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Upper temperature limits for growth and diazotrophy in the thermophilic cyanobacterium HTF *Chlorogloeopsis*

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Abstract. The effect of temperature and oxygen on diazotrophic growth of the thermophilic cyanobacterium HTF (High Temperature Form) *Chlorogloeopsis* was investigated using cells grown in light-limited continuous culture at a dilution rate of $0.02\ h^{-1}$. Diazotrophy was more sensitive to elevated temperatures than growth with combined nitrogen. The maximum temperature for growth of cultures gassed with CO_2-enriched air was more than 55 °C but less than 60 °C with N_2 as the sole nitrogen source, but between 60 °C and 65 °C when nitrate was present in the medium. The effect of temperature on nitrogenase activity, photosynthesis and respiration in the dark was determined using cells grown at 55 °C. Maximal rates of all three processes were observed at 55 °C and rates at 60 °C during short-term incubations were not less than 75% of the maximum. However, nitrogenase activity at 60 °C was unstable and decayed at a rate of $2.2\ h^{-1}$ under air and at $0.3\ h^{-1}$ under argon. Photosynthesis and respiration were more stable at 60 °C than anoxic nitrogen fixation. The upper temperature limits for diazotrophic growth thus seem to be set by the stability of nitrogenase.

Key words: *Chlorogloeopsis* – Continuous culture – Cyanobacterium – Nitrogen fixation – Oxygen effects – Photosynthesis – Respiration – Temperature effects – Thermophile

The capacity to fix dinitrogen is distributed among a wide variety of prokaryotes, including several thermophilic types. The present upper temperature limit for nitrogen fixation is 65 °C observed with the methanogenic archaea *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* (Belay et al. 1984; Fardeau et al. 1987). Nitrogenase activity at 60 °C has been reported in *Clostridium thermosaccharolyticum* (Bogdahn and Kleiner 1986) and for heterotrophs in microbial mats under anaerobic conditions (Wickstrom 1984).

Cyanobacteria capable of nitrogen fixation have been observed in hot spring mats at 55 °C but not at 60 °C. (Stewart 1970; Wickstrom 1980). These are usually classified as *Mastigocladus* sp. and are heterocystous forms with cell division in two planes. Castenholz (1978) reported that, at the highest temperatures, a type designated as HTF (High Temperature Form) *Mastigocladus* was found. This form has cell division in two planes but does not form branched filaments like the *Mastigocladus laminosus* found at slightly lower temperatures, and thus in the classification system of Rippka et al. (1979) it belongs to the genus *Chlorogloeopsis* (Castenholz 1989). HTF *Chlorogloeopsis* forms mats in hot springs with a combined nitrogen source up to a temperature which may be constantly as high as 62–64 °C and specific enrichment is possible at 60 °C in the light without combined nitrogen. In contrast, *Mastigocladus laminosus* has an upper limit for diazotrophic growth of 57–58 °C (Castenholz 1978).

At present, there is little information about nitrogen fixation by thermophilic cyanobacteria in pure culture. *Mastigocladus laminosus* has been reported to have an optimum for nitrogen fixation at 45 °C and an upper limit for diazotrophic growth of 55 °C (Miyamoto et al. 1979) but HTF *Chlorogloeopsis* has not been previously investigated. We report here the effects of temperature and oxygen on diazotophic growth and nitrogen fixation in HTF *Chlorogloeopsis*.

Material and methods

Organism and culture methods

A culture (W-St. Helens 88-HTF) containing HTF *Chlorogloeopsis* together with *Chloroflexus* sp. and heterotrophic bacteria, collected from thermal streams on Mount Saint Helens, Washington State, USA in 1988, was received from R. W. Castenholz. An axenic clonal strain, DM, was obtained by repeated serial dilution of sonicated cultures in $BG\text{-}11_0$ medium (Allen 1968) and incubation at 52 °C.

