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Influence of Temperature and pH on the Growth of the Thermophilic Cyanobacterium *Mastigocladus laminosus* in Continuous Culture

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The thermophilic cyanobacterium *Mastigocladus laminosus* was grown in a steady state continuous culture. Regulation and data sampling of the turbidostat was done with a microcomputer. Growth performance was measured as a function of temperature and pH from 30 to 62°C and between pH 4 and 10. The temperature optimum was between 45 and 55°C. Under optimal conditions the filaments contained predominantly 8 cells with a high phycocyanin content but at the temperature extremes and at high pH, 2- and 4-cell filaments containing little phycocyanin were more abundant.

Key words: Continuous culture — Cyanobacteria — Growth performance — *Mastigocladus laminosus* — pH — Temperature — Thermophily.

The thermophilic cyanobacterium *Mastigocladus laminosus* is a cosmopolitan which grows in hot springs under a wide range of environmental conditions; at temperatures from 45 up to 65°C and at pH values between 6.5 and 10.5 (Schwabe 1960, Binder et al. 1972). Usually *Mastigocladus* as all cyanobacteria satisfies its need for energy with oxygenic photosynthesis through photolithotrophic growth as higher plants but it may also grow photoorganotrophically. In the dark cyanobacteria show respiratory activities (Stanier and Cohen-Bazire 1977, Binder 1982). Normally the cells of *Mastigocladus* form filaments, however, under extreme conditions they may appear unicellular. Biochemical and physiological investigations of whole cells, cell free membranes and isolated proteins have shown that their structures and functions are also dependent on the growth conditions (Ono and Murata 1979, Bohler and Binder 1980, Wolf et al. 1981, Hirano et al. 1981). These observations have demonstrated the importance to define exact growth conditions before the physiology of the organism can be described.

In the present paper controlled growth of a continuous culture of *Mastigocladus laminosus* in a microprocessor-regulated turbidostat is studied as functions of temperature and pH.

Materials and Methods

Mastigocladus laminosus strain Rr has been isolated from a hot spring in Sudur Reykir, Iceland (Binder et al. 1972) and was cultivated in medium D of Castenholz (1970). Cells grown in a batch culture at 50°C and pH 6, stored at −80°C were used as inoculum. The 2 liter turbidostat system used has been described earlier (Frischknecht et al. 1979). A schematic view is shown in Fig. 1.

The bioreactor system was connected to an Apple II computer (Fig. 1). The measuring signals standardized to 0–20 mA for CO_2 concentration at the air inlet and outlet, temperature,

