Section 3.2

Biochemical characterization of nitrate assimilation in *A. platensis*

Nitrate taken up from the environment is transported into the cells by an active transport system (NRT) and reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) prior to fixation into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway. The enzymes and the genes of nitrate assimilatory pathway are characterized in some of the cyanobacteria (Luque and Fochammer, 2008). It has been known for a long time that nitrate and its downstream metabolites are involved in the regulation of enzymes and genes as well and their effects have been studied on nitrate uptake, nitrate transport, synthesis and activity of NR and NiR in many cyanobacteria (Herrero *et al*., 2001; Luque *et al*., 1994).

However, there is a paucity of information regarding the role of nitrate and nitrite in *A. platensis*, except for the report of Jha *et al.* (2007) that NR activity in *Spirulina platensis* (strain ARM 728) was induced by nitrate and a report of inhibition of NR by nitrite and ammonium ions (Ali *et al*., 2008). Therefore, present investigation was carried out to study the influence of various N-sources (nitrate, nitrite, glutamine and ammonia) in the regulation of NR and NiR activities in *Arthospira platensis* PCC 7345. Transcriptional studies on these as well as GS and NRT have also been done using the information obtained from their cloning and sequencing, and reported in the section 3.7 later in this chapter.
3.2.1. Effect of nitrate removal on NR and NiR activity in *A. platensis*

8-9 days old mid-log phase *A. platensis* culture was harvested, washed and resuspended in a N-free medium for 6 h and NR and NiR specific activities were checked as described in Materials and Methods. A steady drop in NR specific activity levels were observed, which fell to 28.8 % of the initial activity after 6 hours; beyond which no further noticeable drop was observed (Fig. 4). Similarly, NiR specific activity decreased to about 74 % at the end of 6 hours, which decreased further to 30.85 % at the end of 12 h (Fig. 4).

![Graph showing NR and NiR activity over time](image)

Fig.4: Effect of nitrate removal on NR & NiR specific activity up to 12 h in *A. platensis*. The cultures growing in normal nitrate (20 mM) supplemented medium were washed, resuspended in N-free medium and assayed for the NR and NiR enzyme activities as described in M & M. The curves were plotted using mean values internal triplicates from three different experiments, along with standard error bars.

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.
3.2.2. Effect of nitrite on NR and NiR in *A. platensis*

A dose response study was carried out with exogenously added nitrite (2, 4 and 8 mM) on the activities of NR and NiR in the presence of normal nitrate concentration (20 mM) in the media (Fig. 5). NR specific activity decreased with increasing concentration of nitrite to 46.66% in 8 mM of nitrite in 6 h, while NiR activity was slightly increased by 12.08% in 2 mM nitrite and remained in that range even at higher concentrations. The inhibition of nitrate assimilatory pathway by nitrite has been reported earlier also in *Synechococcus* (Herrero *et al.*, 1981).

![Graph showing NR and NiR specific activity](image)

**Fig. 5: Effect of 2, 4, and 8 mM nitrite on NR and NiR specific activity in *A. platensis*.** The cultures grown in normal nitrate medium were supplemented with 2, 4, 6 mM nitrite for 6 hours and assayed for enzyme activity as described in M & M. The mean specific activities from three independent experiments are plotted with standard error bars.
3.2.3. Effect of ammonium on NR and NiR in *A. platensis*

A dose response study was carried out with exogenously added ammonia (2, 4 and 8 mM) on the activities of NR and NiR in the presence of normal nitrate concentration (20 mM) in the media (Fig. 6). The NR specific activity decreased with increasing concentration of ammonium and is reduced to 36.36 % in 8 mM of ammonium in 6 h, while the NiR activity decreased to 51.5 %. Ammonium is known to be an inhibitor of the NR and NIR activities (Maudeno *et al.*, 1988) and similar results of inhibition of NR activity were shown earlier by Ali *et al.* (2008).