Abbreviations: chl, chlorophyll a; DCMU, N′-(3,4-dichlorophenyl) N,N-dimethylurea; Taps, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid

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Culture purity was confirmed by phase contrast microscopy and plating on BG-11_0 containing tryptone (1 g/l), yeast extract (1 g/l) and glucose (1 g/l).

The cyanobacteria were grown in continuous culture at 55 °C at a dilution rate of 0.02 h^{-1} in a bioreactor similar to that described by Iversen et al. (1989) which was equipped with a condenser on the gas exit to reduce the loss of water from the culture. The growth medium was BG-11_0 supplemented with 27 nM $NiCl_2$ and 5 mM $NaHCO_3$. In some experiments 1.5 g/l $NaNO_3$ was added as a nitrogen source. The culture (0.8 l) was stirred at 300 rpm and sparged with 1% CO_2 in air at a rate of 0.5 l/min. The pH measured during growth was 7.8–8.0. The culture was illuminated by two 32 W circular fluorescent tubes (full spectrum Vita Lite, Duro-Test Corp., North Bergen, N.J., USA) which gave an average photosynthetic photon flux density (400–700 nm) at the inside of the glass vessel of 120 μmol m^{-2} s^{-1}. Growth on the inner surface of the bioreactor was minimized by including about 50 glass beads (5 mm diameter) and vigorously shaking the glass vessel with a circular motion for a few minutes each day so that the beads exerted a scouring effect on surface growth.

Acetylene reduction assays

All measurements were made with cells directly removed from the bioreactor and maintained at 55 °C before assays. *In vivo* nitrogenase activity was measured using the acetylene reduction assay in 60 ml vials closed with rubber serum stoppers. The gas phase was made up of 20% acetylene in argon with a total pressure of 1 atm at room temperature. The vials were incubated in a thermostatted waterbath with a reciprocal shaker and illuminated from the bottom by 4 fluorescent tubes. The light intensity (photosynthetic photon flux density [400–700 nm]) was adjusted to 200 μmol m^{-2} s^{-1} which gave maximal rates of acetylene reduction. Reactions were started by a addition of cyanobacterial suspension.

Ethylene production was determined by gas chromatography using an HNU 321 gas chromatograph fitted with a flame ionization detector and a HayeSep T column (length: 1 m, i.d.: 2 mm). The carrier gas was N_2 and the column temperature was 100 °C. The flow rate of the carrier gas was adjusted to give retention times for ethylene and acetylene of 30 s and 50 s respectively. The signals from the GC were captured by a microcomputer for automatic calculation of peak areas. The gas phase in the vials was sampled through a narrow bore needle with a syringe at room temperature and in the calculation of absolute rates of acetylene reduction it was assumed that the amount of water vapor withdrawn from the vial was insignificant.

Measurements of inactivation by oxygen

In experiments where the short term effect of a temperature shift on nitrogenase activity was investigated, 60 ml vials containing teflon-coated stirring bars were filled at room temperature with a gas phase (1 atm) of argon or air. Six ml of the gas phase was exchanged with argon-flushed cyanobacterial suspension containing 10 μM DCMU at 55 °C. The vials were then placed on a multipoint stirring apparatus set at 300 rpm which was submerged in a waterbath at the desired temperature. The cyanobacteria were incubated in the dark for the appropriate time at various temperatures and then returned to a waterbath at 55 °C. The gas phase was then exchanged with argon and illumination started. Activity measurements were started by addition of acetylene corresponding to 20% by volume.

Measurements of oxygen evolution and uptake

Oxygen was measured by membrane introduction mass spectrometry using a quadrupole mass spectrometer (Hal 100, Hiden Analytical Ltd., Warrington, UK) connected through flexible vacuum tubing to an inlet with 10 holes (0.4 mm) convered with silicone tubing (Cox 1987). The signals from the mass spectrometer at $m/z = 32$ was recorded by a microcomputer and calibrated at the desired temperature using water in equilibrium with a bubble of air. The vapor pressure of water was included in the calculations and the solubility data for oxygen published by Wilhelm et al. (1977) were used.