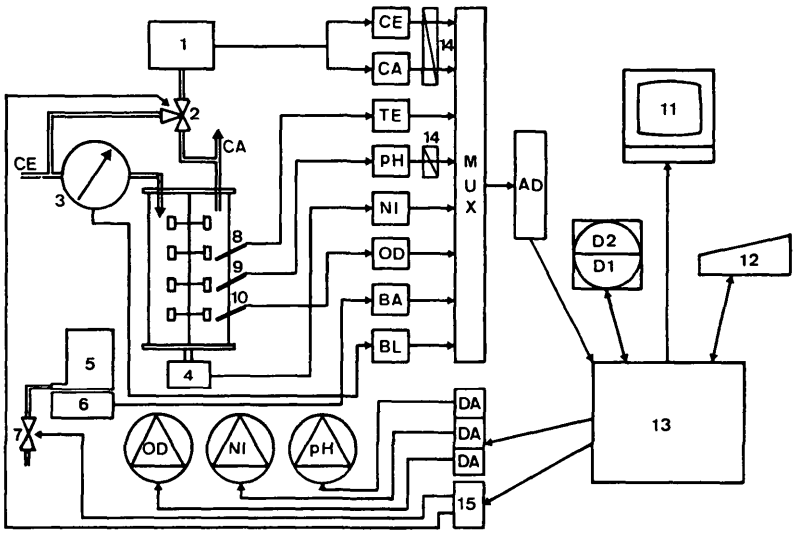

Fig. 1 Scheme of the computer regulated fermenter. (1) IR analyzer for CO_2 concentration; (2) Valve; (3) Gas meter; (4) Stirring motor (level regulation); (5) Weighing flask for culture outlet (production); (6) Digital balance; (7) Valve; (8) Temperature measurement (Pt-100 thermistor); (9) pH electrode; (10) CdS-photoresistor; (11) Monitor; (12) Printer; (13) Computer (Apple II); (14) Amplifier; (15) Reed relay; (D1/D2) Floppy disk drive; (AD) Analog digital converter; (DA) Digital analog converter; (MUX) Multiplexer; (CE) CO_2 concentration inlet; (CA) CO_2 concentration outlet; (TE) Temperature regulation; (pH) pH regulation with peristaltic pump; (NI) Level regulation with peristaltic pump (regulated by stirring motor); (OD) Regulation of turbidity with peristaltic pump (regulated by photoresistor); (BA) Input of electric balance; (BL) Aeration rate.

pH, medium volume, optical density, productivity, balance and aeration rate were sampled under computer control by a Burr Brown MP 21 Analog Input Microperipheral. The 8 bit resolution of this device was sufficient using a sampling rate of 1 kHz and calculating the mean value for each channel from 256 samples. Except for absorption, aeration rate and volume measurements, amplifiers available from the market were used. The optical density probe was a Valvo RPY 71 photoresistor inserted in a glass tube and electrically connected to a Wheatstone bridge. The signal peaks generated by the gas bubbles between light source and probe were damped in a first amplifier stage wired as voltage follower with a 10 μF condenser and a 1 megohm potentiometer at the input. The second stage allowed gain and zero point adjustments. The sensitivity of the absorption measurement was somewhat better compared to the control values taken from a Uvikon 810 photometer (see below).

For level control the current taken up by the stirrer motor was measured as an indirect value for the level depending torque. Assuming a constant aeration rate and a stirrer speed above 1,500 rpm the error for the medium volume was about 10 ml. The aeration rate was measured by a wet gas meter Wohlgroth L-1Cu. To the axis of the gas meter an impulse generator was mechanically coupled delivering 100 pulses per revolution which corresponds to 1 litre of gas. The pulses were counted during 1 min and the result used for the calculation of the flow rate.

Since the computer was used to calculate the pump flow rates (pH control, feeding and outflow) knowledge about the pump setpoints was necessary. Therefore the three pumps were controlled by the computer in a direct digital control mode (DDC). The setpoints were transmitted by a Burr Brown MP 11 D/A converter. The tendency for setpoint oscillation was suppressed resetting the setpoint automatically to the half amplitude after each period.

The valves 2 and 7 (Fig. 1) were switched by digital outputs. Valve 7 emptied the weighing flask whenever the content reached 500 g. Valve 2 switched every 5 min from inlet to outlet CO_2 concentration, measured in a Beckman Model 865 Infrared Analyzer. In order to avoid ground loops, pH meter, stirrer motor and infrared analyzer were galvanically decoupled by isolation amplifiers. All sampled data were stored in selectable intervals on floppy disks for evaluation purposes.

A maximum of 20 fluorescence lamps (Sylvania Daylight, 8 W) surrounding the fermenter were used for illumination giving a maximal light intensity of 25 W/m^2 on the surface of the culture. The dry weight of the culture was determined after sampling cells on a membrane filter (Sartorius SM-40, pore size 0.45 μm) and drying at 105°C (Strickland and Parsons 1978). Absorption spectra were measured on a Uvikon 810 spectrophotometer for the determination of the content of chlorophyll (430 nm), phycocyanin (627 nm) and turbidity (550 nm). Cell number and size were determined microscopically.

