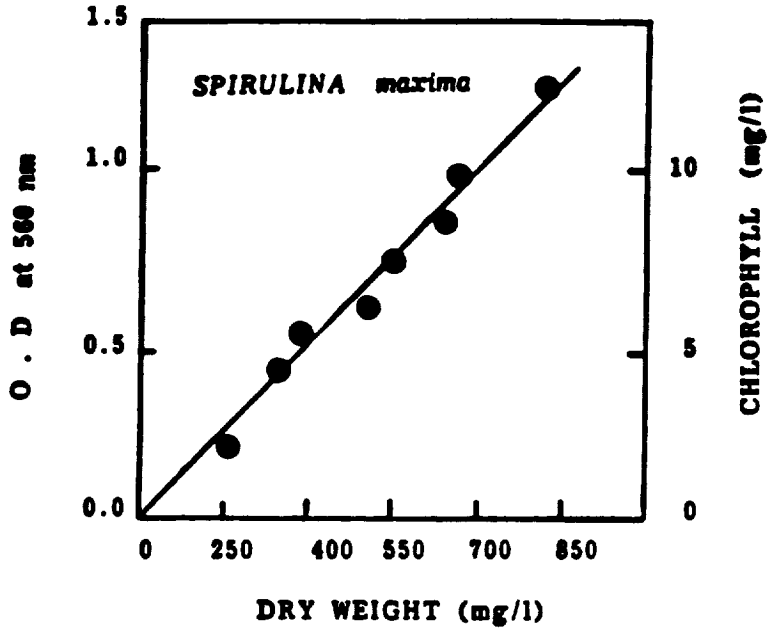

Figure 10. Optical Density versus Dry Weight S. maxima and Total Chlorophyll

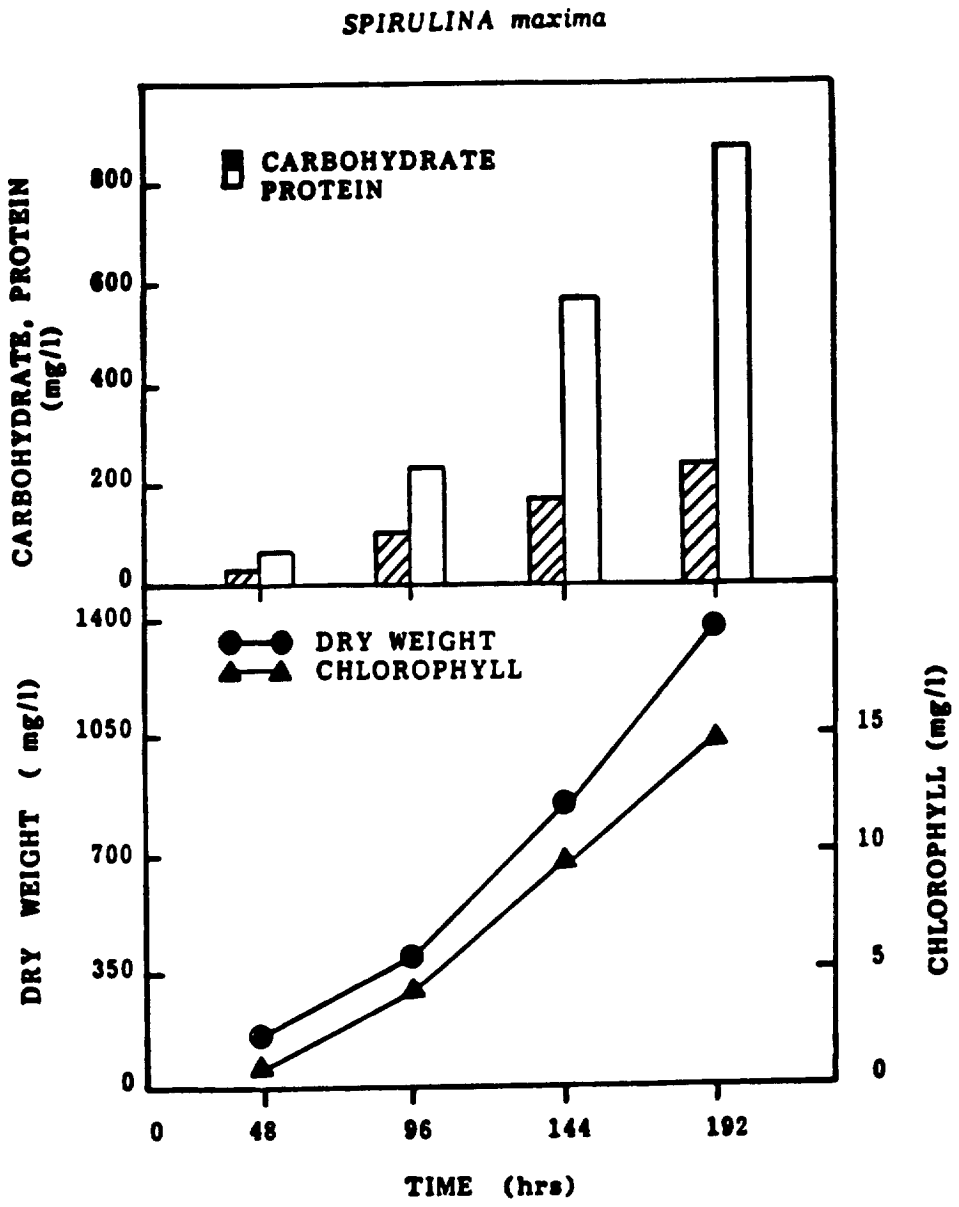

Figure 11. Physiological Characteristics of S. maxima under Optimum Growth Conditions