Fig. 6: Effect of 2, 4, and 8 mM ammonia on NR and NiR specific activity in *A. platensis*. The cultures grown in normal nitrate medium were supplemented with 2, 4, 6 mM ammonium ions for 6 hours assayed for enzyme activity as described in M & M. The mean specific activities from three independent experiments are plotted with standard error bars.
3.2.4. Effect of glutamine on NR and NiR in *A. platensis*

A dose response study was carried out with exogenously added glutamine (2, 4 and 6 mM) on the activities of NR and NiR in the presence of normal nitrate concentration (20 mM) in the media (Fig. 7). The NR specific activity decreased with increasing concentration of glutamine and was reduced to 41.28% in 6 mM of glutamine in 6 h, while the NiR specific activity was decreased to 37%. It has been speculated earlier in the literature that the inhibitory effect of ammonium may be acting through glutamine (Flores and Herrero, 1994).

![Graph showing effect of glutamine concentration on NR and NiR activity](image)

**Fig. 7: Effect of glutamine concentration (2, 4, 6 mM) on NR and NiR specific activity.** The cultures grown in normal nitrate medium were supplemented with 2, 4, 6 mM glutamine for 6 hours and assayed for enzyme activity as described in M & M. The mean specific activities from three independent experiments are plotted with standard error bars.
3.2.5. Kinetics of the effect of downstream N metabolites on NR and NiR activities

In order to understand whether the kinetics of the effect of downstream N metabolites were similar between metabolites/enzymes, effects of 4 mM each of nitrite, ammonium and glutamine on NR and NiR activities were studied in the presence of nitrate (20 mM) after 3, 6 and 9 h. As evident from Fig. 8, though all 3 metabolites inhibited of NR activity substantially even at 3 h, glutamine had the most drastic effect, followed by nitrite, while ammonium ions had a more subdued effect. This trend was also true in the kinetics of inhibition at later time points, as indicated by the slope of the graph for each of the metabolites. The inhibitory effect of nitrite reached a plateau 6 h, whereas that of glutamine and ammonium continued up to 9 h and possibly beyond. In case of ammonium or glutamine, NR activity reduced to 51.5 % and 38 %, respectively after 9 h. It can be inferred from the kinetic analysis that maximum reduction in NR activity after 9 was observed for glutamine, followed by nitrite and ammonium respectively.

As is evident from Fig. 9, all 3 metabolites showed inhibition of NiR activity, though the inhibitory effect of nitrite was not seen for nitrite up to 3 h, while glutamine and ammonium had a drastic effect on NiR activity. NiR activity decreased to 43.02 %, 36.38 % and 60 % after 9 h in the presence of nitrite, ammonia and glutamine respectively (Fig. 9). The kinetics of inhibition was similar for ammonium and glutamine for 3 h, but the rate of inhibition was different at later time points and the maximum reduction in activity was caused by ammonium as is clear from the slope of the graph.
Fig. 8: Effect of nitrite (4mM), ammonium (4mM) and glutamine (4 mM) on NR activity with time in *A. platensis*. The cultures grown in normal nitrate medium were supplemented with 4 mM each of nitrite, ammonium and glutamine and assayed for enzyme activity as described in M & M. The curves were plotted using mean values internal triplicates from three different experiments, along with standard error bars.

Fig. 9: Effect of nitrite (4mM), ammonium (4mM), and glutamine (4 mM) on NiR activity with time in *A. platensis*. The cultures grown in normal nitrate medium were supplemented with 4 mM each of nitrite, ammonium and glutamine and assayed for enzyme activity as described in M & M. The curves were plotted using mean values internal triplicates from three different experiments, along with standard error bars.
Section 3.3

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

The growth, productivity and protein content in plants depend critically on their nitrogen metabolism and N use efficiency (NUE), which in turn depends on the optimal flux of the enzymes of nitrate uptake and assimilation. Whether this is also true of a non-N-fixing, nitrate utilizing, high-protein cyanobacterium like *A. platensis* was sought to be explored in terms of the specific activities and stabilities of the nitrate assimilatory enzymes in this study. Recently, Ali *et al.* (2008) reported that a local strain of *Spirulina platensis* (ARM 728) had higher specific activities and stabilities of NR, NiR and GS in crude extracts at RT, in comparison to a higher plant like rice.

The present study sought to validate these results in the strain PCC 7345 at RT and also explored thermotolerance at higher temperatures, by comparing the specific activities of NR, NiR and GS between *A. platensis* and rice, in crude as well as in G-25 fractionated extracts. The extracts were prepared as described in materials and methods, aliquoted into different microfuge tubes and pre-incubated at different temperatures (4, 25, 40, 60 and 80 °C) for different durations (5, 15, 30, 45 and 60 min) before assaying them separately for NR, NiR and GS at 25 °C.