Results

A growth curve of *Mastigocladus* under optimal conditions in a batch culture is given in Fig. 2. The generation time during the exponential phase was 7 h. The pre-exponential phase with a cell concentration of 6–12 $\times$ 10^6 cells per ml (0.15–0.2 g dry weight per liter) was chosen for the continuous culture. Under these conditions CO_2 was not limiting, i.e. the fluctuation of

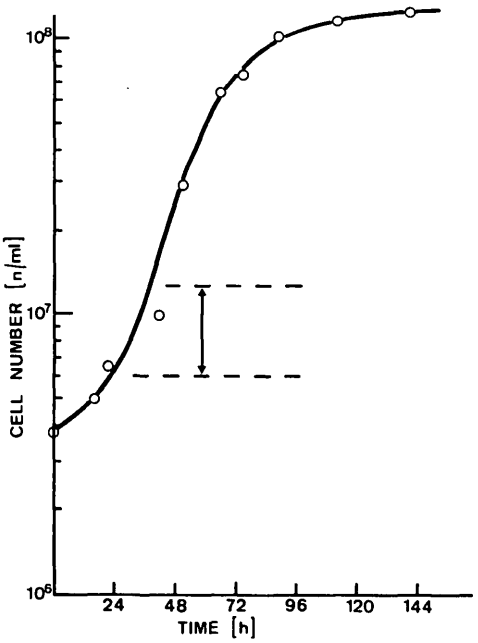

Fig. 2

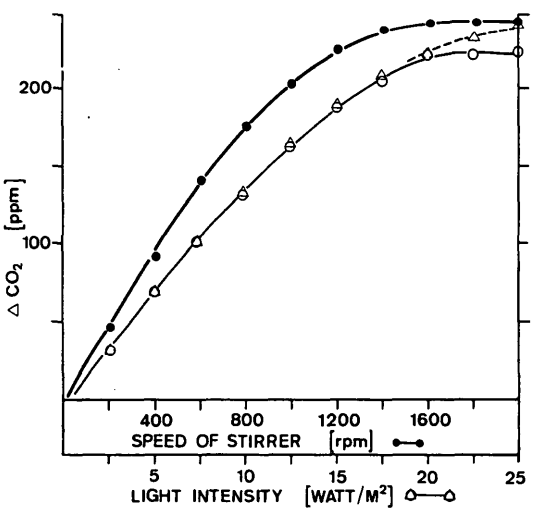

Fig. 3

Fig. 2 Growth curve of a batch culture. The cell number was determined in the microscope as given in **Materials and Methods.** Culture conditions: 50°C and pH 7.2. The arrow indicates the phase of the culture in which the continuous culture was kept thereafter.

Fig. 3 Influence of light intensity and stirrer speed on CO_2 uptake in the continuous culture. Influence of light intensity at culture density A_{550}=0.68 (-○-) and at culture density A_{550}=2.1 (--△--); influence of stirrer speed (-●-). Other conditions were the same as in Table 1.

Table 1 Conditions for the steady state continuous culture

Parameters	Values
Dry weight	0.15–0.2 g/liter
A at 550 nm	0.6–0.8
Specific cell number	6×10^6–1.2×10^7/ml
Stirring speed	2,000 rpm
Illumination	20 lamps (25 W/m²)
CO_2 conc. at air inlet	350–450 ppm

the CO_2 concentration in the inlet and outlet were parallel and a rest concentration of CO_2 (100–200 ppm) in the gas outlet was always kept (Fig. 4). Fig. 3 shows that a stirring speed of 2,000 rpm was not limiting i.e. a faster speed does not increase the CO_2 uptake. Thus the culture was aerated with air only (350–450 ppm CO_2). In addition, this low CO_2 concentration enabled an exact measurement of the CO_2 uptake. For optimal growth the light of 25 W/m^{-2} was saturating (Fig. 3). The growth conditions discussed above are summarized in Table 1.

