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MASS CULTURE OF *APHANIZOMENON FLOS-AQUAE* RALFS EX BORN. AND FLAH. VAR. *FLOS-AQUAE* (CYANOBACTERIA) FROM KLAMATH FALLS, OREGON, USA, IN CLOSED CHAMBER BIOREACTORS.

Habte Jebessa Debella[1]

ABSTRACT: *Aphanizomenon flos-aquae* Ralfs ex Born. and Flah. var. *flos-aquae* (Cyanobacteria) was sampled from Upper Klamath lake and subjected to batch culture of 15-litre bioreactors. Growth characteristics viz. doubling time (Dt), specific growth rate (μ), temperature requirement, biomass and chlorophyll-a concentrations were studied during exponential growth phase at a fixed light intensity of 40-45 micromole in a CT medium. CT growth medium when supported by CO_2 from a chemical byproduct in a connected chemical reactor resulted in better growth characteristic than either ASM or ASM-1 in literature. A relationship of dry weight (dw) and absorbance of the culture in CT medium was developed for calculations without the absolute need of the time-consuming dry weight measurements. Maximum measured dry weight obtained at this growth condition after 120 hours is 151 g/m^3. The best growth temperature studied in this work is 20°C with a supply of CO_2. Results are compared with previous findings in the literature and recommendations are made for mass cultivation.

Key words/phrases: CT medium, Mass culture, Strain NH-5, Var *flos-aquae*

INTRODUCTION

Although mass culture of some cyanobacteria of the Order Oscillatoriales for industrial and human food-supplement started as early as the 1950's, *Aphanizomenon flos-aquae* has not been one of them. Some workers conducted culture of *A. flos-aquae* in small glass bottles at different times for different experimental purposes (O'Flaherty and Phinney, 1970; Smith and Foy, 1974) in a nutrient medium named ASM or ASM-1 (Gorham *et al.*, 1964). There is no report in the available literature that *A. flos-aquae* has been mass cultured so far.

Lately, the discovery of a neurotoxin named Saxitoxin in some rare strains of *Aphanizomenon* such as strain NH-5 (Mahmood and Carmichael, 1986), attracted the attention of many workers and the species came under close scrutiny. Studies of *A. flos-aquae* by highly sensitive methods from field samples of Klamath Falls repeatedly showed that this particular species was free of any toxin and hence was marketed after being harvested and

[1] Addis Ababa University, College of Education PO Box 1176, Addis Ababa, Ethiopia
e-mail: hdebella@bio.aau.edu.et

processed under high-tech procedures. The processing plant was equipped with the state of the art filtration, freeze-drying and packaging equipment. A detail of the facility is discussed in Carmichael *et al.* (2000).

Under natural conditions, phytoplankton organisms in open water are prone to contamination by other unwanted alga. When such contamination involves toxic species of phytoplankton such as *Anabaena flos-aquae*, (Mahamood and Carmichael, 1987) and others (Carmichael and Falconer, 1993), quality control issues make harvesting of wild type algae difficult. Moreover, lake history tells us that lake phytoplankton composition can drastically change due to environmental changes, age of the lake and human influences, replacing the normally existing species by other species. If such occurrence happens to Klamath lake, companies relying on wild *A. flos-aquae* for the industrial production of human food supplements will certainly face serious problems. Therefore, the objective of this study was to establish an optimum culture condition of *A. flos-aquae* from Klamath Falls under controlled laboratory conditions as a first attempt for large-scale mass culture of the algae for human food supplement and industrial purposes.

