3

Spirulina: Growth, Physiology and Biochemistry

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Introduction

Algal physiology and biochemistry have been reviewed and discussed quite extensively in the last decades. The excellent contributions by Lewin (1962), Fogg (1975) and Carr and Whitton (1973) are just a few examples of textbooks which cover a wide range of topics related to algal physiology and biochemistry. The aim of this chapter is to point out relevant areas in which *Spirulina* has been used as a model organism or studies whose data can be of significant importance in further understanding the growth, physiology and biochemistry of *Spirulina*, especially when grown in outdoor conditions.

Growth Rate: The Basics

The growth rate of *Spirulina* follows the common pattern of many other microorganisms which undergo a simple cell division without any sexual or differentiation step. Thus, under 'normal' growth conditions the specific growth rate *(μ)* is described by the following equation

$$\mu = \frac{t}{x}\frac{\mathrm{d}x}{\mathrm{d}t} \tag{3.1}$$

where x is the initial biomass concentration. The way to calculate the specific growth rate of microalgae has been described in many publications (Vonshak, 1986, 1991; Stein, 1973). The most commonly used formula is:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \tag{3.2}$$

where x_1 and x_2 are biomass concentrations at time intervals t_1 and t_2. The simple equation that combines the specific growth rate *(μ)* and the doubling time (d.t.) or

the generation time *(g)* of a culture is:

$$g = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} = \text{d.t.} \tag{3.3}$$

These equations are true for the logarithmic or exponential phase of growth in batch cultures. When growing an algal culture in a continuous mode such as in a chemostat or turbidostat the equations are modified so that

$$\mu = D = \frac{1}{v} = \frac{dv}{dt} \tag{3.4}$$

where v is the total volume of the culture and dv/dt is the dilution rate.

More detailed studies on growth kinetics of *Spirulina* were performed by Lee et al. (1987) and Cornet et al. (1992a, b). The studies included elaborate in-depth details of mathematical modeling, which are beyond the scope of this chapter.

Growth Yield and Efficiency of Photoautotrophic Cultures

Many of the studies on *Spirulina* that attempted to estimate its growth yield and photosynthetic efficiency were limited, mainly because most of the cultures were not axenic (bacteria-free). Developing procedures to obtain an axenic culture of *Spirulina* led the way to this kind of study (Ogawa and Terui, 1970). The first assessment of quantum yield for *Spirulina* using cultures grown at different dilution rates was in a Roux bottle. The opalescent plate method was used to measure the light energy absorbed by the cells and to assess the growth yield, Y_{kcal}, i.e. the amount of dry algal biomass harvested per kcal light energy absorbed. Calculated values of Y_{kcal} ranged from 0.01 to 0.02 g cell $kcal^{-1}$. These values corresponded to a *Y* value of 6–12 per cent. In a much later study (Ogawa and Aiba, 1978) where assimilation of CO_2 was used to estimate the quantum requirement of *Spirulina* cultures grown at steady state conditions, it was found that the value was about 20 quantum mol^{-1} CO_2, which corresponds to a *Y* value of 10 per cent. This is in good agreement with the Y_{kcal} values of 0.01-0.02 reported earlier.

The relation of the specific growth rate to the specific absorption rate of light energy was used to establish a mathematical equation describing the growth of *Spirulina* in a batch culture (Iehana, 1987). The equation indicates that the specific growth rate increases linearly with the increase of the specific absorption rate of light energy in culture with a high cell concentration. In an earlier work, Iehana (1983) analyzed the growth kinetics of *Spirulina* when grown as a continuous culture under light limitation. The kinetic analysis was done by comparing the relationships between the extinguished luminous flux in the culture and the growth rate. Under fixed luminous conditions, the specific growth rate of *Spirulina* was proportional to the extinction rate of the luminous flux per cell concentration. The obtained equation simulated growth in the exponential phase. When cell concentration was kept constant, the equation was comparable to Michaelis-Menten type kinetics. Two other groups, Lee et al. (1987) and Cornet et al. (1992a,b) have published detailed studies on attempts to establish a mathematical model for the growth of *Spirulina* under a variety of growth conditions. It seems that they all fit well the experimental growth data under normal steady state conditions, where light is either limiting or is at its saturation level. These models

should be modified if stress conditions, such as photoinhibition or environmental stress (i.e. temperature or salinity), are introduced.

Mixotrophic and Heterotrophic Growth

The isolation of an axenic culture of *Spirulina* enabled the use of different organic sources to stimulate growth, in heterotrophic or mixotrophic modes. Ogawa and Terui (1970) were the first to report that an axenic culture of *Spirulina* grown on a mineral medium enriched with 1 per cent peptone had a higher growth rate in the logarithmic phase and in the linear phase than the culture grown on minimal medium. There was a 1.2–1.3 fold and 1.85–1.93 fold increase, respectively.

The effectiveness of the peptone was higher in light-limited cultures. Addition of glucose also affected the growth yield and a combination of 0.1 per cent peptone and 0.1 per cent glucose doubled growth yield compared with that obtained without any organic carbon source. Cultures of *Spirulina* grown on glucose were used to further analyze the autotrophic and heterotrophic characteristics of the cells.

Stimulation of the growth rate in the presence of glucose suggests that respiratory activity occurs in *S. platensis* even in light. Photosynthetic (O_2 evolution) and respiratory (O_2 consumption) activities were examined using 100-h and 250-h cultures grown on glucose, either in the light or aerobic-dark conditions in the presence or absence of 5 mM DCMU (3-(3,4-dichlorophenyl)-1,dimethylurea, a potent inhibitor of photosynthesis). Respiratory activity clearly indicated that the rate of O_2 consumption was unaffected by light, irrespective of DCMU presence. In the presence of DCMU no photosynthetic activity was detected. Heterotrophically grown cells also showed lower photosynthetic activity. This might be due to the fact that the contents of pigments such as chlorophyll-a , carotenoids and phycocyanin in the cells were lower than in the autotrophic and mixotrophic cultures. Results indicated that in mixotrophic conditions, autotrophic and heterotrophic growth functions independently in *S. platensis* without interaction (Marquez et al., 1993). In a recent publication (Marquez et al., 1995), the potential of heterotrophic, autotrophic and mixotrophic growth of *Spirulina* was evaluated. Most of the results agree with those previously published, except that in this case, heterotrophic growth of *Spirulina* was observed in cultures grown in the presence of glucose. Perhaps in previous studies, the cultivation time was not long enough. Marquez et al. (1995) also suggest that CO_2 produced from heterotrophic glucose metabolism might be used photosynthetically, together with bicarbonate from the culture medium.