For CO_2 measurements it was necessary to pump the medium first in a reactor which was run under identical conditions as the main fermenter (pH, aeration, temperature, stirring) but without organisms, in order to preequilibrate CO_2, bicarbonate and carbonate in the medium.

Growth performance was measured from pH 4 to pH 10. At each pH the temperature profile from 30 to 62°C was taken and each state was kept for 36 h. Good growth has been observed between pH 5 and 9, whereas at pH 4 and pH 10 the cells were dying rapidly. An example for the influence of temperature change on CO_2 uptake of the cells is given in Fig. 4. Switching from 42 to 46°C, the CO_2 concentration in the outlet decreased from 260 ppm to 190 ppm. The curve shows that the new steady state was reached only after 12–14 h. The similar time dependence was also found after a pH change (data not shown). Hence, steady state data were taken between 24 and 36 h after the change of pH and temperature.

The influence of pH and temperature on the doubling time of the cells in the continuous

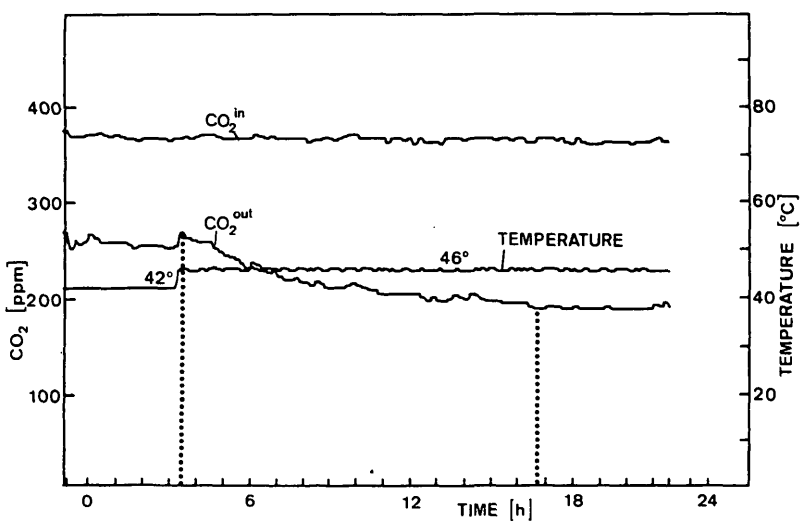

Fig. 4 Time course of the change of CO_2 uptake after a temperature jump in the continuous culture. Direct computer plot of the CO_2 concentration in the air inlet and outlet as well as the temperature during 24 h. The temperature jump was from 42 to 46°C at pH 7.

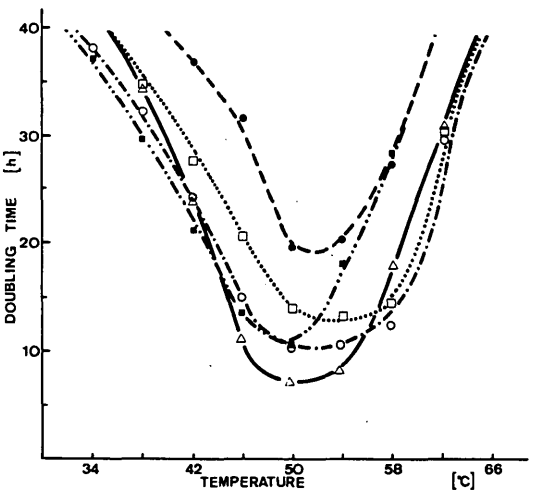

Fig. 5 Doubling time of the cells as a function of temperature and pH in the continuous culture. The doubling time is calculated according to the following formula:

$$t_d = \ln 2/D$$

(t_d) doubling time in h; (D) dilution rate. (--●--) pH 5; (···□···) pH 6; (-△-) pH 7; (---○---) pH 8; (----■----) pH 9.