3.3.1 Thermotolerance of nitrate reductase in *A. platensis*

NR had maximal specific activity at 4 and 25 °C, and remained stable for at least an hour in the extracts of both *A. platensis* and rice. At higher temperatures, *A. platensis* NR revealed higher thermotolerance than that of rice (Fig. 10). For example, at 60 °C and 80
°C, the specific activity of rice NR declined to negligible levels within 5 min indicating total inactivation, whereas in *A. platensis*, even after 30 min, NR retained full activity at 40 °C, over half the activity at 60 °C, and 28% of the initial activity at 80 °C. The data presented clearly indicate that the kinetics of thermal inactivation of each of the enzymes was significantly slower in *A. platensis* (top panel) than in rice (bottom panel), especially after 30 minutes of preincubation at 60 and 80 °C. In view of the fact that earlier attempts to enhance the stability of higher plant NR by genetic manipulation met with limited success (Campbell *et al.*, 2006), our finding that *A. platensis* could be a natural source for stable and thermotolerant NR bears significance.

Fig. 10: Thermotolerance of NR activity in *A. platensis* (top) and rice (bottom). Crude extracts from *A. platensis* cultures and rice leaves were pre-exposed to a range of temperatures (4, 25, 40, 60 & 80 °C) and NR activity was assayed after different time intervals (5, 15, 30, 45, 60 min) as described in Materials and Methods. The mean specific activities from three independent experiments are plotted with standard error bars.
3.3.2. Thermotolerance of nitrite reductase in *A. platensis*

NiR activity remained stable for at least an hour in the extracts of both *A. platensis* and rice at 4, 25 and 40 °C. At higher temperatures, *A. platensis* NiR revealed higher thermotolerance than that of rice, though to a lesser degree than in NR (Fig. 11). Its specific activity in rice declined to 45 % and 37 % of the initial activity in 1 h at 60 and 80 °C respectively, whereas in *A. platensis*, NiR retained over 73 % and 64 % of its initial activity under similar conditions. Ali et al. (2008) did not observe significant differences in the half lives of NiR at RT between *A. platensis* and rice (13 and 12 h respectively), whereas our present data at higher temperatures clearly bring out that thermotolerance is an advantage of NiR in *A. platensis* as compared to that of rice.

![Graph](image)

**Fig. 11:** Thermotolerance of NiR activity in *A. platensis* (top) and rice (bottom). Crude extracts from *A. platensis* cultures and rice leaves were pre-exposed to a range of temperatures (4, 25, 40, 60 & 80 °C) and NiR activity was assayed after different time intervals (5, 15, 30, 45, 60 min) as described in Materials and Methods. The mean specific activities from three independent experiments are plotted with standard error bars.
3.3.3. Thermotolerance of glutamine synthetase in *A. platensis*

The specific activity of GS, in general, was higher in *A. platensis* than in rice, indicating the difference in steady state abundance of the enzyme, corroborating earlier results from a different strain (Ali et al., 2008). At 4, 25 and 40 °C, the enzyme remained nearly stable for at least an hour in both organisms. At higher temperatures, *A. platensis* GS revealed higher thermotolerance than that of rice (Fig. 12). Its specific activity in rice declined to 55.62 % and 28.1 % of the initial activity in 1 h at 60 and 80 °C respectively, whereas in *A. platensis*, GS retained over 76.7 % and 56.8 % of its initial activity under similar conditions. It was observed that at 60°C, the specific activity of *A. platensis* GS increased marginally by about 10 % in 30 min, but the significance of this, if any, is not clear. However, it does not affect the main result that the *A. platensis* enzyme (GS) is more thermotolerant than that of rice.