These results have to be further investigated in other *Spirulina* strains so that it can be established whether these characters are universal for most of the *Spirulina* strains or specific to the strain used by the researcher.

Response to Environmental Factors

The Effect of Light

Without doubt, light is the most important factor affecting photosynthetic organisms. Due to the prokaryotic nature of *Spirulina,* light does not affect the differentiation or development processes. Nevertheless, *Spirulina*, like many other algae grown

photoautotrophically, depends on light as its main energy source. The photosynthetic apparatus and its components are described in Chapter 2. The response of outdoor cultures to light and the important role that light and photosynthesis play in productivity in mass cultivation of *Spirulina* are discussed in detail in Chapters 5 and 8. In this section, we will examine the effect of light on laboratory cultures of *Spirulina*, and the way cells respond and adapt to different levels of light.

Effect on growth

Most of the laboratory studies on the response of *Spirulina* to light were performed under photoautotrophic growth conditions, using a mineral medium and bicarbonate as the only carbon source. The first detailed study on the response of *Spirulina maxima* to light was done by Zarrouk in 1966. In his somewhat simple experiment, he reached the conclusion that growth of *S. maxima* is saturated at levels of 25–30 klux. Since not much information is given on the way light was measured and the light path in the vessel cultures, it is very difficult to compare these results with more recent ones. From data obtained in the author's laboratory, growth of *Spirulina platensis* became saturated at a range of 150–200 µmol $m^{-2}s^{-1}$. This is about 10 to 15 per cent of the total solar radiance at the 400–700 nm range. This value is highly dependent on growth conditions and correlates with the chlorophyll to biomass concentration. Another experimental parameter which determines this response is the light path of the culture. Therefore, it is highly recommended that when attempting to establish the maximal specific growth rate μ_{max}, a turbidostat system should be employed. In such a manner, we have estimated the μ_{max} of *Spirulina* to be in the range of 8–10 h. The use of a turbidostat system also eliminates nutritional limitation or self-shading problems.

Effect on photosynthesis

The most common way to study the photosynthetic response of algal cultures to light is through the measurement of the photosynthesis *(P)* versus irradiance *(I)* curves. A typical *P—I* curve is shown in Figure 3.1. The saturation and compensation points are

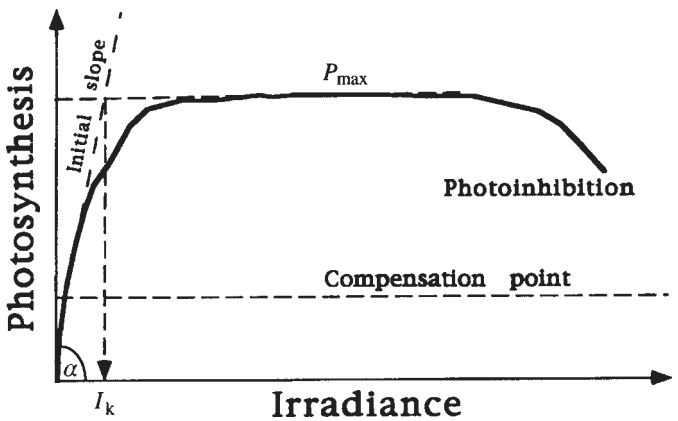

Figure 3.1 Schematic diagram of a photosynthesis *(P)* versus irradiance curve, showing the typical photosynthetic parameters. For more details see text.

the most important parameters. In the dark, the rate of oxygen evolution or carbon fixation will be negative because of respiration. As irradiance is increased, a point is reached when the photosynthetic rate is just balanced by respiration. This is the compensation point. As irradiance is further increased, the rate of photosynthesis increases linearly. Eventually, the curve levels off, as photosynthesis becomes saturated, reaching a maximum, P_{max}. The initial slope, α, is a useful indicator of quantum yield, i.e. photosynthetic efficiency.

The saturation irradiance may be also defined by the value of I_k, which represents the point at which the extrapolation of the initial slope crosses Pmax. Exposing *Spirulina* cultures to high photon flux densities above the saturation point may result in a reduction of the rate of photosynthesis, a phenomenon defined as photoinhibition. The classical view that photoinhibition is observed only at high irradiance values today seems to be a very simplistic one. It will be discussed later how photoinhibition may be observed even at relatively low irradiance levels when other environmental stresses are introduced.

The P_{max} and I_k levels are highly dependent on growth conditions. *Spirulina* cultures grown at high or low light intensities will have different P_{max} and I_k values. Changes in these values may represent the culture's ability to photoadapt to the different light environments. Furthermore, the P_{max} and I_k values may be used as a tool for screening strains of *Spirulina* which have a better photosynthetic performance under outdoor conditions. An example for such a screening process is given in Table 3.1, a summary of experiments carried out in the author's lab, indicating different photosynthetic parameters in three different *Spirulina* strains. Although the strains were grown under the same temperature and light conditions, they have different α and I_k values. The fact that the cultures have a similar growth rate, μ, under laboratory conditions may be meaningless for the outdoor cultivation systems. For the outdoor conditions, strains with different P_{max} or I_k may have different productivities since they differ in their ability to utilize the high solar irradiance available outdoors.

Table 3.1 Photosynthetic parameters obtained from *P* versus *I* curves of three *Spirulina* isolates, grown under the same laboratory conditions

	Spirulina strain		
Parameters	BP	P4P	Z19/2
μ	0.048	0.043	0.044
I_k	145 ± 15	115 ± 13	165 ± 15
P_{max}	625 ± 8	614 ± 5	645 ± 13
α	4.8 ± 0.4	6.3 ± 1.0	3.85 ± 0.7

μ is specific growth rate (h^{-1}).
I_k is irradiance at the onset of light saturation ($\mu Em^{-2}s^{-1}$).
P_{max} is maximal rate of light-saturated photosynthesis,
α is initial slope of the *P-I* curve ($\mu mol\ O_2\ h^{-1}\ mg\ Chl^{-1}$)/ ($\mu Em^{-2}s^{-1}$).
All strains were grown under laboratory conditions, constant temperature of 35°C and constant light, 120 $\mu molm^{-2}s^{-1}$.

Light stress—photoinhibition

Photoinhibition, as mentioned earlier, is defined as a loss of photosynthetic capacity due to damage caused by photon flux densities (PFD) in excess of that required to saturate photosynthesis. The phenomenon of photoinhibition has been studied extensively and is well documented in algae and higher plants (Critchley, 1981; Greer et al., 1986; Kyle and Ohad, 1986; Öquist, 1987; Powles, 1984).