Cell suspensions (10 ml) were removed from the bioreactor and transferred to a thermostatted measuring chamber of the type described by Jensen and Cox (1988). Taps NaOH pH 8.0 was added from a 0.5 M stock solution to a final concentration of 10 mM. Before starting an experiment, the suspension was degassed by evacuating the stirred suspension for 1 min to avoid bubble formation as a result of oxygen evolution during the experiment. The maximum capacity for oxygen evolution was determined under continuous illumination with yellow light from two diametrically opposed projectors. The light intensity was adjusted with neutral density filters to give the maximum rate of O_2 production (photosynthetic photon flux density (400–700 nm) of 1.2 mmol m^{-2} s^{-1} on the surface of the reaction chamber). Respiration in the dark was measured by following the consumption of oxygen evolved during a period of illumination.

Chlorophyll a determination

One-milliliter samples were extracted with 9 ml methanol for 10 min at 60 °C. The concentration of chlorophyll a was determined from the absorbance of the centrifuged extract at 665 nm using an extinction coefficient of 74.5 l g^{-1} cm^{-1} (Mackinney 1941).

Results

Effect of temperature on growth in nitrogen-containing and nitrogen-free media

The cyanobacteria were routinely grown at 55 °C using a dilution rate of 0.02 h^{-1} which was between a half and one third of the estimated rate of exponential growth under non-light limited conditions. Under these conditions a cell yield of 8 ± 1 mg/l chl was obtained in the light-limited continuous culture. Attempts were made to obtain a stable culture at 60 °C by raising the temperature in steps of 1 °C at approximately 24 h intervals, starting from 55 °C. This was successful with nitrate in the growth medium, where a stable culture was maintained for several weeks at 60 °C. Under diazotrophic conditions washout of the culture was observed starting at 56–57 °C. Attempts to obtain a stable culture with nitrate at 65 °C were carried out in the same way, but growth at this temperature could not be obtained.

These experiments were performed with a gas phase containing 1% CO_2 in air. The critical maximum temperature for diazotrophic growth was apparently a few degrees higher when the gas phase was 1% CO_2 in N_2 although growth at 60 °C could still not be obtained (results not shown). Since the cyanobacteria produce oxygen during photosynthesis, the culture is not anoxic even if a gas phase without oxygen is used. We estimated a steady state value of around 50 μM for the dissolved O_2 concentration in the steady state, on the basis of measurements on samples withdrawn from the bioreactor.

Effect of temperature on nitrogenase activity

Cells grown diazotrophically at 55 °C were used to investigate the temperature dependence of acetylene

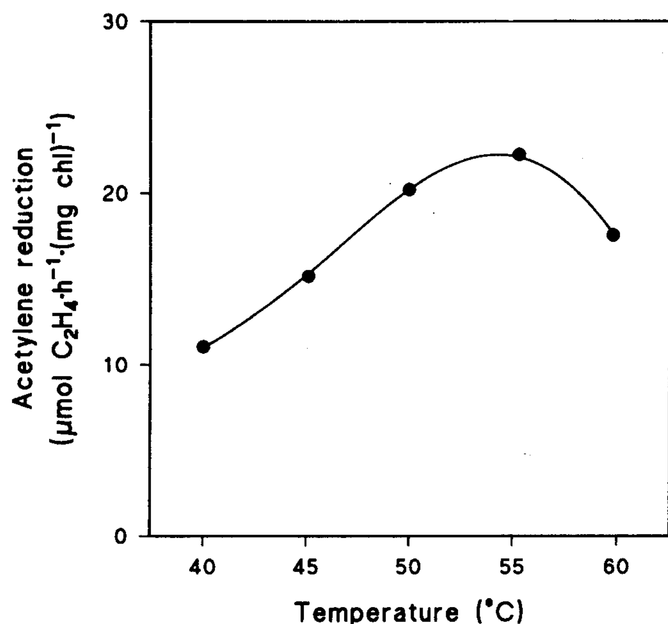

Fig. 1. The effect of temperature on nitrogenase activity measured under anoxic conditions. Cyanobacterial suspensions (7.5 µg/ml chl) were flushed with argon and buffered by addition of Taps/NaOH pH 8.0 from a 0.5 M stock solution to a final concentration of 10 mM. 10 µM DCMU was added from a 10 mM solution in ethanol to inhibit oxygen production. Assays were started by additon of 6 ml cyanobacterial suspension to the vial. Further details are given in the 'Methods' section