MATERIAL AND METHODS

A non-toxic strain of *Aphanizomenon, Aphanizomenon flos-aquae* Ralfs ex Born. and Flah. var. *flos-aquae* (Cyanobacteria) (hereafter *A. flos-aquae*) was grown in CT nutrient medium (Watanabe and Nozaki, 1994), made up with double distilled water. Water samples were obtained from Upper Klamath lake near Klamath Falls, Oregon in 1999. Samples examined under an inverted microscope were found to contain typical colonies of *A. flos-aquae*, *Microcystis aeruginosa* and some unidentified green algae. Single colonies of *A. flos-aquae* were removed, washed and transferred aseptically to sterile media in test tubes. Several colony isolates were incubated in the laboratory until growth was achieved. The cultures are axenic. ASM-1 (Gorham *et al.*, 1964) and CT media (Watanabe and Nozaki, 1994) were evaluated and the best one was used to culture *A. flos-aquae*. After a few weeks, those test tubes containing a dense population of *Aphanizomenon* were transferred into 500 ml glass bioreactors containing 500 ml sterile media. Cells at exponential growth stages were then transferred into four-liter cylindrical glass bioreactors scaling up. Final experimental volumes were made in 15 L volume, cylindrical transparent glass bioreactor (Bellco Biotechnology, Inc, USA). This bioreactor had two side arms with 45 mm screw caps and 100 mm center neck. Its outside diameter was 294 mm with approximate height of 460 mm. One of the side arm caps was modified to

make sampling outlet while the other was used for air inlet. The center neck was used as air outlet. Samples were taken using 60 ml polyethylene syringes through an inserted polyethylene hose, which was introduced through one of the side caps. The inserted polyethylene was capped using a 0.20 μm pore-size Pall Gelman Sciences Acrodisc Filter. CO_2 was produced in another 4 L reactor from a chemical reaction between $NaHCO_3$ and dilute HCl as follows:

$$2NaHCO_3 + 2HCl \rightarrow 2H_2O + 2Na^+ + 2Cl^- + 2CO_2(g).$$

The pH of the solution in which CO_2 was produced was constantly kept below 5 so that CO_2 gas was always available for the culture. The solution was changed every 24 hours. An air-pipe-line from a remote air compressor was connected to this bottle and the bottle's air outlet was connected to an empty similar reactor by an interface to which the final culture bioreactor was fused. Experiments were made in a digital incubator except at 26±1°C for which the incubator was not necessary. Cultures were grown at the same light intensity (40 - 45 micromole. $m^{-2}s^{-1}$) but variable temperatures (20, 24, 26 and 28 °C). Such low light intensity was chosen in an attempt to mimic the natural environment of Klamath Falls. Light intensities were controlled either by increasing or decreasing the number of cold white florescent lamps in the incubator units. Light intensity was measured outside the flask. Light was supplied on 12:12 hour diel cycle.

Chlorophyll-a was measured by cold methanol extraction according to APHA (1992). Phaeophytin pigment concentration was calculated as in ISO 10260 (1992 (E)). Biomass was measured by filtering 50 ml of culture through a pure dried and weighed GF/C filter paper either at 60°C for 24 hours or at 105°C for 2 hours in an electric oven. The weight difference was taken and converted to standard mass units. Light intensity was measured using Quantum Radiometer/Photometer, Li-COR Model L1-188A (LAMBDA Instruments, Lincoln, Nebraska).

Establishment of the basis of calculations of yields (dry weight)

To calculate dry weight directly from absorbance values, the relationship of dry weight to absorbance was obtained by measuring absorbance values at 436 nm and measuring the dry weight of the same sample. Linear regression analysis showed that they were directly correlated ($R^2 = 0.9884$). Expected dry weight can be calculated either from equation of the curve or can be directly read from the relationship (Fig. 1).

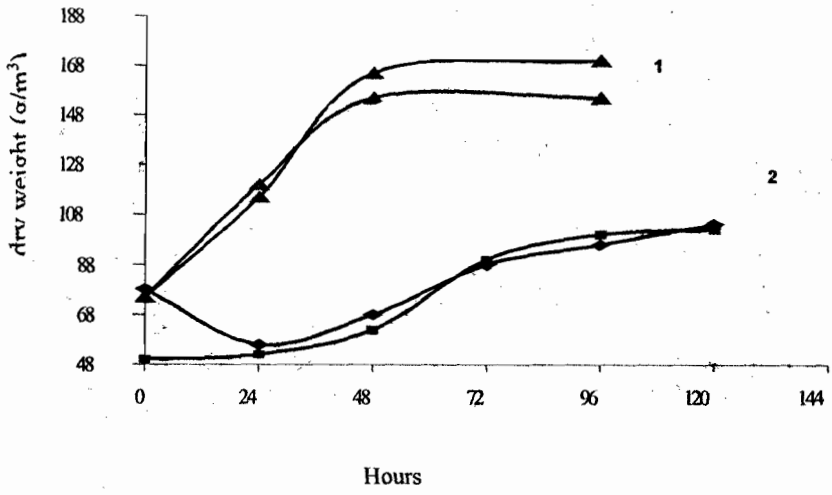

Fig. 1. Growth characteristics of *A. flos-aquae*. Numbers 1 and 2 refer to 28 and 20 degree Celsius growth temperatures, respectively. No CO_2 added.