The phenomenon of photoinhibition in laboratory *Spirulina* cultures was first studied by Kaplan (1981), who observed a reduction in the photosynthetic activity when cells of *Spirulina* were exposed to high light under CO_2-depleted conditions. It was suggested that the reduction of the photosynthetic activity was due to the accumulation of H_2O_2.

A much more elaborate study on the photoinhibitory response was carried out in our laboratory (Vonshak et al., 1988a). We demonstrated that different strains of *Spirulina* may differ in their sensitivity to the light stress. At least in one case it was found that this difference was most likely due to a different rate of turnover of a specific protein, D1, which is part of the PS II (see Chapter 2). The different response of *Spirulina* strains to a photoinhibitory stress may be a genotypic characteristic as well as arising from growth conditions. We also found that cultures grown at high light intensity exhibit a higher resistance to photoinhibition, as demonstrated in Figure 3.2. Cultures grown at 120 and 200 $\mu molm^{-2}s^{-1}$ were exposed to a HPFD of 1500 $\mu molm^{-2}s^{-1}$. Indeed, the cells grown in strong light do show a higher resistance to light stress. It should be emphasized that the photoinhibition not only affects the P_{max} level, but actually has a stronger effect on light-limited photosynthetic activity. This can be shown by comparison of the *P-I* curves of the control and the photoinhibited cultures of *Spirulina* (Figure 3.3). It can be seen that photoinhibited cultures have a lower photosynthetic efficiency. Therefore, they are more light-limited than the control cultures, i.e. requiring more light in order to achieve the same photosynthetic activity. The implications for outdoor cultures of this observation will be discussed in Chapter 5.

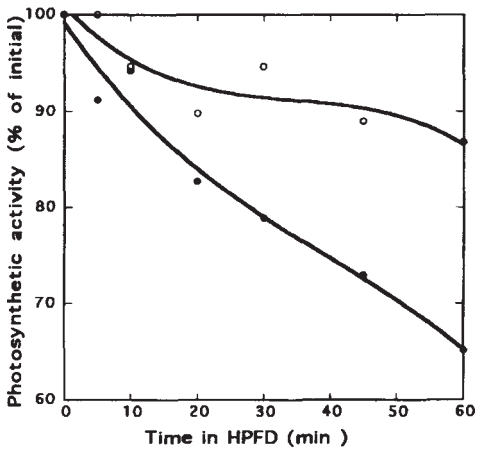

Figure 3.2 The effect of growth irradiance on the response of *Spirulina* to HPFD. Cells were grown at 120 (●) or 200 (○) $\mu molm^{-2}\ s^{-1}$ for 4 days and then diluted to the same chlorophyll concentration and exposed to HPFD of 1500 $\mu molm^{-2}s^{-1}$.

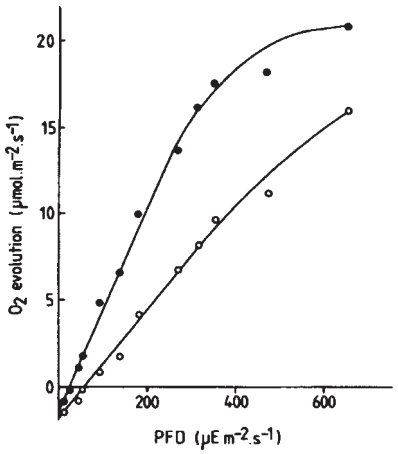

Figure 3.3 Photosynthesis versus irradiance curves of control (●) and photoinhibited (○) *Spirulina* cultures.

Effect of Temperature

While light is considered the most important environmental factor for photosynthetic organisms, temperature is undoubtedly the most fundamental factor for all living organisms. Temperature affects all metabolic activities. Temperature also affects nutrient availability and uptake, as well as other physical properties of the cells' aqueous environment.

Effect of temperature on growth

Spirulina was originally isolated from temporal water bodies with a relatively high temperature. The usual optimal temperature for laboratory cultivation of *Spirulina* is

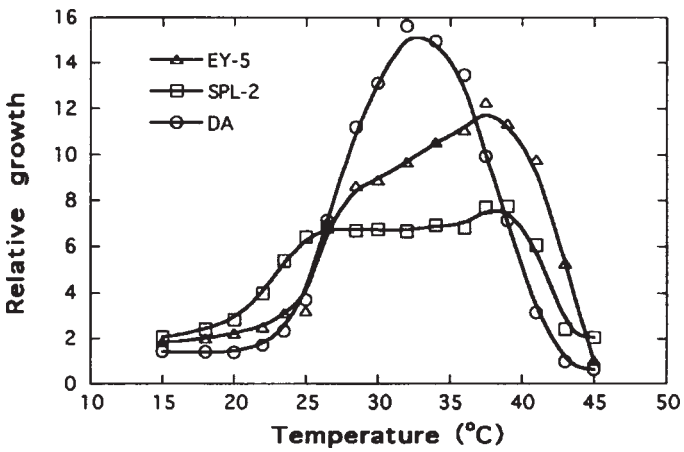

Figure 3.4 The response of three *Spirulina* isolates to temperature, as measured by the increase in chlorophyll concentration in cultures incubated in a temperate gradient block of 15–45°C illuminated continuously at 150 μmolm^{-2}s^{-1}.

in the range 35–38°C. However, it must be pointed out that this range of temperature is arbitrary. Many *Spirulina* strains will differ in their optimal growth temperature, as well as their sensitivity to extreme ranges. In our laboratory, many strains of *Spirulina* are maintained and tested for their physiological responses. In Figure 3.4, three isolates of *Spirulina* are compared. The cells were incubated under constant light in a temperature gradient block. The increase in chlorophyll was measured after a certain period of incubation. The three strains differed significantly in their response to temperature. The one marked DA has a relatively low temperature optimum of 30–32°C, while the one marked EY-5 grows well at temperature of up to 40–42°C. The isolate marked SPL-2 is characterized by a relatively wide temperature optimum. This is just one example of the variations observed. Obtaining strains with a wide temperature optimum could be of high monetary value in the outdoor cultivation industry since we believe that temperature is one of the most important limiting factors in outdoor production of *Spirulina*. Specific strains which fit the local climatic conditions should be used.