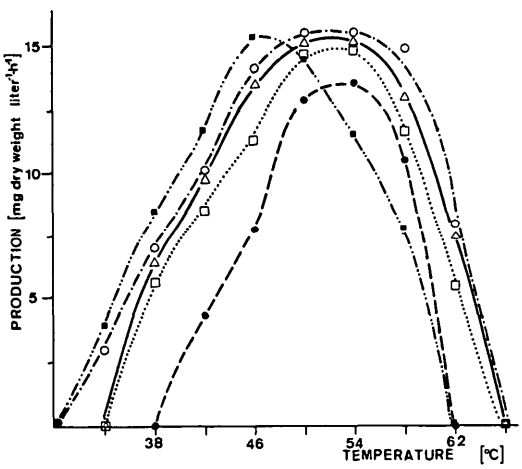

Fig. 6

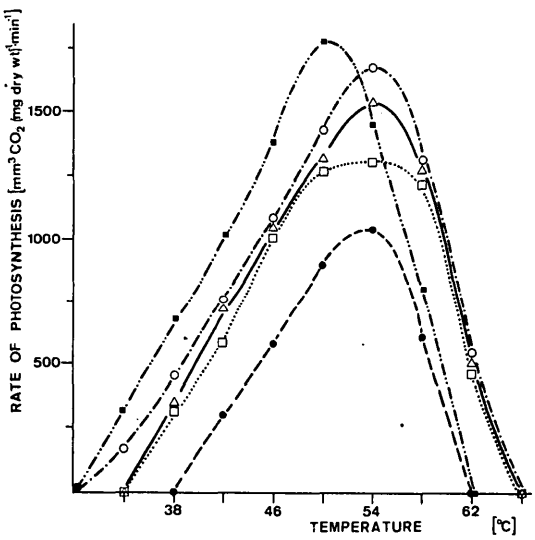

Fig. 7

Fig. 6 Biomass production as a function of temperature and pH of the continuous culture. The biomass production is calculated according to the following formula:

$$P = W \times D$$

(P) biomass production in mg dry weight liter^{-1} h^{-1}; (W) dry weight in mg liter^{-1}; (D) dilution rate. The symbols of the curves are explained in Fig. 5.

Fig. 7 Rate of photosynthesis as a function of temperature and pH in the continuous culture. The rate is calculated from the CO_2 uptake of the cells and is defined in the following formula:

$$L_{N,CO_2} = \frac{R \cdot C_1 \cdot \Delta CO_2}{W \cdot V_L} \text{ in mm}^3 \text{ g dry weight}^{-1} \cdot \text{min}^{-1}.$$

$$C_1 = \frac{P_Z \cdot T_S}{T_Z \cdot P_S}$$

(L_{N,CO_2}=specific gas exchange rate, R=aeration rate, $\Delta CO_2 = CO_2$ concentration inlet minus CO_2 concentration outlet in ppm, W=dry weight in g liter^{-1}, V_L=culture volume, P_Z=barometer pressure in mm Hg, P_S=standard pressure=760 mm Hg, T_Z=temperature of air, T_S=standard temperature=273°K). The symbols of the curves are explained in Fig. 5.

culture is shown in Fig. 5. The shortest doubling time of 6.8 h was observed at pH 7 at 50°C. At pH 8 a much broader minimum of about 10 h was observed, i.e. from 46 up to 58°C. The dry weight of the cells shows also a broad maximum between 48 and 56°C at a pH from 6 to 8 (Fig. 6). This maximum was clearly shifted and narrower at pH 9 (shifted to 48°C) and at pH 5 (shifted to 54°C).

