Chapter 4
Discussion
_*Spirulina*_ is well known for its nutritional and biotechnological importance and has received considerable attention for its unique ability to survive in highly alkaline and saline conditions (Vonshak, 1997). Being a non-N₂-fixing cyanobacterium, *Spirulina* carries out oxygenic photosynthesis and meets its nitrogen requirement only through nitrate assimilation pathway, attaining protein content as high as 60 %. The nitrate assimilation pathway is poorly characterized in this organism, as nitrogen fixation demanded most of the research attention in cyanobacteria. This is in spite of the understanding that the molecular basis for high protein content in *Spirulina* must be rooted in nitrate assimilation and N use efficiency of this organism. The present work characterizes some aspects of nitrate assimilation in *Spirulina* (*Arthrosira platensis* strain PCC 7345) at the level of enzymes and genes of the nitrate assimilatory pathway.

**Physiological studies on nitrate assimilation in A. platensis**

Nitrogen is an essential element required for the growth of *A. platensis*, and the form and content of inorganic nitrogen could have a bearing on its growth parameters. As the axenic strain used for the present work (*Arthrosira platensis* PCC 7345) was not supported by any previously published work on nitrate assimilation, it was essential to establish the associated growth and physiological parameters for the present work. The effect of different nitrogenous sources on different growth parameters viz., protein content, optical density and chlorophyll content were analyzed in batch cultures grown in shake-flasks. These experiments were done by resuspending cultures grown in the recommended (BG11 + ASN111) growth medium with fresh media containing one source of N at a time. The optimum concentration for all
parameters of growth was found to be 20 mM nitrate, though it was retained even at 30 mM and was only slightly lower at 10 mM (Fig. 2). Higher nitrate concentrations (>30 mM) led to precipitation of the media, while lower concentrations (<10 mM) adversely affected the growth parameters. The experiments on all other sources of N were done using a concentration of 4 mM based on biochemical parameters described in Chapter 3.2.

When NH₄Cl was used as the sole source of N, OD and chlorophyll were similar to that obtained with 20 mM nitrate, but protein content was lower. However, when 4 mM NH₄NO₃ was used as a dual N source, all growth parameters were as high as in nitrate (20 mM) alone, in line with the reports of Zarrouk (1966) and Paoletti et al. (1975). Glutamine and KNO₂ had adversely impacted all growth parameters when used as the sole nitrogen sources, with nitrite having more drastic effects than the glutamine (Fig. 3). This is in line with the findings that nitrite ions are toxic to the cells and affect photosynthetic electron transport chain (Almeida et al., 1995) and that amino acids and other organic nitrogen sources are very poor source of nitrogen for cyanobacteria (Kapp et al., 1975).

In addition, the cultures grown in nitrite and glutamine also showed signs of chlorosis. It is known that in photosynthetic organisms, N limitation triggers ordered degradation of phycobilisomes, ribosomes and thylakoid membranes, resulting in chlorosis (Saha et al., 2003). Furthermore, nitrogen limitation leas to catabolisation of lipids and fatty acids, resulting in their reduction, as found in Arthrospira platensis and Anacystis nidulans (Poirreck et al., 1984).
Standardization of the growth of *A. platensis* in different nitrogen sources has been an important criterion in the mass cultivation of *A. platensis* for various useful products, though much of the emphasis was on finding economical nutrient sources for industrial production of cultures. Previous studies have reported the use of cheaper nitrogen sources like ammonia (Boussiba, 1989; Sassano *et al.*, 2007) organic waste residues (Chuntapa *et al.*, 2003) and urea (Stanca and Popovici, 1996; Danesi *et al.*, 2002; Torre and Carlos, 2003; Luis *et al.*, 2004; Soletto *et al.*, 2005) for the cultivation of *A. platensis* in the batch and fed-batch cultures for high yields. Costa *et al.* (2001) studied the growth response of *Arthrospira platensis* in different nitrogen sources in addition to sodium nitrate in a bioreactor in an attempt to find a cheaper alternative source and reported that highest biomass was obtained in sodium nitrate followed by ammonium nitrate and urea.