Effect of temperature on photosynthesis and respiration

The net productivity of an algal culture is directly correlated to the gross rate of CO_2 fixation or O_2 evolution (photosynthesis) and the rate of respiration. Photosynthesis and respiration are dependent on temperature, but only CO_2 fixation and O_2 evolution are both light- and temperature-dependent. A detailed study on the response of a *Spirulina* strain marked M-2 was performed by Torzillo and Vonshak (1994). The O_2 evolution rate of *Spirulina* cells measured at different temperatures is shown in Figure 3.5. The optimal temperature for photosynthesis was 35°C; however, growth at 28 per cent and 23 per cent of the optimum were measured at the extreme minimum and maximum temperatures tested: 10°C and 50°C, respectively. The effect of temperature on the dark respiration rate of *Spirulina* was also measured, by following the O_2 uptake rate in the dark. A temperature-dependent exponential relationship was obtained, with the respiration rate increasing as temperature increased (Figure 3.6).

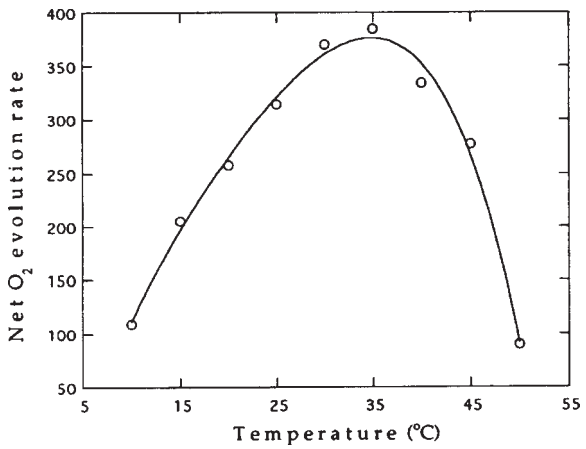

Figure 3.5 The effect of temperature on the gross O_2 evolution rate (photosynthesis) (μmol O_2 mgchl^{-1} h^{-1}) of *Spirulina platensis* cells. Cells were allowed to equilibrate at each temperature for 15 min before the measurement.

The temperature-dependent dark respiration rate was given by

$$R=0.771e^{(0.0636T)}$$

where R is the respiration rate (µmol O_2 mg^{-1} chl h^{-1}) and T is the temperature (°C). At 50°C and 15°C dark respiration rates dropped almost to zero. An Arrhenius plot for respiration showed an activation energy of 48.8 kJmol-1 for *Spirulina.* The temperature coefficient (Q_{10}) of the organism in a temperature range was calculated by the following equation, deduced from the Arrhenius equation (Pirt, 1975):

$$\log Q_e = \frac{E_a}{2.303R} \frac{10}{(T+10)T} \qquad (3.5)$$

where E_a is the activation energy (kJmol-1) and R is the universal gas constant (8.31 JK-1mol^{-1}). A Q_{10} of 1.85 was calculated for the range 20–40 °C. The respiration-to-photosynthesis ratio in *Spirulina* was 1 per cent at 20 °C and 4.6 per cent at 45°C. These rather low values confirm the general assumption that cyanobacteria have low respiration rates (van Liere and Mur, 1979). The respiration-to-photosynthesis rates measured in these experiments were found to be much lower than those reported for outdoor cultures of *Spirulina*, where up to 34 per cent of the biomass produced during the daylight period may be lost through respiration at night (Guterman et al., 1989; Torzillo et al., 1991). However, it must be noted that respiration rate is strongly influenced by light conditions during growth. In our *Spirulina* strain, the respiratory activity had a much higher temperature optimum than the photosynthetic activity. Nevertheless, the photosynthetic activity of the cells was more resistant to the temperature extremes than dark respiration at the minimum and maximum temperatures tested.

Interaction of temperature and light

Deviation from the optimal growth temperature has an inhibitory effect on the photosynthetic capacity. This reduction in activity represents a limitation that is

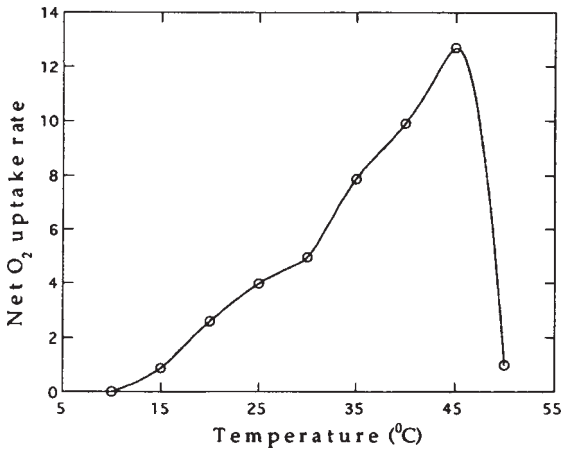

Figure 3.6 The effect of temperature on O_2 uptake rate in the dark (respiration) O_2 $mgchl^{-1}$ h^{-1}) of *Spirulina platensis* cells. Cells were allowed to equilibrate at each temperature for 15 min before the measurement.

immediately overcome after a shift back to the optimal temperature, if no other damage was done. The kinetics of recovery from low-temperature incubation indicate that some repair mechanism must take place before the original photosynthetic activity is reached. This observation was made only when the cultures were incubated at a low temperature in the light. *Spirulina* cultures incubated at a low temperature in the dark seemed to acquire their original photosynthetic activity as soon as they were transferred to 35°C without any lag period. It is thus suggested that *Spirulina* cultures grown at less than the optimal temperature are more sensitive to photoinhibition than those grown at the optimal temperature. The latter will be better able to handle excess light energy, since they have a higher rate of electron transport, an active repair mechanism and more efficient ways of energy dissipation. As shown in Figure 3.7, cultures exposed to HPFD at 25°C, a temperature below the optimal, were much more sensitive to HPFD stress, as compared with cultures exposed to the HPFD at 35°C. The difference was more pronounced with prolonged exposure time to high irradiance. This fits well with the overall concept of photoinhibition, i.e. that any environmental factor which reduces the rate of photosynthesis may encourage photoinhibition. Jensen and Knutsen (1993) have demonstrated that the increased susceptibility of *Spirulina* to HPFD at low temperatures may also be due to a lower rate of protein synthesis, affecting cells' recovery from light stress.

Many other factors interact with temperature and probably affect the growth and productivity of *Spirulina.* Solubility of gases in the medium and availability of nutrients are some of these. More detailed and extensive work is required in order to better understand these interactions.