RESULTS

Calculation of specific growth rate (μ) and the doubling time (Dt)

To calculate the specific growth rate (μ), the least squares regression line was fit to the logarithm of dry weight versus time in hour and converted to per day basis (Guillard, 1973). Growth conditions were analyzed at a fixed light intensity (40-45 micromole m^{-2} s^{-1}) and variable temperatures. Growth temperatures were 20, 24, 26, and 28°C (Fig. 2 and 3).

The equation used to calculate the doubling time was drawn from the exponential growth phase of cultures during the first five days (Figure. 2), as follows unless otherwise mentioned:

$N = N_o e^{\mu t}$

Log N= Log N_o+0.301μt, where N = Final dry weight at time t and N_o = Initial dry weight at the beginning of the experiment. This equation has the same meaning as:

$Y = ai + a_\mu t$,

Where, $\mu = 3.322a_\mu$, per day for equations derived in base 2 logarithms. For details, see Guillard (1973) (Fig. 4).

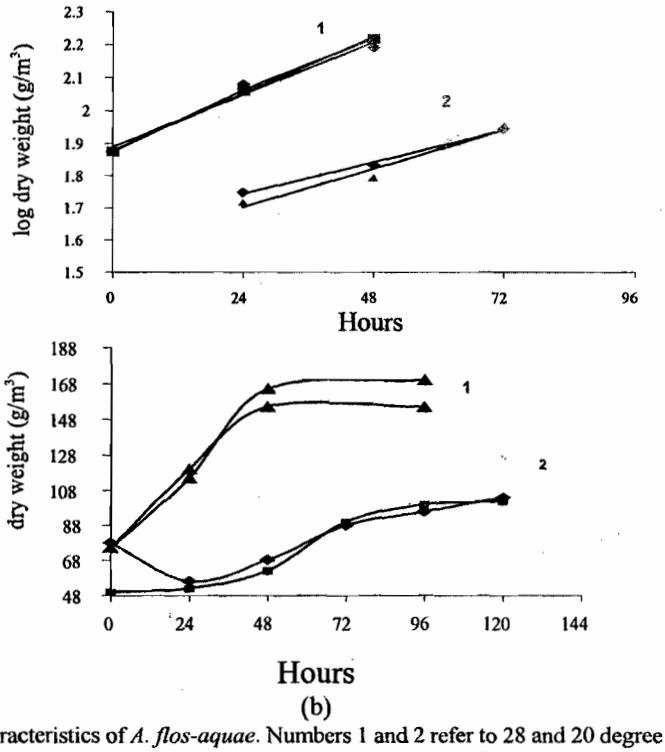

Fig. 2. Growth characteristics of *A. flos-aquae*. Numbers 1 and 2 refer to 28 and 20 degree Celsius growth temperatures, respectively. No CO_2 added.

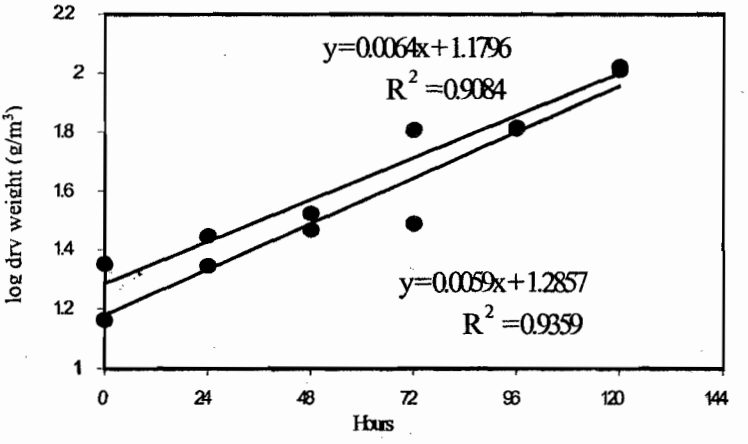

Fig. 3. Dry weight derived from Chlorophyll-a concentration on logarithmic scale; the two curves represent replicate samples. Cultures were grown at 26 +/- 0.5 degree Celsius.