There were other studies on the influence of light, temperature and pH on the growth, morphology and photosynthesis in *Arthrospira platensis* have been reported (Boussiba, 1989; Vonshak *et al.*, 1992; Jensen and Knusten, 1993; Wu *et al.*, 2005) but few reports on the influence of different N sources in batch cultures of *Arthrospira platensis*. Therefore, the effect of various nitrogen metabolites on the growth parameters of *Arthrospira platensis* PCC 7345 in the present study were useful to standardize the conditions for further characterization of nitrate assimilation.
Biochemical studies on nitrate assimilation in A. platensis

In cyanobacteria, nitrate is the most common form of combined inorganic nitrogen in the largely nitrogen-deficient natural environment. Nitrate and its downstream metabolites like nitrite, ammonium and glutamine are involved in the transcriptional regulation of N-assimilatory genes, as well as post translational regulation of their enzymes (Guerrero et al., 1981; Herrero et al., 2001; Luque and Forchhammer, 2008). Indirect regulation through the photosynthesis and respiration mechanisms is also known (Flores and Herrero, 2005). In general, in nitrogen fixers, nitrate assimilation is inducible by nitrate/nitrite (Herrero et al., 1981 and 1984; Bagchi et al., 1985; Herrero and Guerrero, 1986; Flores and Herrero, 1994, Luque et al., 1994), whereas in non-nitrogen-fixing cyanobacteria, nitrate assimilation is believed to be constitutive (Palod et al., 1990).

No such studies were conducted on A. platensis till recently, when Jha et al. (2007) reported that NR activity was depleted by withdrawal of nitrate from the medium and was induced rapidly by reintroduction of nitrate. This was the first report of nitrate induction of nitrate assimilation in a non-N-fixing cyanobacterium, contrary to the popular notion of constitutive expression (Palod et al., 1990). In the present study, this finding on NR activity was verified in a different strain of A. platensis (PCC 7345), as well as extended to NiR activity and transcript levels of NRT, NR, NiR and GS.

It was found that removal of nitrate from the growth medium led to a 72 % decrease in NR specific activity in 6 hours, while NiR specific activity decreased
more slowly, by 68% in 12 hours (Fig. 4). These data clearly suggest that nitrate is
essential for the NR activity and NiR activities, which was later confirmed to be
induction at the transcriptional level (Fig. 61). Thus, the present study goes beyond
the findings of Jha et al. (2007) on nitrate induction of NR activity on a different
strain, to report nitrate regulation of NR and NiR activities and transcript levels in A.
*platensis*. These results show for the first time that nitrate induces multiple genes and
enzymes of nitrate assimilation and that therefore, nitrate may play the role of a signal
in metabolic regulation in *A. platensis*, a phenomenon well known in higher plants
(Raghuram et al., 2006).

The interaction of various downstream N metabolites with nitrate induction of
N assimilation in *A. platensis* was studied by resuspending cultures grown in the
recommended (BG11 + ASN111) growth medium with fresh media containing one
downstream metabolite of N, along with 20 mM nitrate.

A dose response study of the effects of nitrite on nitrate regulation of NR
and NiR activities revealed that nitrite (4 mM) inhibited the induction of NR
specific activity to half its control level in 6 h, while NiR activity was unaffected
(Fig. 5). The inhibitory role of nitrite on NR expression was also found at the
transcriptional level (Fig. 62), indicating regulation of gene expression. The effect
of nitrite on NiR RNA level was less pronounced. Similar studies of the effect of
ammonium ions (4 mM) and glutamine (4 mM) showed that they inhibited NR
specific activity by half of the control level, though higher concentrations of
ammonium ions (8 mM) and glutamine (6 mM) were required to inhibit NiR
specific activity by half the control value in 6 h (Fig. 6 & 7). Ammonium ions also inhibited nitrate induction of NiR RNA levels, though not NR RNA (Fig. 63). Glutamine had an opposite effect, with a clear inhibition of NR RNA level, while the NiR RNA levels remained unaffected (Fig. 64). The kinetics of the effect of downstream N metabolites were broadly similar between various metabolites/enzymes tested in this study. At 4 mM, all three metabolites (nitrite or ammonium or glutamine) inhibited NR and NiR activities in the presence of nitrate (20 mM), and the effect was substantial even at 3 h (Fig. 8), except for the slower effect nitrite on NiR (Fig. 9).