Response to Salinity

Cyanobacteria inhabit environments which vary drastically in their saline levels. In the last 15 years many studies were published on the response of cyanobacteria to

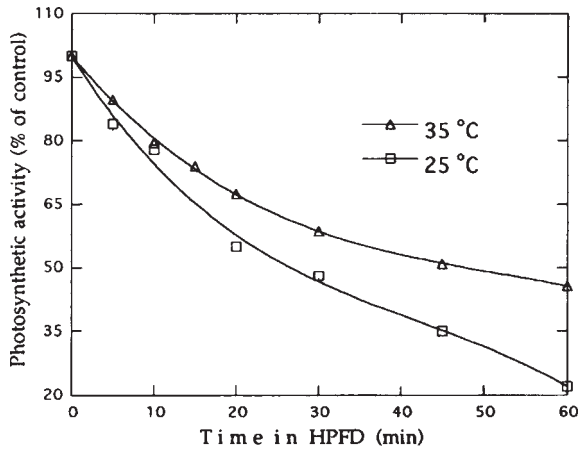

Figure 3.7 The effect of temperature on the response of *Spirulina* to a HPFD stress. Cultures incubated at 35°C or 25°C were exposed to 2500 $\mu molm^{-2}s^{-1}$. At time intervals, the reduction in photosynthetic activity was measured.

different saline environments: the specific role of organic compounds as osmoregulants (Borowitzka, 1986), modification in photosynthesis and respiration activity (Vonshak and Richmond, 1981), and variations in the protein synthesis pattern (Hagemann et al., 1991). Different *Spirulina* species have been isolated from a variety of saline environments. We will describe the work done using strains isolated from alkaline and brackish water. Since the exact taxonomic position of the marine strains of *Spirulina* is still not clear, we will not discuss their response, although it has been a subject of a detailed study (Gabbay and Tel-Or, 1985).

Effect of salinity on growth

Exposure of *Spirulina* cultures to high NaCl concentrations results in an immediate cessation of growth. After a lag period, a new steady state is established. A typical growth response curve to NaCl is shown in Figure 3.8, where changes in biomass concentrations of three *Spirulina* cultures exposed to control, 0.5 and 0.75 MNaCl are presented. As can be seen, not only is growth inhibited for at least 24 h after the exposure at the high NaCl concentration, but a decrease in biomass is observed after which a new steady-state exponential growth rate is established. The new growth rates after adaptation are slower and inversely correlated to the increased NaCl concentration in the medium (Vonshak et al., 1988b). A decrease in the growth rate because of salt stress has also been demonstrated in other cyanobacteria, such as *Anacystis* (Vonshak and Richmond, 1981) and *Nostoc* (Blumwald and Tel-Or, 1982). It is worth noting that the length of the time lag is exponentially correlated to the degree of stress imposed on the cells. This lag period in many cases is associated with a decline in chlorophyll and biomass concentrations in the culture (Vonshak et al., 1988b).

The response of *Spirulina* to salinity with regard to degree of growth inhibition, adaptability to salt levels and the rate of adaptation varies widely, depending on the strain used in the study. In Table 3.2, an example is given for two strains of *Spirulina* exposed to different salt concentrations. The changes in growth rate and doubling time after adaptation indicate that the M2 strain seems to be more resistant to the salt stress than the 6MX strain.

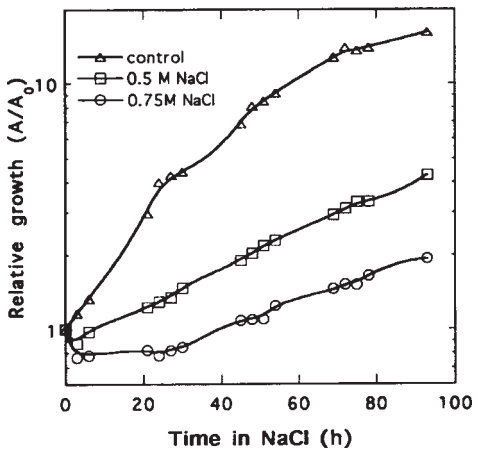

Figure 3.8 The growth response of *Spirulina* to increased concentrations of NaCl in the growth medium (NaCl concentrations indicated are above the normal level in the growth medium).

Table 3.2 Specific growth rates and doubling time of *Spirulina* strains grown under salinity stress at 35°C

	M2		6MX	
Treatment	Specific growth rate (h^{-1})	Doubling time (h)	Specific growth rate (h^{-1})	Doubling time (h)
Control	0.063	11.0	0.059	11.8
+0.50 M NaCl	0.044	15.9	0.026	26.2
+0.75 M NaCl	0.034	20.3	0.018	39.4

Effect of salinity on photosynthesis and respiration

It has been suggested that exposure to high salinity is accompanied by a higher demand for energy by the stressed cells (Blumwald and Tel-Or, 1982). Changes in the photosynthetic and respiratory activity of *Spirulina* were measured over a period of 30 min to 48 h, after exposure to 0.5 and 1.0 M NaCl. These changes were compared with changes in biomass concentration as an indicator of growth (Figure 3.9). A marked decrease in the photosynthetic oxygen evolution rate was observed 30 min after exposure to the salt at both concentrations (Figure 3.9a). This decline was followed by a recovery period, characterized by a lower steady-state rate of photosynthesis. Recovery at 0.5 M NaCl was faster than at 1 M NaCl (after 1.5 vs 3.0 h) and leveled off at 80 per cent of the control activity vs about 50 per cent respectively. Respiratory activity also dropped rapidly immediately after salt application at both concentrations (Figure 3.9b). Activity was restored ten times faster at 0.5 M than at 1.0 M NaCl and continued to increase to twice the control level at 1.0 M NaCl.

The immediate inhibition of the photosynthetic and respiratory systems after exposure to salt stress was explained by Ehrenfeld and Cousin (1984) and Reed et al. (1985). They showed that a short-term increase in the cellular sodium concentration was due to a transient increase in the permeability of the plasma membrane during the first seconds of exposure to high salt. It has been suggested that the inhibition of photosynthesis arising from the rapid entry of sodium, might be the result of the detachment of phycobilisomes from the thylakoid membranes (Blumwald et al., 1984). Elevated activities of dark respiration in cyanobacteria because of salinity stress have previously been reported (Vonshak and Richmond, 1981; Fry et al., 1986; Molitor et al., 1986). This high activity may be associated with the increased level of maintenance energy required for pumping out the toxic sodium ions.

