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Perspective of nitrate assimilation and bioremediation in *Spirulina platensis* (a non-nitrogen fixing cyanobacterium): An overview

Authors Info

Q. Fariduddin^{1*}, P. Varshney¹
and A. Ali²¹Plant Physiology and Biochemistry
Section, Department of Botany,
Aligarh Muslim University,
Aligarh-202 002, India²Department of Life Sciences,
University of Mumbai,
Mumbai-400 098, India*Corresponding Author Email :
qazi_farid@yahoo.com

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Abstract

Cyanobacterium, *Spirulina platensis*, has been used for many centuries as a food product and also has important applications in industry and environmental remediation. Apart from being a very good source of essential nutrients such as provitamins, minerals, polyunsaturated fatty acids, *S. platensis* is also characterized by its high protein content. The nitrate assimilation genes of *S. platensis* is organised in a systemic operon with the structure: *nirA* (nitrite reductase)–permease gene(s)–*narB* (nitrate reductase). Genomic localisation in *S. platensis* is different from the operons found in other cyanobacteria due to the presence of both types of nitrate transporters (*nrtP* and ABC types). Both nitrate uptake and transcription of the nitrate assimilatory genes in *S. platensis* are regulated. However, the mechanism of regulation of nitrate assimilatory genes is different from other non-nitrogen fixing cyanobacterial species. In the last decade, it has also been exploited for its capacity to decontaminate water and environmental pollution caused by hazardous materials. Role of *S. platensis* in removal of toxic metal ions from the polluted effluent have taken more importance in this area because of their small size have a high surface area-to-volume ratio and therefore provide a large contact area for metal binding. The biosorption processes have been studied extensively using *S. platensis* microbial biomass as biosorbent for heavy metal ions removal because of adherent advantage of mass cultivation. All of these desirable physiological characteristics and applications have made *S. platensis* as a model organism to understand nitrogen metabolism. This review deals with recent advances in the characterization of nitrate assimilation and bioremediation potential of *S. platensis*.

Introduction

Spirulina platensis is non-nitrogen fixing, nitrate-utilising filamentous cyanobacteria best known for its high protein content (70% by dry weight) and has remarkable nutraceutical, environmental and biotechnological significance (Vonshak, 1997; Gumbo and Nesamvuni, 2017). There are few studies on the induction, activity, stability and thermotolerance of N-assimilatory enzymes in *S. platensis* (Jha *et al.*, 2007; Ali *et al.*, 2008; Lochab *et al.*, 2010 and 2014) but it is not understandable how the organism control its nitrogen metabolism to produce so much protein. The N-metabolic pathway and its regulation responsible for its high protein content remain to be well characterized at the gene or protein level.

Nitrate assimilation is the major process of nitrogen acquisition in non-N₂-fixing cyanobacteria (Guerrero *et al.*, 1981). Accordingly the N metabolism and its regulation has been studied well in many cyanobacterial organisms like *Synechocystis* sp. PCC 6803 which does not have high protein content or other environmental applications. Moreover, recent advances in the genomic sequencing of *S. platensis* opened the new vistas for further research in non-nitrogen

fixing cyanobacteria (Fujisawa *et al.*, 2010; Janssen *et al.*, 2010; Carrieri *et al.*, 2011; Cheevadhanarak *et al.*, 2012).

For the assimilation of nitrate, an organism needs to have an active transporter for nitrate (NRT), nitrate reductase (NR) and nitrite reductase (NiR). NRT is essential for the transport of the substrate into the cell, and NR and NiR are required for the subsequent two-step reduction of nitrate into ammonium. In the organisms capable of nitrate assimilation, the presence of nitrate generally exerts positive effects on the expression of the genes encoding NRT, NR, and NiR (Siverio, 2002; Fernandez and Galvan, 2008; Krouk *et al.*, 2010; Ali *et al.*, 2011). On the other hand, reduced nitrogen sources such as ammonium and glutamine, have negative effects on the nitrate assimilatory genes (Ali *et al.*, 2010). Both cyanobacteria and plants respond to these nitrogen signals, but the mechanism of signal perception and transduction in cyanobacteria are quite different from those in higher plants.