Table 1 Growth characteristics of *A. flos-aquae* under different conditions.

Growth temperatures (C°)	Doubling time (hours)	Specific growth rate, (μ)= 3.322 x slope [a]	Average doubling time (Dt), hours	Logarithmic regression equation [a]	R^2
20	73.53	0.0136	66.89	1. Y=0.0041x+1.6454	0.9934
	60.24	0.0166		2. Y=0.005x+1.5826	0.9589
20	52.81	0.0189	51.08	3. Y = 0.0057x + 1.5465	0.9804
	49.35	0.0203		4 .Y = 0.0061x + 1.5074	0.9690
26	46.95	0.0213	48.99	5. Y=0.0064x+1.1796[a]	0.9084
	51.02	0.0196		6. Y=0.0059x+1.2857[a]	0.9359
28	42.92	0.0233	44.29	7. Y=0.0071x+1.8799	0.9976
	45.66	0.0219		8. Y=0.0066x+1.8906	0.9719

[a] = Equations are derived from converted Chlorophyll-a concentration to dry weight (APHA, 1992) (Figure.4). [b] = CO_2 from a chemical reaction byproduct is applied.

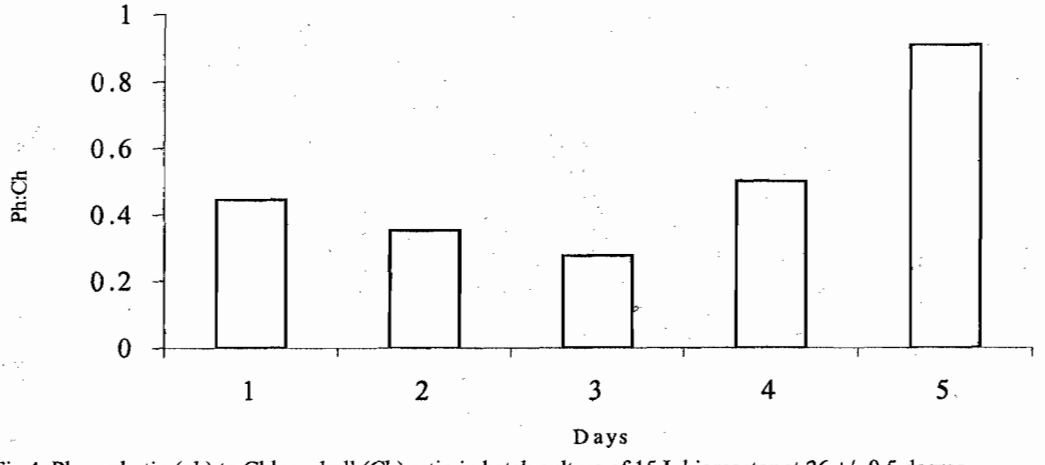

Fig.4. Phaeophytin (ph) to Chlorophyll (Ch) ratio in batch culture of 15 L bioreactor at 26 +/- 0.5 degree Celsius.

Guillard (1973) recommends the use of base 2 logarithm for alga cultures, which yields inversely μ as divisions per day. In this equation, since time was given in hours, μ was multiplied by 24 to provide divisions per day. That is 0.0136 x 24 = 0.33 divisions per day or 72.73 hours per division is required under this given condition (Fig. 5 and 6).