Osmoregulation and strain-specific response of Spirulina *to salinity*

During the course of adaptation to salinity, an osmotic adjustment is required. In *Spirulina*, a low molecular weight carbohydrate accumulates. This has been identified as a nine-carbon heteraside named Glucosyl-glycerol, as well as trealase (Martel et al., 1992). We compared biomass composition of two *Spirulina* strains grown under salt stress conditions; a significant change in biomass composition was observed, mainly reflected in the increase in carbohydrates and a decrease in the

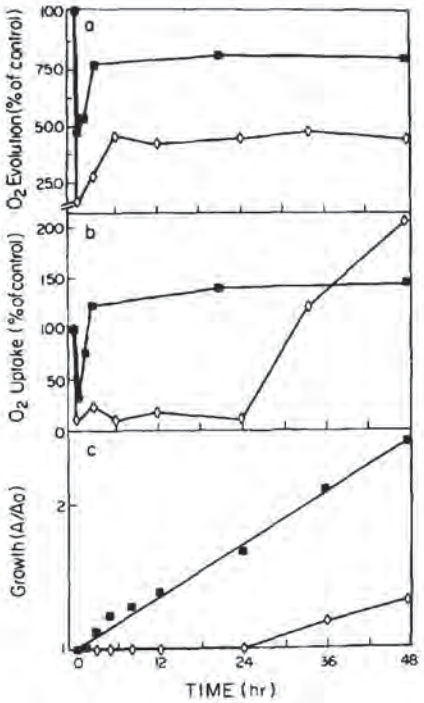

Figure 3.9 Effect of NaCl on photosynthesis (a), respiration (b) and growth (c) in *Spirulina* exposed to ■—■ 0.5 M and ◇—◇ 1.0 M NaCl. 100 per cent activity values for apparent photosynthesis and respiration were 663 and 70 μmol O_2 mgchl^{-1} h^{-1}, respectively

protein level (Table 3.3). These changes are correlated with the degree of stress imposed, i.e., a higher level of carbohydrates at a higher salt concentration. The difference in the level of carbohydrates accumulated by the two strains may also reflect a difference in their ability to adapt to salt stress.

Interaction with light

Photosynthetic activity of *Spirulina* declines under salinity stress even if the cultures are grown continuously in the saline environment and adapt to the new osmoticum. This decline is associated with a modification in the light energy requirement, i.e.

Table 3.3 Biomass composition of *Spirulina* strains grown for 95 h under salinity stress

Strain	Treatment	Chlorophyll (% dry weight)	Protein (% dry weight)	Carbohydrate (% dry weight)
M2	Control	2.6	56.1	35.5
	+0.50 M NaCl	1.9	44.1	46.7
	+0.75 M NaCl	1.3	41.1	55.8
6MX	Control	1.6	60.2	30.0
	+0.50 M NaCl	1.0	23.9	64.4
	+0.75 M NaCl	0.5	22.0	61.4

Table 3.4 Photosynthetic characteristics of *Spirulina* grown under control and salinity conditions

	Control	0.68 M Nacl
μ	0.048	0.024
I_k	160	250
P_{max}	625	285
α	3.9	1.1
R	24	63

μ=specific growth rate (h^{-1}).
I_k=light saturation ($\mu Em^{-2}s^{-1}$).
P_{max}=saturated rate of photosynthesis ($\mu mol\ O_2\ h^{-1}\ \mu g\ Chl^{-1}$).
R=dark respiration (ìmol O_2 uptake $h^{-1}\mu g\ Chl^{-1}$).
α=initial slope of the *P-I* curve ($\mu mol\ O_2\ h^{-1}\ mg\ Chl^{-1}$)/($\mu Em^{-2}s^{-1}$).

less light is required for the saturation of photosynthesis. Comparison of the *P* vs *I* curve for control and salt stress cultures shows that all the parameters have been modified (Table 3.4), indicating a reduction in the P_{max} as well as the photosynthetic efficiency, known as the initial slope a. The amount of light required to saturate photosynthesis (I_k) increased, again indicating that photosynthesis operates at a significantly lower efficiency when *Spirulina* is exposed to salt stress. It is also worth noting that the dark respiration rate is increased by 2.5 fold, which may be how cells produce the extra energy required to maintain their internal osmoticum. This reduction in the ability to use light energy absorbed by the photosynthetic apparatus increases the sensitivity of the salt-stressed cells to photoinhibition. When salt-stressed cultures of *Spirulina* are exposed to HPFD, a much faster decline in photosynthesis is observed, as compared with the control (Figure 3.10). Control cultures exposed to a photoinhibitory stress lose about 40 per cent of their photosynthetic activity after 60 min exposure, in 0.5 M and 0.75 M NaCl cultures: a

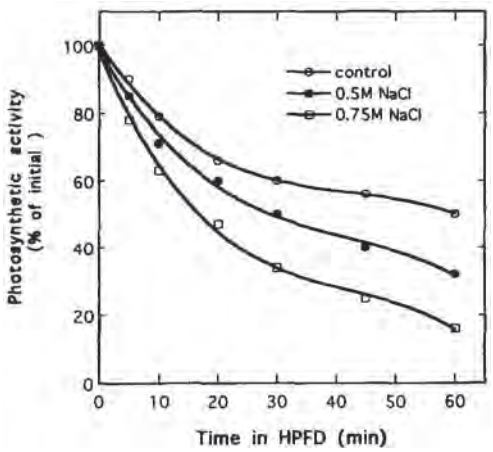

Figure 3.10 The response of *Spirulina* cells grown at different NaCl concentrations to a photoinhibitory stress. Cells were allowed to adapt to the salinity stress and only then exposed to a HPFD of 2500 $\mu mol\ m^{-2}s^{-1}$

60 per cent and 80 per cent reduction is observed, respectively. Most likely, salinity-stressed cells are less efficient in handling light energy (Table 3.4) and also have a lower rate of protein synthesis. Since recovery from photoinhibition is associated with the ability to synthesize specific protein associated with PS II (Vonshak et al. 1988a), a reduction in the level of the protein synthesis affects the repair mechanism.

Conclusions

Although it has been almost more than 20 years since the commercial application of *Spirulina* was proposed, relatively very little has been done to study the basic physiology of *Spirulina*. The original work of Zarrouk (a Ph.D. thesis written in France in 1966) was never published in a scientific journal, which was most unfortunate since it contained virtually unknown, valuable information about *Spirulina.* The unclear situation of the systematics of *Spirulina* (Chapter 1) has made comparative physiological studies even more difficult. We have tried to summarize most of the recent findings on *Spirulina* growth physiology; we believe that further in-depth study to identify *Spirulina* strains and measure their response to environmental factors is required. We also believe that with the new molecular biology studies of *Spirulina,* more information and a better understanding will be achieved.

Biochemistry

Introduction

The use of *Spirulina* as an experimental tool in biochemical studies has been very limited. The number of publications related to metabolic pathways and enzyme isolation is low. This dearth of information shows that the main interest in *Spirulina* is for its biotechnological application. *Spirulina* does not fix nitrogen and does not develop differentiated cells like heterocysts or akinates as part of the filament. Since most of the isolates do not form colonies when grown on a solid support, genetic manipulation of *Spirulina* is difficult. Moreover, only a few laboratories have reported the isolation of axenic (bacteria-free) cultures.