The rate of photosynthesis calculated from the CO_2 uptake of the cells increased when the pH was increased from 5 to 8. In this pH range the temperature maximum lied at 54°C, whereas at pH 9 the maximum was lowered to 50°C (Fig. 7). The same temperature dependency was seen for the biomass production. It is striking that the rate of CO_2 uptake at 54°C increased between pH 6 and 8 while the dry weight measured for the calculation of the biomass production was almost constant. This may be explained by the fact that the CO_2 uptake measurement included the net CO_2 taken up by the cell, while for biomass production only the carbon within the cell was measured. At higher pH values soluble secondary products (e.g. slime) were possibly excreted into the medium and were not trapped on the membrane filter for dry weight measurement.

The change of pH and temperature not only altered the growth performance but also the number of cells per filament and the pigmentation. Between pH 6 and 7 and the optimal

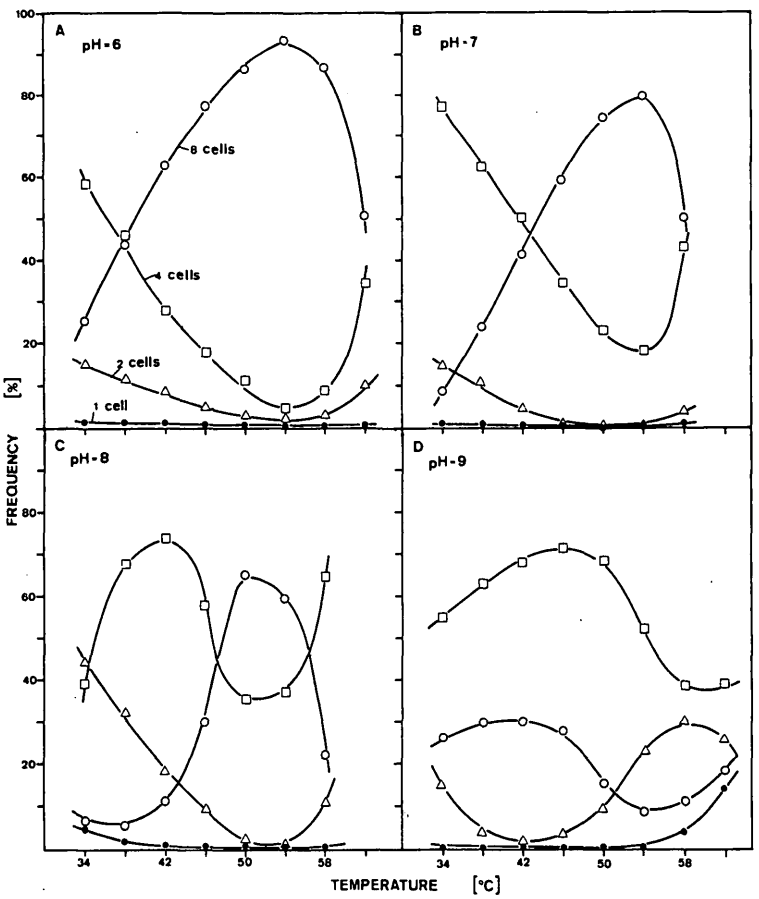

Fig. 8 Number of cells per filament as a function of temperature and pH in the continuous culture. The relative frequencies are given in % of the total number of cells. Length of filaments: (●) single cell; (△) 2 cells; (□) 4 cells; (○) 8 cells and more.

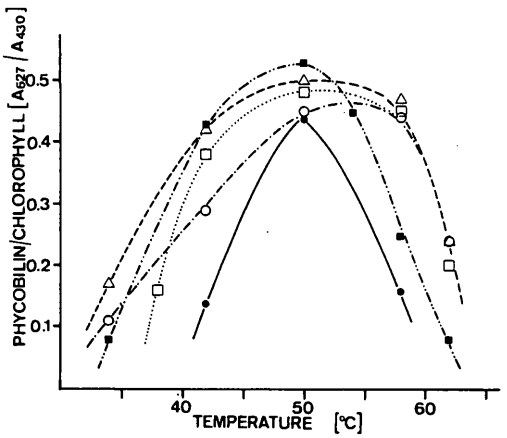

Fig. 9 Influence of temperature and pH during growth on the phycobilin to chlorophyll ratio of the cells. The phycobilin to chlorophyll ratio is measured as the absorbance ratio at 627 nm to 430 nm. The symbols of the curves are given in Fig. 5.

temperature (54°C), 80–95% of the filaments consisted of 8 cells and more (Fig. 8), whereas towards the upper and lower temperature extremes, 2 and 4 cells per filaments were more frequent.