Different responses to nitrate have been reported in literature between the non-nitrogen fixing (nitrate utilizing) and N_2 fixing cyanobacteria. In non-nitrogen fixers, high levels of NR have been reported in media lacking combined nitrogen (Palod *et al.*, 1990), suggesting constitutive expression, whereas in N_2 fixers, nitrate assimilation is known to be a substrate inducible system (Flores and Herrero, 1994). However, positive regulation of nitrate assimilation (*nir* operon) by nitrite has been reported in nitrate-limited conditions in unicellular, non- N_2 fixing *Synechococcus* sp., strain PCC7942 (Aichi *et al.*, 2004). There is a paucity of information regarding the role of nitrate and nitrite in other non- N_2 fixing cyanobacteria in general and *S. platensis* in particular. Nitrate assimilation in *S. platensis* is not well characterized, despite its direct link with its high protein content. It can also be used as a model organism to study the regulation of nitrate assimilation pathway and the relationship between nitrogen metabolism and protein content.

Comparison of nitrate assimilation in cyanobacteria (*S. platensis*) with higher plants : *S. platensis*, whose biochemical machinery for N-metabolism resembles with that of higher plants like rice, produces at least 10-fold higher protein levels than that of rice, indicating a correspondingly high capacity for nitrate utilization. In a recent report, nitrate has been shown to confer halotolerance to NaCl-tolerant mutant of *Spirulina* (Gupta *et al.*, 2017). However, the biochemical and molecular basis for such huge differences in nitrogen use efficiency and protein productivity are not known. Many cyanobacteria also have the capability of fixing nitrogen due to the development of heterocysts, which are specialized cells within the filaments that exclude oxygen and sustain a reducing environment (Slonczewski and Foster, 2009). However, unlike other cyanobacteria, *S. platensis* lacks these structures and can, therefore, not complete the process of nitrogen fixation that differentiate *S. platensis* with other N_2 fixing cyanobacteria.

Unlike higher plants, cyanobacteria usually do not encode multi gene families. With much smaller numbers of transporters, enzymes, and regulators, the *S. platensis* nitrate assimilation pathway and its regulation appears to be much simpler than those in higher plants. However, interactions between the regulatory proteins and pathways make up a complex network and sometimes give rise to different phenotypes in different species or strains, some of which may be of physiological significance but others may not.

A remarkable difference between *S. platensis* and higher plants is found in the balance between the positive and negative effects of the nitrogenous compounds. The positive effects of nitrate may be observed in the presence of ammonium in higher plants (Krouk *et al.*, 2010) whereas the effect of ammonium overrides that of nitrate/nitrite in most cyanobacteria. The tight regulation by ammonium seems to be related to the higher nitrogen content in cyanobacteria. During steady-state growth under nitrogen-replete conditions, the rate of nitrogen assimilation in cyanobacteria is about 20% of CO_2 assimilation, which means that over 30% of the reducing equivalents generated by oxidation of water are used for nitrate assimilation (Flores *et al.*, 1983). Since the proportion of the reducing power to be used for nitrogen assimilation can be reduced to 10% of the total by assimilating ammonium, it is reasonable for cyanobacteria to show a strong preference for ammonium over nitrate.

Moreover, cyanobacteria and higher plants are quite different in their mechanisms of nitrogen signal perception and transduction. In cyanobacteria the signal for the ammonium-promoted negative regulation is the depletion of cellular 2-OG. Conversely, nitrogen limitation results in the accumulation of 2-OG, which leads to the activation of nitrate assimilatory genes and proteins (Muro-Pastor *et al.*, 2001). The positive effect of nitrate is mediated by nitrite, which activates a limited number of genes directly related to nitrate assimilation (Aichi *et al.*, 2001). This contrasts with the activation by both nitrate and nitrite of a large number of genes in higher plants (Wang *et al.*, 2003). The roles of signalling pathways in the regulation of nitrate assimilatory enzymes have been also well characterized in higher plants as compared to cyanobacterial nitrate assimilation pathway (Ali *et al.*, 2007).