Elongation Factor

EF-Tu, the elongation factor that binds aminoacyl-tRNA to the ribosome, plays an important role in the biosynthesis of proteins. It has been purified from a number of bacteria, as well as from chloroplast of higher plants and green algae.

The EF-Tu of *S. platensis* was first isolated by Tiboni and Ciferri (1983). It appeared to be very similar to the protein isolated from bacteria. The estimated molecular weight of *S. platensis* EF-Tu is about 50 000, similar to that reported for the EF-Tu of Gram-positive bacteria. The protein was first isolated using a Sephadex G-100 column, and an EF-Tu-containing fraction was identified by assaying GDP binding activity. Further purification was performed by an affinity chromatography step using GDP sepharase.

EF-Tu may be used in evolutionary studies to explore phylogenetic relations between *Spirulina* and other prokaryotic groups. Attempts made by the isolators to ascertain immunological similarity between *S. platensis* EF-Tu and the protein from bacteria or chloroplasts were unsuccessful since no immunoprecipitate was observed when antisera to *E. coli* or spinach chloroplast EF-Tu were tested with crude or purified preparations of *S. platensis* EF-Tu.

Nitrite Reductase

Nitrite reduction is considered to be the final step of assimilatory nitrate reduction, in which nitrite is reduced to the level of ammonia in the following way:

$$NO_2^- + 6e^- + 8H \rightarrow NH_4^+ + 2H_2O$$

This reaction is catalyzed by the enzyme nitrite reductase (NiR).

Ferredoxin-dependent NiR (Fd-Nir) (ammonia: ferredoxin oxidoreductase, EC 1.7.7.1) has been purified from higher plants and extensively characterized. This enzyme was isolated from *Spirulina* by Yabuki et al. (1985). After breaking of the cells in a tris buffer by sonication, purification was carried out, inducing an hydrophobic chromatography anion exchange. Affinity chromatography was executed. The main steps of this process and the degree of purification are give in Table 3.5.

Yabuki et al. (1985) report that the enzyme was stable for more than a month when stored at 4 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl and 10 per cent v/v glycerol. The assay method for nitrite reductase was done in a total volume of 1 ml, 20 mmol of Tris-HCl buffer, pH 7.5, 2 mmol of sodium nitrite, 3 mmol of methyl viologen, 3.75 mg of sodium dithionite, and the enzyme preparation. The reaction was carried out at 35°C. One unit of nitrite reductase is the amount of enzyme that reduces 1 mmol of nitrite per min under these assay conditions.

The absorption spectrum of this enzyme had six major peaks at 278, 402, 534, 572, 588 and 658 nm. This spectrum is different from that reported for spinach Fd-NiR. The nature of the visible spectrum suggests the presence of siroheme, which has been detected in enterobacterial NADPH-sulfite reductase and also in spinach sulfite reductase.

Table 3.5 Summary of the purification steps for nitrite reductase from *Spirulina*

Step	Total activity (units)	Specific activity (units [mg protein]$^{-1}$)	Purification (fold)
Crude extract	919	0.046	1
Acetone 25–75%	737	0.16	3.5
DEAE-cellulose	312	0.26	5.7
Butyl Toyopearl	216	0.40	8.7
Sephadex G-75	195	2.2	47.8
DEAE-cellulose	195	25.3	550
Fd-Sepharose 4B	78	30.0	652
Fd-Sepharose 4B	70	194	4217

Data extracted from Yabuki et al. (1985).

The molecular weight of NiR from *Spirulina* was 52 000 dalton, the same size as that of the enzyme isolated from *Anabaena.* The K_m values were 2.0×10^{-4}M (nitrite), 2.0×10^{-5} M (*Spirulina* ferredoxin), and 4.0×10-4 M (methyl viologen). Both prokaryotic and eukaryotic Fd-NiR had K_m values of the same order of magnitude. The pH-activity curve obtained with Tris-HCl buffer was rather broad and had an optimum at around pH 7.8.

Ferredoxin-sulfite Reductase (Fd-SiR)

The enzyme hydrogen-sulfide ferredoxin oxidoreductase, EC 1.8.7.1, which catalyzes the reduction of sulfite to sulfide with NADH, reduced ferredoxin or methyl viologen as an electron donor, was isolated from *Spirulina* by Koguchi and Tamura (1988). Their purification process yielded a highly purified enzyme, up to a homogenous band in electrophoresis test. The purification steps and the increase in purity are summarized in Table 3.6. The highly purified enzyme catalyzes the reduction of sulfite using physiological concentration of ferredoxin as an electron donor.

Comparison of the absorption spectrum of *Spirulina* Fd-SiR to that of the b-subunit (a siroheme-containing protein) of NADPH-sulfite reductase from *Escherichia coli* suggests that these enzymes are very similar in their chromophoric properties.

The molecular weight of *Spirulina* Fd-SiR obtained by native gel electrophoresis was 60 000 dalton. This value is nearly equal to those reported for MV-SiRs cited above. When tested in SDS-polyacrylamide gel electrophoresis, the purified Fd-SiR showed a molecular weight of 63 000 dalton. Koguchi and Tamura (1988) suggested that *Spirulina* Fd-SiR has a single 63 000 dalton molecular weight subunit and is composed of two identical subunits at high ionic strength.

Cytochrome b_6f

The cytochrome b_6f complex participates in electron transfer and proton translocation in photosynthesis and respiration. The b_6f complex transfers electrons

Table 3.6 Summary of the purification steps for ferredoxin-sulfite reductase

Step	Activity (units)	Specific activity (units [mg of protein]$^{-1}$)	Purification (fold)
Crude extract	720	0.066	1
Acetone (0–70%)	440	0.74	11.2
1st DEAE-cellulose	375	0.75	11.4
2nd DEAE-cellulose	238	2.5	37.9
DEAE BIO-GEL	126	11.4	172.7
Butyl-Toyopearl	83.7	22.6	342
Sephadex G-100	25.4	39.7	601
Fd-Sepharose	7.8	49.0	741

Data extracted from Koguchi and Tamura (1988).

between the two photosystems (from plastoquinol to plastocyanin), and in cyclic electron flow around photosystem I. The b_6f complex from *Spirulina* was isolated by Minami et al. (1989). The purification steps are summarized in Table 3.7.

It is worth noting that a high efficiency of recovery was obtained when hepatyl thioglucoside was used for solubilization of the thylakoid membranes. It seems that the procedure described has some advantages over the traditional sucrose gradient purification procedure.