reduction. In preliminary experiments, a series of different acetylene concentrations were used and the results were fitted to the Michaelis-Menten equation using non-linear regression. K_m for acetylene reduction at 55 °C was 0.6 mM when an argon atmosphere was used. Thus using 20% acetylene by volume (corresponding to 5.2 mM acetylene in solution at 55 °C) (Wilhelm et al. 1977) the concentration is well above the value for K_m. When acetylene in air was used, higher K_m values were observed and activities measured at temperatures higher than 55 °C were unstable. Measurements of activity were therefore performed under anoxic conditions, where the activity was sufficiently stable in the temperature range employed. Generation of photosynthetic oxygen in the light was avoided by the presence of 10 µM DCMU which was shown to completely inhibit O_2 evolution. This did not result in reductant limitation for at least 2 h at the growth temperature since the rate of acetylene reduction was constant for this period and identical to that in the absence of DCMU.

The effect of temperature on nitrogenase activity is shown in Fig. 1. The optimum temperature is 55 °C and the activity at 60 °C, where diazotrophic growth could not be observed, is 79% of the maximum rate.

Effect of temperature on photosynthesis and respiration

The effect of temperature on photosynthesis and respiration was also studied using cells grown diazotrophically at 55 °C. Oxygen exchange was measured in a closed reaction vessel using membrane introduction mass spectrometry.

In preliminary experiments, rates of oxygen evolution and oxygen uptake were observed during several consecutive light-dark cycles in which the peak oxygen concentration in each cycle was increased. The rate of oxygen uptake in the dark period increased with increasing oxygen concentration. At 55 °C, the rate with 200 µM O_2 was 1.9 times the rate observed at 50 µM; at 60 °C the corresponding ratio was 1.6. Similar effects of oxygen concentration on respiration rates have been previously reported in *Anabaena variabilis* and attributed to the existence of a mixture of vegetative cell respiration (saturated at relatively low O_2 concentrations with an estimated K_m of about 1 µM) and heterocyst O_2 uptake with a low apparent affinity for oxygen (Jensen and Cox 1983).

Results from a typical experiment are shown in Fig. 2 where the cyanobacteria were subject to several light-dark cycles to obtain information about both initial activity and its stability at elevated temperatures. Both photosynthesis and subsequent dark O_2 uptake were quite stable at temperatures up to 60 °C; estimated rates of inactivation at 60 °C were $0 \pm 0.2\ h^{-1}$ (Fig. 2). However, at 65 °C, inactivation was so rapid that measurements of this type were not possible.

The effect of temperature on the rates of photosynthetic and respiratory O_2 exchange was investigated at a series of temperatures. Maximum photosynthesis was observed between 55 °C and 60 °C with no significant difference between the two temperatures; the rate at 40 °C was 54% of the maximum. Respiration was measured as