![Graph showing thermotolerance of GS activity in A. platensis and rice](image)

**Fig. 12:** Thermotolerance of GS activity in *A. platensis* (top) and rice (bottom). Crude extracts from *A. platensis* cultures and rice leaves were pre-exposed to a range of temperatures (4, 25, 40, 60 & 80 °C) and GS activity was assayed after different time intervals (5, 15, 30, 45, 60 min) as described in Materials and Methods. The mean specific activities from three independent experiments are plotted with standard error bars.
3.3.4. Thermotolerance of *A. platensis* enzymes following gel filtration

In order to verify the possible role of salts, small proteins and any other small molecules in conferring thermotolerance to the *A. platensis* enzymes, its crude extracts were subjected to Sephadex G-25 gel filtration at 4 °C and again tested for thermal tolerance of NR and NiR at 80 °C for 30 min and 1 h. The data shown in Fig. 13 clearly indicates that gel-filtered extracts retained their original thermotolerance levels for both the enzymes. GS was always assayed in gel-filtered extracts, as crude extracts were not suitable for direct use in the assay because of the precipitation observed in the reaction due to the presence of small molecules and salts in the crude extract. Therefore, it is very likely that thermotolerance is a property inherent to these enzymes in *A. platensis*, though it can only be confirmed after their complete characterization.

![Graph showing thermotolerance of NR and NiR](image)

*Fig. 13: Thermotolerance of NR and NiR before and after Sephadex G-25 gel filtration of *A. platensis* extracts.* The enzymes were assayed in crude or G-25-purified extracts pre-incubated at 25 °C or 80 °C for 30 and 60 min as described in Materials and Methods. For the calculation of relative specific activities, the absolute specific activity at 4 °C was taken as 100 % for both fresh and purified extracts. The actual activities were 1.80 and 2.44 IU for NR and 30.24 and 31.19 for NiR before and after fractionation, respectively. The means of replicates were plotted along with SD bars.
In conclusion, NR, NiR and GS in A. platensis revealed relatively higher thermotolerance 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 activities respectively. The thermotolerance of all three nitrate assimilatory enzymes renders A. platensis an attractive natural source of sturdy enzymes for various applications.
Section 3.4

Preliminary assessment of bioremediation potential of *A. platensis* for nitrate removal

Cyanobacteria and algae are increasingly being explored for the bioremediation of polluted water sources, including nitrate removal (Hu *et al.*, 2000). Preliminary evidence for the use of *Arthrospira platensis* in decontamination of nitrate and phosphate from swine waste was established in the beginning of the last decade (Kim *et al.*, 2000; Lodi *et al.*, 2003). However, there were no attempts to utilize its potential for the removal of excess nutrients from potable waters and other sources rich in inorganic pollutants and low in organic contaminants. A detailed biochemical characterization of the regulation of the nitrate assimilatory enzymes by nitrate and downstream N metabolites presented in section 2 demonstrated that both NR and NiR are inducible enzymes in *A. platensis* with higher activity in the presence of nitrate. The thermotolerant nature of N-assimilatory enzymes in *A. platensis* has been shown in section 3.

This section deals with studies aimed at determining the viability of *A. platensis* in different water sources and its ability to remove nitrate ions from potable water sources, without adding any other nutrient exogenously. For this purpose, tap water, ground water and Yamuna river water in and around the GGSIP university campus were autoclaved in conical flasks and used. The nitrate content was analyzed before and after inoculation with *Arthrospira platensis* PCC 7345 at a cell concentration of 4 mg/ml chlorophyll content as described in Material and Methods. Nitrate contents were analyzed periodically up to 15 days.
3.4.1. Nitrate removal from tap water

Tap water had an initial nitrate content of 38.76 nmoles/ml before inoculation. Following the inoculation of *Arthrosira platensis* PCC 7345, the change in the level of nitrate ions in tap water over 15 days is shown in Fig. 14. There is a reduction in nitrate level from 40 nmoles/ml to 5 nmoles/ml (87.5 %) after 12 days, following which there was a sharp increase in nitrate levels due cell lysis, as described later.

![Graph showing nitrate concentration over 15 days with two cultures](image)

**Fig. 14:** Change in nitrate concentration following inoculation of *Arthrosira platensis* PCC 7345 in tap water over 15 days. The curve was plotted using mean values internal triplicates from two different experiments, along with standard error bars. Culture 1 and culture 2 represents two independent tap water samples inoculated with the same strain.