The purified complex contained a small amount of chlorophyll and carotenoid. At least four polypeptides were present in the complex: cytochrome *f* (29 kDa), cytochrome b_6 (23 kDa), iron-sulfur protein (ISP, 23 kDa), and a 17 kDa polypeptide. Each polypeptide was separated from the complex and treated with 2-mercaptoethanol or urea. The absorption spectra of cytochrome b_6 and cytochrome *f* were similar to those of *Anabaena* and spinach, as expected. The complex was active in supporting ubiquinol-cytochrome *c* oxidoreductase activity. Fifty per cent inhibition of activity was accomplished by 1 mM dibromothymoquinone (DBMIB). The k_m values for ubiquinol-2 and cytochrome *c* (horse heart) were 5.7 mM and 7.4 mM, respectively.

The isolation of the complex and research on its structure and function may enhance understanding of the major metabolic activities in *Spirulina*, as well as providing information on the evolutionary development of photosynthesis and respiration in cyanobacteria.

ATPase activity

The ATP synthase activity of *Spirulina* was studied by several groups, most dealing with the enzyme associated with the thylakoid membrane, also known as the ATPase coupling factor, F_1.

The latent ATPase activity in photosynthetic membranes of oxygen-evolving organisms is strikingly different from other ATPase activities. Hicks and Yocum (1986) demonstrated that the latent cyanobacterial ATPase activity in *Spirulina* membrane vesicles and F1 was elicited by treatments that stimulate chloroplast activity. They also showed that ATP acted both as an inhibitor and as an allosteric effector of CaATPase activity in *Spirulina* F_1.

Using a homogenization step for breaking the trichomes, followed by sonication, Owers-Narhi et al. (1979) obtained photosynthetic membranes from *Spirulina platensis* which contained the latent Ca^{+2}-ATPase. The purification steps used are summarized in Table 3.8.

Table 3.7 Summary of purification of the cytochrome b_6f complex from *Spirulina*

Purification step	Cyt *f*/protein (nmol mg $^{-1}$)	Specific activity (μmol mg l $^{-1}$)	Purification (fold)
Extraction	0.05	1.3	1
Solubilization	0.17	8.2	6.3
Ammonium sulfate fractionation	0.82	31.5	24.2
DEAE column chromatography	12.2	87.2	67

Data extracted from Minami et al. (1989).

Table 3.8 Partial purification of Ca $^{+2}$-ATPase activity from *Spirulina*

Fraction	Protein (mg)	Activity (units[a])	Sp. Activity (units mg $^{-1}$)	Purification (fold)
Crude extract	267	16040	0.04	1.0
EDTA Supernatant	177	10610	0.12	3.0
$(NH_4)_2SO_4$ Precipitate	71	4280	0.13	3.3
DEAE Eluate	43	2600	0.651	6.1

[a]One unit is defined as 1 µmol of phosphate released per minute.

Lerma and Gomez-Lojero (1987) used *Spirulina maxima* cells in their studies. They claimed that the ATPase activity of *S. maxima* membranes did not display persistent latency as was reported for *S. platensis*. The enzyme was readily activated by similar methods used to activate the chloroplast LF1 and showed a requirement for Mg^{2+}. The activity of ATPase reported in this study was much higher than in the one using *S. platensis* cells (Table 3.9).

Bakels et al. (1993) have recently reported in detail the unusual thermodynamic properties and activation mechanism of ATPase activity in coupled membrane vesicles isolated from *Spirulina platensis*. The nature of this activity is discussed in detail in relation to the alkalophilic nature of the cells.

Although most of the work relating to ATPase activity was done on the photosynthetic membrane and the coupling factor, it should be mentioned that ATPase activity was detected in other membrane fractions of *Spirulina*. The most recent were reported by Xu et al. (1994) describing an ATPase activity associated with the plasma membrane fraction of *Spirulina*. The activity was Mg $^{+2}$-dependent and could be stimulated by 50 mM of NaCl or KCl. Optimal pH reported was relatively high, 8.5, as compared with higher plant plasma membrane ATPase. This observation further supports the unique alkalophilic characteristics of *Spirulina*.

Acetohydroxy Acid Synthase (AHAS)

The enzyme acetohydroxy acid synthase (EC 4.1.3.18) is known as the first common enzyme in the biosynthesis of valine, leucine and isoleucin. It is considered to be a

Table 3.9 Purifiaction of ATPase from *Spirulina maxima*

Fraction	Total activity (µmol Pi min $^{-1}$)	Specific activity ([µmol Pi min $^{-1}$][mg protein] $^{-1}$)	Purification (fold)
Membranes	275	0.125	1.0
30–60% fraction of $(NH_4)_2SO_4$ precipitation	89.2	2.22	17.8
AF_1 after gradient centrifugation	81.43	3.62	29.0
AF_1 after ion-exchange chromatography	32.49	8.12	65.0

conserved protein, with high sequence similarities between bacteria, yeast and higher plants.

Two isoforms of acetohydroxy acid synthase were detected in cell-free extracts of *Spirulina platensis* by Forlani et al. (1991) and separated both by ion-exchange chromatography and by hydrophobic interaction. Several biochemical properties of the two putative isozymes were analyzed. It was found that they differed in pH optimum, FAD (flavin adenine dinucleotide) requirement for both activity and stability, and in heat lability. The results were partially confirmed with the characterization of the enzyme extracted from a recombinant *Escherichia coli* strain transformed with one subcloned *S. platensis.* AHAS activities, estimated by gel filtration, indicate that they are distinct isozymes and not different oligomeric species or aggregates of identical subunits.

Concluding Remarks

The biochemistry of *Spirulina* was previously reviewed by Ciferri (1983) and Ciferri and Toboni (1985). Although these reviews were published over ten years ago, the amount of information generated since then is fairly poor. Although a few unique physiological characteristics of *Spirulina* such as its alkalophilic nature were found, very little was done to study the biochemistry of the major metabolic activities. Little research has been carried out on the lipid and fatty acid metabolism of *Spirulina* (see Chapter 10). Some of the claims of the beneficial health properties of *Spirulina* are attributed to the relatively high content of ?-linolenic acid in the cells. What is the nature of this rather high content? Exploring the reason for the high rate of *?*-linolenic acid accumulation may help not only in revealing the biochemistry of fatty acid metabolism in *Spirulina,* but may also have an impact on modifying the chemical composition as well as the selection of strains studied for production of specific chemicals.

There is no doubt that much more research has to be done. Development of genetic and molecular biology tools for *Spirulina* will greatly aid biochemical studies.

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