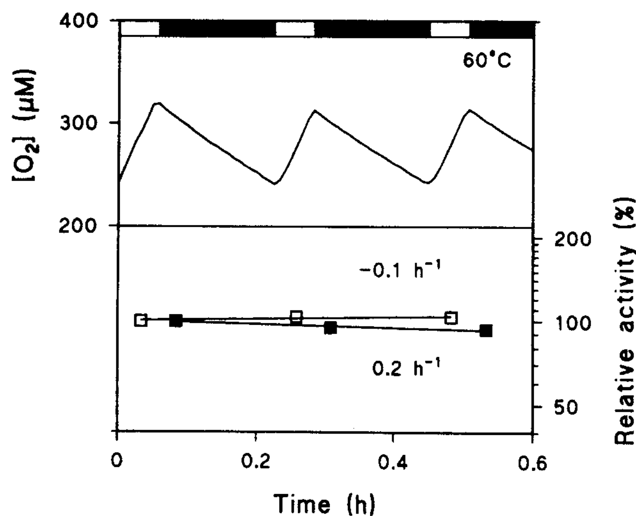

Fig. 2. Changes in O_2 concentration in a suspension of cyanobacteria during several light-dark cycles. The suspension (7.5 µg ml chl) contained Taps NaOH pH 8.0 at a final concentration of 10 mM. The *open bars* indicate that the suspension was illuminated and the *black bars* indicate darkness. Rates of photosynthesis (□) and respiration (■) were determined from the measured oxygen concentration by linear regression over an interval of 3.5 min before and after the peak oxygen concentration. The rates of photosynthesis were normalized to the activities found in the first cycle and rates of inactivation were estimated using a non-linear fit to an exponential decay. Further details are given in the 'Methods' section

the rate of uptake when the O_2 concentration was 200 μM and thus contains contributions from both vegetative cells and heterocysts. Maximal rates were found around 55 °C, but activity declined to 76% of the maximal activity at 60 °C. At 40 °C the respiration rate was 57% of the maximal activity.

Effect of temperature on the stability of nitrogenase activity in the presence and absence of oxygen

The effect of oxygen on the stability of nitrogenase activity was investigated by exposure of stirred cell suspensions to different temperatures under atmospheres of air or argon before the rate of acetylene reduction at 55 °C under anoxic conditions was determined. Loss of activity could be fitted to an exponential decay and the rate of deactivation given as the first-order rate constant in units of reciprocal time. The results from an experiment performed at 60 °C are shown in Fig. 3. Nitrogenase activity is clearly more sensitive to preincubation at 60 °C when oxygen is present, where a rate of inactivation of 2.2 h^{-1} was observed. This rate is much higher than the comparable rates of inactivation for photosynthesis and respiration. The effect of temperature on the observed first-order rate of inactivation of nitrogenase is shown in Fig. 4. Activity was stable between 45 °C and 55 °C but significant loss of activity occurred at low temperatures in the presence of oxygen (half-life of 50 min at 25 °C) emphasizing the need to protect the cyanobacteria from exposure to laboratory temperatures in the presence of air. Rapid inactivation was observed at high temperatures; at 65 °C the half-life of nitrogenase activity was 1.3 min under a gas phase of air compared to 14 min under anoxia.

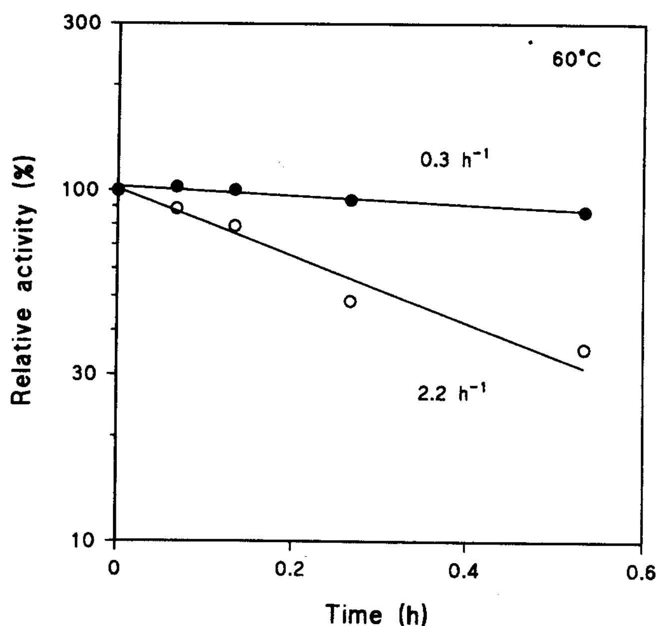

Fig. 3. The effect on nitrogenase activity of short-therm incubation of cyanobacterial suspensions under atmospheres of argon (●) and air (○). Residual activities after the incubation of the cyanobacterial suspension at 60 °C were measured at the growth temperature of 55 °C using the acetylene reduction assay. Results were normalized to the activities found in control suspensions. The rates of inactivation were estimated using a non-linear fit to an exponential decay. Further details are given in the 'Methods' section