3.4.2. Nitrate removal from the Ground water

Ground water had an initial nitrate content of 200.6 nmoles/ml before inoculation. Following the inoculation of *Arthrosira platensis* PCC 7345, the change in the level of nitrate ions in tap water over 15 days is shown in Fig. 15. There is a reduction in nitrate level to 148 nmoles/ml (27 %) after 9 days, following which there was a sharp increase in nitrate levels due cell lysis, as described later.
Fig. 15: Change in nitrate concentration following inoculation of *Arthospira platensis* PCC 7345 in ground water over 15 days. The curve was plotted using mean values internal triplicates from two different experiments, along with standard error bars. Culture 1 and culture 2 represents two independent ground water samples inoculated with the same strain.

3.4.3. Nitrate removal from the Yamuna river water

Yamuna water had an initial nitrate content of 10.88 nmoles/ml before inoculation. Following the inoculation of *Arthospira platensis* PCC 7345, the change in the level of nitrate ions in tap water over 15 days is shown in Fig. 16. There is a reduction in nitrate level to 2-3 nmoles/ml (71.97 %) after 12 days, following which there was a sharp increase in nitrate levels due cell lysis, as described later.

Fig. 16: Change in nitrate concentration following inoculation of *A. platensis platensis* PCC 7345 in Yamuna water over 15 days. The curve was plotted using mean values internal triplicates from two different experiments, along with standard error bars. Culture 1 and culture 2 represents two independent Yamuna water samples inoculated with the same strain.
Fig. 17: A photograph showing the cultures of *Arthospira platensis* in different stages of the experiment. The figure on the left panel shows the culture at zero time point before inoculation. The stage of the culture as observed on 15\textsuperscript{th} day was shown in the right panel, where 8A represents culture grown in Tap water, 8B represents culture grown in Ground water and 8C represents culture grown in Yamuna water.

In the present study, maximum growth was observed in the cultures growing in Yamuna water, followed by ground water and the least in tap water (Fig. 17). The culture in Tap water turned pale yellow at the end of 15 days, remained green in ground water and biomass increase was visible in Yamuna water. Analysis of chlorophyll content in cell pellets recovered by centrifugation revealed that the maximum content was in Yamuna water (1.31 mg/ml) and the minimum content was in tap water (1.1 mg/ml).

In conclusion, analysis of nitrate removal by *Arthospira platensis* from various water sources varying in composition in terms of inorganic salts and organic nutrients revealed viability of cells for up to 8-9 days. Maximum growth and chlorophyll content was observed in the Yamuna water and analysis of nitrate estimation revealed a reduction in nitrate levels to 87.5 \%, 71.97\% and 26.33 \% in tap water, Yamuna water and ground water respectively. This indicates the potential of *Arthospira platensis* PCC 7345 for the bioremediation of potable water sources. More comprehensive studies using continuous fed-batch cultures might help offer better insights.
Section 3.5

PCR cloning of N assimilatory genes in *A. platensis*

In order to understand the molecular biology of nitrate assimilation and protein content in *A. platensis*, cloning and characterization of the genes of nitrate uptake and assimilation became necessary. The nitrate assimilatory pathway in cyanobacteria generally consists of clusters of related genes encoding the nitrate transport system (*nrtABCD* or *nrtP*) and nitrate assimilatory enzymes (*narB* for nitrate reductase and *nirA* for nitrite reductase). These are usually co-transcribed as a *nir* operon and represented as *nirA-nrtABCD-narB* operon (Omata, 1995; Frias et al., 1997), though the operon type of arrangement is not universal in all cyanobacteria and the situation in *Arthrosira* is not known at all. The other genes downstream of nitrate assimilation are glutamine synthetase (*glnA*) and glutamate synthase (*glsF*), which are very well characterized in all the recently sequenced cyanobacteria (Luque and Forchammer, 2008), but not in *Arthrosira platensis* PCC 7345. Therefore, this study undertook the PCR cloning and sequencing of nitrate reductase, nitrite reductase, nitrate/nitrite/ammonium transporters, glutamine synthetase and glutamate synthase from the genomic DNA isolated from *Arthrosira platensis* strain PCC 7345.

3.5.1. DNA Isolation and analysis

DNA was isolated from *Arthrosira* cultures using the modified protocol for isolating DNA from filamentous cyanobacteria (Wu et al., 2000) with minor modifications as described in Materials and Methods. The quality and quantity of the isolated DNA