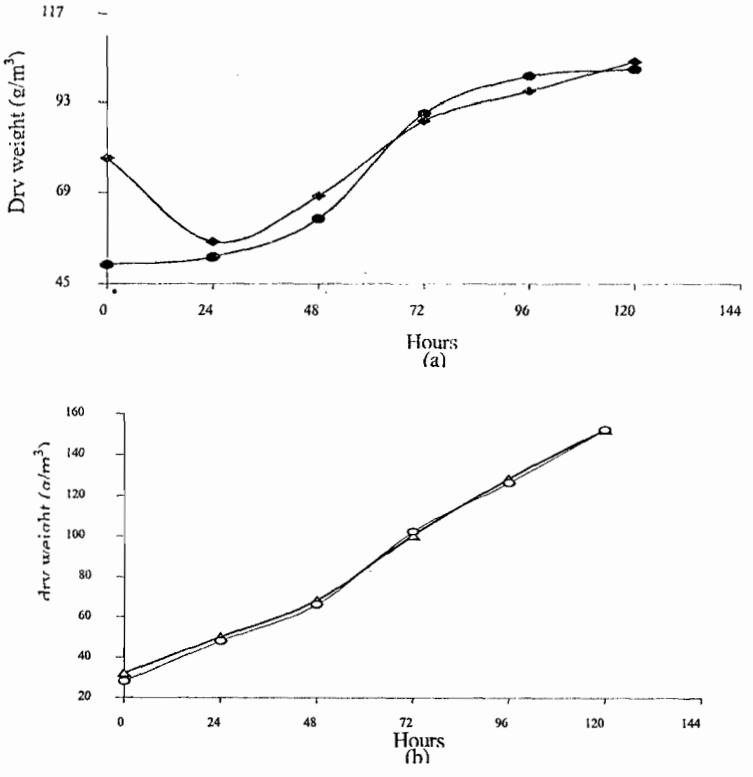

Fig. 5. Growth of *A. flos-aquae* at 20+/-0.5 degree Celsius (a) No extra CO_2 added (b) Extra CO_2 added.

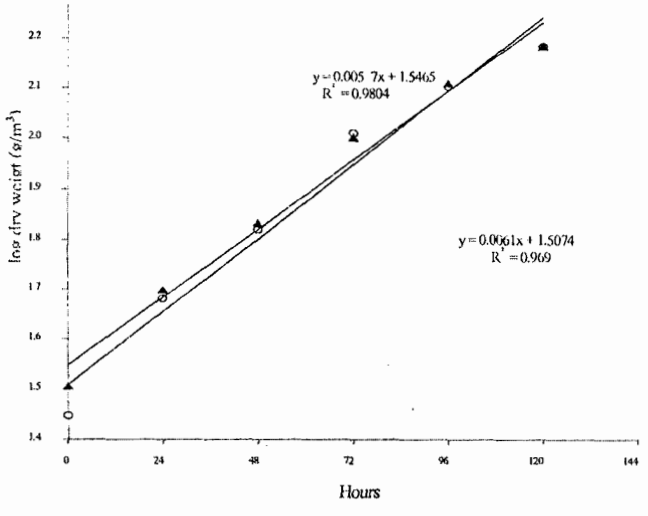

Fig. 6. Addition of CO_2 from a chemical reaction by-product improved growth of *A. flos-aquae* at 20+/-0.5 degree Celsius.

DISCUSSION

Different approaches were used to detect growth characteristics and the physiology of cultures of *A. flos aquae* in 15 L bioreactors. Biomass of culture during the exponential growth period (the first 120 hours) was considered and only the part of the curve that showed the exponential or logarithmic phase has been taken and fitted to the points by the least squares regression analysis. All the correlation coefficients were greater than 0.93 (Table 1). The experiments were conducted until the exponential growth curves reached stationary phases (Fig. 2 and 5). Growth curves without and with gaseous CO_2 showed marked differences at the same temperature (Fig. 5 (a) and (b)). Application of CO_2 removed the lag phase (which is clear from Fig. 6) and improved the growth). This can best be explained by taking dry weight per unit time in both cases. Comparison of productions under the same condition but different temperatures (20 and 28° C) showed marked difference. At 28° C, the curve showed no lag-phase and the stationary-phase point was much higher than at 20°C. Even though the initial innoculum played a role in this graph, there should be further investigation of such a high temperature production for cold-water organisms (Fig. 2). The physiology of the culture was not good during the first 24 hours but started to improve as the exponential growth ascended. This has been shown by the Phaeophytin to Chlorophyll-a ratio at 26 ± 0.5°C (Fig. 4). This is practically possible because the inoculum must adapt to the artificial medium as it is first exposed to laboratory conditions. Different growth temperatures affected the specific growth rates and then the doubling times. Whitton (1973) compared growth conditions of different strains of *A. flos-aquae* all in ASM culture media in small culture flasks. The doubling time and light intensity requirements varied extensively among the strains. On the contrary, there is no data on *A. flos-aquae* grown in CT nutrient medium or on a large scale. CT medium for the strain of *A. flos-aquae* from Klamath falls showed much shorter doubling time when compared to ASM-1 or ASM with similar results in literature (Table 2). Smith and Foy (1974) showed that different buffers had different effects on the doubling time and growth characteristics of different cyanobacteria including *A. flos-aquae*. They found that buffers having higher pKa values, more than 7.0, were much better for algal growth than those with lower pKa value. They thus, concluded that ASM-1 medium was the best medium for all the strains they studied. Since different strains of *A. flos-aquae* from different localities showed different growth characteristics and different doubling times, it is difficult to compare these results with the present work. Whether the doubling time is a reflection of genetic dissimilarity or environmental effect (growth medium and