The inhibition of NR activity by nitrite and ammonium in *A. platensis* was reported earlier by Ali *et al.* (2008). The present study corroborated their findings on NR and extended them to NiR as well as to their transcriptional levels. In other cyanobacteria, ammonium is known to be an inhibitor of NR (Herrero *et al.*, 1981; Kobayashi *et al.* 1997 and Aichi *et al.* 2001) and NiR activities (Herrero and Guerrero, 1986), nitrate transport element binding protein (Madueno *et al.*, 1988) and GS activity (Vega-Palas *et al.*, 1990). It was also shown that the inhibitory effect of ammonium may be acting through glutamine (Flores and Herrero, 1994). Whether the inhibitory effect of ammonium and glutamine is due to nitrate uptake, NR down regulation, or uncoupling of photosynthesis or due to a derivative has not been established. This has not been resolved comprehensively in any cyanobacterial strains, and there is a controversy on whether ammonium acts on NR indirectly by eliminating nitrate transport or directly on the enzyme at the levels of synthesis or post-translational modification (Luque and Forchammer, 2008).
The present study shows that all three downstream metabolites tested had their effects on NR or NiR gene expression, with nitrite affecting both, while ammonium and glutamine affecting only one of the two genes, despite the fact that they both inhibited NR and NiR at the enzyme level. Though the effect of downstream metabolites on nitrate uptake have not been measured in this study, it seems unlikely that differential effects on NR and NiR by ammonium and glutamine can be explained in terms of their influence on nitrate uptake. The differences in the effect of these metabolites at the RNA level and activity level of NR/NiR also cannot be explained in terms of nitrate uptake, since nitrate has a positive effect on both the NR and NiR at the level of RNA as well as activity. It seems more likely that the downstream metabolites have their own effects on NR/NiR gene expression and/or activity.

**Thermotolerance of NR, NiR and GS in A. platensis**

Since *A. platensis* is able to convert more nitrogen into protein than any higher plant, biochemical and molecular characterization of N assimilatory enzymes are of considerable interest. Moreover, purified nitrate assimilatory enzymes are used in environmental testing (Patton *et al.*, 2002; Campbell *et al.*, 2006), nitrate decontamination (Mellor *et al.*, 1992), meat processing (Gotterup *et al.*, 2007), biosensors (Chen *et al.*, 2008) and other applications (Angeby *et al.*, 2002). There is no known natural source for sturdy and stable nitrate assimilatory enzymes, and attempts have been made to genetically engineer higher plant NR for applied uses (Campbell *et al.*, 2006). There are several reports of enhancing the thermostability of
GDH from micro-organisms (Khan et al., 2005; Hamza and Engel, 2007). Moreover, there has been a renewed interest in the thermal behaviour of enzymes for their biotechnological and environmental applications (Eisenthal et al., 2006). In addition to the search for new thermostable enzymes from thermophiles, various attempts have been made for improving the thermostability of the enzymes from mesophilic organisms (Menendez-Arias, 1989). However, the factors influencing thermostability of enzymes are not fully understood (Matthews, 1993).