Distribution and characterization of genes related with nitrate assimilation in *S. platensis* : The distribution of the genes associated with nitrate assimilation in *S. platensis* PCC7345 genomes has been analyzed bioinformatically (Ohashi *et al.*, 2011) but complete annotation of genes in *S. platensis* awaits their independent molecular characterization. Table 1 shows the distribution of the genes associated with nitrate assimilation and its regulation in *S. platensis*. In cyanobacteria, the nitrate assimilatory pathway usually in the form of a cluster of genes encoding the nitrate assimilatory enzymes (*narB* for nitrate

reductase and *nirA* for nitrite reductase) and nitrate transport system (*nrtABCD* or *nrtP*) which are co-transcribed as a *nir* operon (*nirA-nrtABCD-narB* operon) (Frias *et al.*, 1997). The operon type of arrangement is not universal. The other genes like glutamine synthetase (*glnA*) and glutamate synthase (*gltS*) are present downstream of nitrate assimilation genes, which have not yet been characterized in *S. platensis*.

The genes of cyanobacterial N-assimilatory pathway are regulated by a nitrogen control mechanism mediated by *ntcA*, a transcriptional activator. The role of ammonium as an inhibitor of *ntcA* has also been studied in great detail in unicellular, non-N₂-fixing in comparison to filamentous non-N₂-fixing cyanobacteria (Muro-Pastor *et al.*, 2001). At high concentrations of ammonium, it inhibits uptake and assimilation of nitrate/nitrite (Kobayashi *et al.*, 1997), as well as transcription of N-assimilatory genes through repression of *ntcA* (Lindell and Post, 2001; Lindell *et al.*, 2005). In N₂-fixing cyanobacteria, the expression of N-assimilatory genes are regulated by the presence of nitrate/nitrite while in case of non-N₂-fixing cyanobacteria, the regulatory role is mainly attributed to repression of *ntcA* by ammonium (Flores and Herero, 2005). Moreover, the role of nitrate /nitrite is not so well understood. The only nitrite regulation known so far operates through *ntcB*, a lys R-type transcriptional activator that upregulates the *nirA* operon in *S. elongatus* strain PCC 7942 (Aichi *et al.*, 2004). The role of PII and pipX regulators has been shown in unicellular non-N₂-fixing cyanobacterium *S. elongatus* PCC 7942 (Espinosa *et al.*, 2006) by enhancing the 2-OG-dependent *ntcA*-mediated upregulation of *nirA* operon. Due to the lack of information regarding the regulators in *S. platensis*, their role in regulating N-metabolism remains to be dissected in detail.

Molecular characterization of nitrate assimilatory pathway remains an explored area of research in the non-N₂ fixing filamentous cyanobacterium *S. platensis*, despite its importance in nutritional and environmental biotechnology. The first comprehensive characterization of the genes of nitrate uptake, assimilation and regulation in *S. platensis* PCC 7345 has been done. The full-length genes of *nrtP*, *narB*, *nirA*, *glnA* and *gltS* and the regulator *ntcA* have been cloned, sequenced and their phylogeny, genomic organization and transcriptional regulation by nitrate and its downstream metabolites have been determined (Lochab *et al.*, 2014). The genes *narB* and *nrtP* were found in a single genomic clone of 5.2 kb with separate reading frames of 2,211 and 1,513 nucleotides, respectively, coding for proteins of

Fig. 1 : Localization of nitrate assimilation genes and gene clusters in Cyanobacteria