condition) is not clear at this point. However, it can be seen that CT nutrient medium is much better than ASM or ASM-1 for *A. flos-aquae* from Klamath Falls in comparison to previous works (Table 2). *A. flos-aquae* from Klamath Falls is perhaps one of the strains of this species that, in nature, produces big flakes of colonies that could be picked by hand. After a few months in the laboratory, this strain lost its bundle-like colony character and dispersed. The mystery behind the formation of such a big colony has not been explained so far.

Table 2 Comparison of growth conditions of different strains of *A. flos-aquae* (data and references taken from Whitton, 1973)

Strains	Reference	Medium	Temp (°C)	Light intensity (micromole)	Doubling time (Dt)
A. flos-aquae (Sawyer's)	Whitton (1973)	ASM-1 modified	20	98	13
A. flos-aquae (Sawyers)	Whitton (1973)	ASM-1	26	98	---
A. flos-aquae (Lough Neagh)	Smith and Foy (1974)	ASM-1 Modified	20	449.2±2.0	20.8-22.7
A. flos-aquae (Klamath)	O'Flaherty and Phinney (1970)	ASM	15,20		110
A. flos-aquae (Klamath)	This work with CO_2	CT	20	40-45	52.81 49.35
	This work with no CO_2	CT	20 26 28	40-45	66.89 48.99 44.29

Increase in temperature increased specific growth rate (μ) and hence decreased doubling time (Dt). Since Dt is inversely related to μ (Table 1), this result is in agreement with results of Gentile and Maloney (1969). From growth characteristics of this cold water alga at 20 °C with CO_2 from chemical reaction product, it can be visualized that increase of biomass can be achieved by manipulating or modifying the CT medium. A chemical or physical parameter that is able to assist *A. flos-aque* form those big colonies will certainly increase biomass. Application of CO_2, from a chemical reaction by-product, reduced the doubling time by a magnitude of 24%. Two modifications were made to enhance algal growth: air was conditioned in interface empty bioreactor before entering the culture medium and CO_2 was added from chemical by-product. There was no change in temperature (20 °C). Conditioning air before it enters the culture reduced the velocity of air. It is not clear whether addition of CO_2 or decrease in the velocity of air reduced the doubling time, thus improving the growth rate. It is obvious, however, that the lag-phase has been completely removed from the curve and the exponential growth continued beyond the 120 hours limit which was not observed in the absence of extra CO_2 application. Culture transfer was made during the

culture's exponential phase (between 72 and 96 hours). The disappointing low biomass of algal production under artificial conditions as pointed out by *Spirulina* researchers (Richmond and Grobbelaar, 1986) is still prevailing. Whether this species will be the next candidate to be mass cultured on large scale for human food supplement entails the question of increasing biomass per given volume or area. To curtail this problem and enhance growth condition of *A. flos-aquae*, it is necessary to study and devise a medium buffered by a high pKa value around 9.5. This is deduced from the fact that 95% of the time the pH of Klamath Falls water is around 9.5, which coincides with the high bloom season of *A. flos-aquae* (Kann, 1998). Low light intensity was used intentionally to mimic the natural environment from which the sample was taken at Klamath lake. It should not be excluded, however, that light intensity can play a role in increasing biomass. Exhaustive investigation of light intensity and temperature requirements should be made to judge the optimum requirement of this strain for large-scale mass culture. Perhaps high light intensity at 20°C with CT medium is a promising prospect. Future focus should study the growth characteristics of *A. flos-aquae* in continuous culture and scaling up of the results to outdoor pilot production facilities under controlled conditions.

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