The ratios of 2-, 4- and 8-cells per filament changed drastically when the pH was increased from 7 to 9 whereby 4-cell filaments get predominant and 8-cell filaments decreased to 10–30%. In general 8-cell filaments were predominant at optimal growth conditions, i.e. at optimal pH and temperature, whereas 2- and 4-cell filaments preponderated at non-ideal and extreme conditions. The cells could be counted at pH 5 at all temperatures because they did not form filaments but rather amorphous aggregations without clear distinction between the individual cells.

Upon changing pH and temperature of the culture, the cells changed their color between blue green and light yellow. The phycobilin to chlorophyll ratio (Ph/Ch=A_{627}/A_{430}) of the experiments discussed above is shown in Fig. 9. These curves demonstrate that at optimal temperatures (45–55°C) the cells had a high phycobilin content (Ph/Ch=0.5) which gave the culture a blue green colour. At the temperature extremes however the phycobilin content decreased to a phycobilin to chlorophyll ratio of 0.2–0.08, thus making the cells to appear yellow.

Discussion

In the natural environment of the hot springs, good growth of *Mastigocladus* is found between 45 and 60°C and at pH values from 7.0 to 9.5 (Binder et al. 1972). This growth spectrum does not fully coincide with the one measured in the fermenter cultures. In the fermenter cells grow at temperatures as low as 34°C. In nature this is not observed probably because below 45°C other species (*Phormidium*, *Oscillatoria*) are more competitive and suppress the growth of *Mastigocladus*. In nature *Mastigocladus* is never found below pH 7, whereas in the fermenter growth is observed as low as pH 5. The reason is not clear yet but one has to keep in mind that the composition of the medium in the fermenter is different from the one of the water in the hot spring. Furthermore it is possible that hot spring water below pH 7 contains inhibitory compounds which prevent growth of cyanobacteria. In the hot springs *Mastigocladus* typically grows well up to pH 9.5, whereas in the fermenter the cells die at this pH. The reason for this discrepancy might also be caused by the difference in the composition of the culture medium and the water of hot springs.

It is demonstrated in the fermenter cultures that *Mastigocladus* as a typical filamentous

cyanobacterium is not susceptible to the shear forces of vigorously stirring. On the contrary, smoothly stirred cultures grow slower but contain longer filaments (unpublished observation). The length of the filaments is not only dependent on this physical stress, but also on the doubling time. In slow growing cultures at non-ideal conditions the cells tend to get more globular and to dissect more into smaller cell aggregates or even to the unicellular form. On the other hand under ideal growth conditions the cells divide rapidly enough to form longer filaments.

The obvious variation of the phycobilin content, which depends on the growth conditions, demonstrates that *Mastigocladus* possesses a sensitive mechanism for adaptation. Yet this regulation is surely not restricted to the phycobili proteins but there exists probably a subtle tuned mechanism in such organisms with broad growth spectra to adapt their physiology to the actual environmental conditions.

The computer regulated fermenter system described here gives the possibility to determine the exact growth parameters of continuous cultures under variable environmental conditions such as pH and temperature. *Mastigocladus* is a well suited cyanobacterium for these investigations: sturdy, insensitive to physical stress and contaminations, fast growing and with a broad temperature and pH spectrum. The well defined cultures of *Mastigocladus* described here represent an ideal starting material for the study of regulation mechanisms and the isolation of membranes and proteins. Such investigations are currently under way.

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