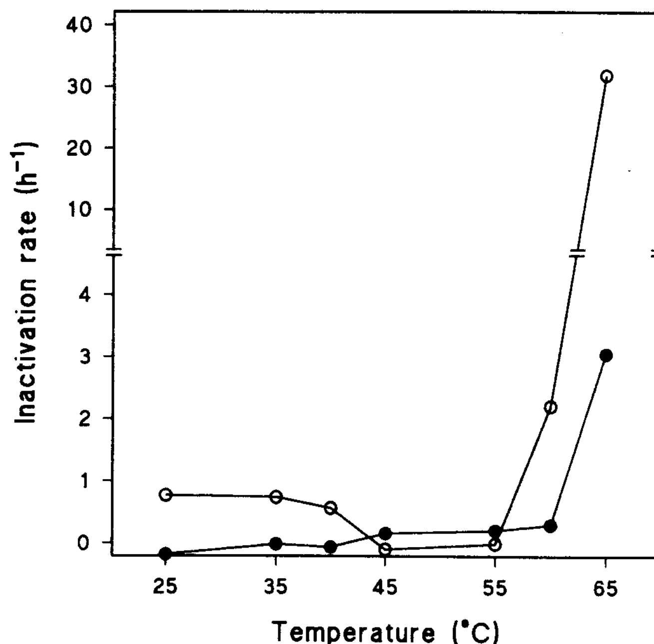

Fig. 4. The effect of temperature on the rate of inactivation of nitrogenase activity under atmospheres of argon (●) and air (○). The results were obtained from a series of experiments similar to that shown in Fig. 3

Discussion

The results presented here show that the need to fix nitrogen sets a lower maximum growth temperature for HTF *Chlorogloeopsis* than growth with nitrate. The upper limit for diazotropic growth is thus determined by some reaction specific to the reduction of N_2 (nitrogenase itself and associated reactions necessary for nitrogenase activity). We have shown that the upper limit is not determined by limitations in the capacity of the cells to fix N_2 in short-term experiments, indicating that the stability of some component involved in nitrogen fixation is the limiting factor. The dramatic effect of O_2 on the rate of inactivation suggests its involvement in the reactions leading to loss of activity.

The rates of inactivation of nitrogenase activity can be compared to the growth rate of the cells in the continuous culture to give an estimate of the number of times nitrogenase will require reactivation in the course of a cell cycle. This would be in excess of 100 for cells grown with atmospheric O_2 at 60 °C, so it is not unexpected that diazotrophic growth was not observed under these conditions.

One strategy used by cyanobacteria for nitrogen fixation in the presence of oxygen is to limit N_2 uptake to periods of darkness (Fay 1992). In this case energy

can only be obtained from respiration, but dissolved O_2 concentrations in the natural environment will be lower. However, the dissolved O_2 concentration needed to obtain maximum respiratory uptake by HTF *Chlorogloeopsis* cells was found to be similar to that causing inactivation of the nitrogen-fixing apparatus. Thus it seems unlikely that nitrogen fixation at the expense of respiration can increase the upper limit for diazotrophic growth by these cyanobacteria.

It is well established that nitrogenase and particularly its dinitrogenase reductase component is rapidly inactivated by O_2 and that diazotrophy is critically dependant on maintaining low O_2 tensions in the immediate vicinity of the enzyme (Fay 1992). Thus, although we have not demonstrated a direct effect on nitrogenase, it seems likely that at elevated temperatures, inactivation becomes more rapid or the protection mechanisms become less effective, until a critical point at which reactivation sets too high a burden on the cell.