Recently, Ali et al. (2008) reported that a local strain of *Spirulina (A. platensis* ARM 728) had higher specific activities and stabilities of NR, NiR and GS in crude extracts at RT, as compared to a higher plant like rice. However, it was not known whether this is true in other strains of this organism, and whether their nitrate assimilatory enzymes survive higher temperatures. Therefore, the thermodurability of NR, NiR and GS was examined in the crude extracts of the *A. platensis* strain PCC 7345 in the present study, in comparison with those of rice.

NR, NiR and GS in *A. platensis* revealed relatively higher thermodurability than those of rice, especially at above-ambient temperatures. Even at 80 °C for 1 h, these enzymes were less affected in *A. platensis* than in rice, retaining 3.4, 1.7 and 3.7 fold higher specific activities respectively (Fig. 10-12). Although the reasons for such higher thermostability have not been explored in detail in the present study, the role of salts, small proteins and other small molecular weight compounds in imparting thermodurability to *A. platensis* enzymes has been ruled out by assessing thermodurability in extracts subjected to G-25 gel filtration (Fig. 13). However, the
role of larger proteins in imparting thermotolerance cannot be ruled out, unless these studies are conducted on purified enzymes to verify whether heat resistance is intrinsic to the enzyme protein. Based on studies conducted in Anabena sp., Rajaram and Apte (2008) showed the involvement of Cpn 60, a member of the heat shock protein family in cyanobacteria. They showed that heat stress caused rapid and severe inhibition of photosynthesis and nitrate reduction in nitrate-supplemented, nitrogen fixing Anabena sp. is directly correlated with the loss of Cpn 60 protein.

The thermal tolerance of all three nitrate assimilatory enzymes renders A. platensis an attractive natural source of sturdy enzymes for various applications. This is also the first report of a single natural source for all three thermotolerant nitrate assimilatory enzymes. The nitrate inducibility of N assimilation genes, higher specific activity and thermotolerance of N assimilatory enzymes may also account, at least in part, to the higher N-utilization capacity and high protein content in this organism. These properties may also be of interest in environmental applications for bioremediation of N-contaminated water sources. Therefore, attempts were also made to study the bioremediation potential of A. platensis as follows.

**Bioremediation studies on A. platensis**

Preliminary evidence for bioremediation had earlier been established in Korea for the use of Spirulina in decontamination of nitrate and phosphate from swine waste (Kim et al., 2000; Lodi et al., 2003). Others have evaluated the efficacy of integrated algae–shrimp culture where Arthrospira platensis was used for water quality control with
semi continuous algae harvest, and other phytoremediation options (Rangsayatorn, 2002). Most of the bioremediation studies on *Spirulina* (*A. platensis*) dealt with sewage waters and polluted industrial effluents, which also tend to contain high levels of organic carbon. There were no studies of significance that attempted to exploit the autotrophic properties of *A. platensis* for bioremediation of potable water sources, which generally have more of inorganic ions and less of organic carbon.

Therefore, the viability of *A. platensis* in different fresh water sources and its ability to remove nitrate ions from water was examined in the current study using tap water, ground water and Yamuna river water, without adding any other nutrient exogenously. The Tap water had the lowest concentration of inorganic and organic nutrients, followed by ground water and Yamuna water. Maximum growth and chlorophyll content of *Arthrospira platensis* was observed in the Yamuna water (Fig. 17). The level of nitrate ions decreased upon inoculation of *Arthrospira platensis* over several days, to 87.5 % in tap water (Fig. 14), 71.97 % in Yamuna water (Fig. 15) and 26.33 % in ground water (Fig. 16). These results indicate the potential of *Arthrospira platensis* in the bioremediation of potable water sources. When the cultures were allowed to grow further, a sharp increase in nitrite levels was observed in water samples, going beyond their original levels, possibly due to cell lysis or leakage of nitrite from the *A. platensis* cells. Though the pattern of nitrate removal was similar in all three types of water samples, quicker cell growth was observed in ground water, as it had the highest initial nitrate content, which supports biomass growth (Lodi *et al.*, 2003).