Protection of nitrogenase in heterocystous cyanobacteria depends on a combination of physical barriers preventing ready diffusion of O_2 into the heterocyst and an active heterocyst respiration which can represent at least half of the total filament activity (Jensen and Cox 1983). There is no evidence to suggest that respiratory O_2 uptake either in heterocysts or vegetative cells is particularly sensitive to an increase in temperature from 55 °C to 60 °C so we consider it unlikely that a general failure of the protective O_2 consumption process is a cause of inactivation. However, the steady-state concentration of O_2 within the heterocyst, and hence the rate of inactivation of nitrogenase, is controlled by the balance between the rate of influx and the properties of the consuming reactions, and small changes in these could lead to significant changes in the intracellular O_2 concentration. Alternatively, nitrogenase itself may undergo conformational changes making it more subject to inactivation as the temperature is raised. If this is a general property of nitrogenase it may be significant that the highest recorded temperatures for nitrogen fixation are for methanogens and clostridia, both confined to anoxic environments where oxygen inactivation is not a problem.

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References

Allen MM (1968) Simple conditions for the growth of unicellular blue-green algae on plates. J Phycol 4: 1–4

Belay N, Sparling R, Daniels L (1984) Dinitrogen fixation by a thermophilic methanogenic bacterium. Nature 312: 286–288

Bogdahn M, Kleiner D (1986) N_2 fixation and NH_4^+ assimilation in the thermophilic anaerobes *Clostridium thermosaccharolyticum* and *Clostridium thermoautotrophicum*. Arch Microbiol 144: 102–104

Castenholz RW (1978) The biogeography of hot spring algae through enrichment cultures. Mitt Int Verein Limnol 21: 296–315

Castenholz RW (1989) Order Stigonematales. In: Staley JT, Bryant MP, Pfennig N, Holz JG (eds) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, pp 1794–1799

Cox RP (1987) Membrane inlets for on-line liquid-phase mass spectrometric measurements in bioreactors. In: Heinzle E, Reuss M (eds) Mass spectrometry in biotechnological process analysis and control. Plenum Press, New York, pp 63–74

Fardeau M-L, Peillex J-P, Delaïch J-P (1987) Energetics of the growth of *Methanobacterium thermoautotrophicum* and *Methanococcus thermolithotrophicus* on ammonium chloride and dinitrogen. Arch Microbiol 148: 128–131

Fay P (1992) Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56: 340–373

Iversen JJL, Nielsen M, Cox RP (1989) Design and performance of a simple, inexpensive, modular laboratory-scale bioreactor. Biotechnol Ed 1: 11–15

Jensen BB, Cox RP (1983) Effect of oxygen concentration on dark nitrogen fixation and respiration in cyanobacteria. Arch Microbiol 135: 287–292

Jensen BB, Cox RP (1988) Measurements of hydrogen exchange and nitrogen uptake by mass spectrometry. Methods Enzymol 167: 467–474

Miyamoto K, Hallenbeck PC, Benemann JR (1979) Nitrogen fixation by thermophilic blue-green algae (cyanobacteria): temperature characteristics and potential use in biophotolysis. Appl Environ Microbiol 37: 454–458

Mckinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 150: 315–322

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cynobacteria. J Gen Microbiol 111: 1–610

Stewart WD (1970) Nitrogen fixation by blue-green algae in Yellowstone thermal areas. Phycologia 9: 261–268

Wickstrom CE (1980) Distribution and physiological determinants of blue-green algal nitrogen fixation along a thermogradient. J Phycol 16: 436–443

Wickstrom CE (1984) Discovery and evidence of nitrogen fixation by thermophilic heterotrophs in hot springs. Curr Microbiol 10: 275–280

Wilhelm E, Battino R, Wilcock RJ (1977) Low-pressure solubility of gasses in liquid water. Chem Rev 77: 219–262