Gene names (*nrtABCD*, *nrtP*, *focA*, *narB*, and *nirA*) in the pentagons represent the putative transcription units in different cyanobacteria. The locations of extra genes in the cluster have been shown by an inverse triangle above the pentagons (gene name not shown here)

737 and 504 amino acids. The deduced amino acid sequence of NrtP protein showed high sequence identity with that of *Trichodesmium erythraeum* IMS101 (84%). The highest sequence identities were found with *Lyngbya* sp. PCC 8106 and *Trichodesmium erythraeum* IMS101 (77%). Characterization of the genomic clones of other nitrate assimilatory genes of *S. platensis* revealed separate ORFs for *nirA* (1,686 bp), *glnA* (1,422 bp) and *gltS* (4,607 bp), indicating the absence of operon architecture. The *ntcA* gene from *S. platensis* PCC 7345 showed a high degree of similarity with previously reported *ntcA* gene sequences from other cyanobacteria, such as *S. platensis* NIES-39 (99%), *Lyngbya* sp. PCC 8106 (96%), *Leptolyngbya* sp. PCC 7376 (90%) and *Trichodesmium erythraeum* IMS101 (89%). The phylogenetic analyses were done using deduced protein sequences of all the five N-assimilatory genes as well as for the regulator *ntcA* showed very high degree of amino acid sequence similarity of the various genes of N uptake and assimilation among different cyanobacteria adapted to different habitats (Fujisawa *et al.*, 2010)

Localization of the genes encoding NRT, NR and NiR in *S. platensis* : The genes of nitrate uptake and assimilation are often clustered as operons in cyanobacteria. The most common arrangement of the nitrate assimilation genes is the operon *nirA*-

Table 1 : Genes related to nitrate assimilation and its regulation with completely sequenced genome in *S. platensis*

Strain	<i>S. platensis</i> PCC7345 (fresh water strain of β -cyanobacteria)
Transporters	NO ₃ ⁻ /NO ₂ ⁻ (<i>nrtABCD</i> / <i>nrtP</i>)
Enzymes	NR (<i>narB</i>) and NiR (<i>nirA</i>)
Sensor/ Regulators	NO ₂ ⁻ , (<i>ntcB</i>), 2-oxoglutarate (2-OG), <i>ntcA</i> , <i>glnA</i> , <i>pipX</i>

nrtABCD-narB (termed the *nirA* operon). The *nir* operon in fresh water cyanobacteria like *Synechococcus elongatus* strains PCC6301 and *Anabaena* sp. is comprised of the genes *nirA-nrtABCD-narB* (Ohashi et al., 2011) (Fig. 1A), whereas marine cyanobacteria like *Trichodesmium* sp. have *nrtP* in place of *nrtABCD* in the operon (Fig. 1B) (Wang et al., 2000; Ohashi et al., 2011), as the NRT encoding gene. Although many cyanobacterial genomes have the genes encoding NiR, NRT and NR in a single operon, there are other arrangements also. The most common among these is separation of the gene encoding NiR from the other genes. The *nirA* gene is separated from *nrtABCD-narB* in *Synechocystis* sp. PCC6803 (Fig. 1C) and from *nrtP-narB* in the marine β -cyanobacterium *Synechococcus* sp. PCC7002 (Fig. 1D). Recent progress in genome sequencing projects has revealed more complex arrangements of the nitrate assimilation genes. Lochab et al. (2014) showed that *S. platensis* PCC7345 has the *nrtABCD* gene cluster in addition to the transcription units carrying *nirA* and *nrtP-narB* (Fig. 1E). The *nrtP* and *narB* are organised together while *nrtABCD*, *nirA*, *glnA* and *gltS* exists in separate genomic locations, indicating that *S. platensis* does not follow the operon architecture typical of other cyanobacteria. This is in spite of the fact that it belongs to the same order Oscillatoriales as *Trichodesmium*. It is interesting and significant that *S. platensis* PCC 7345 has both types of transporters, *nrtP* as well as *nrtABCD*, which are otherwise known to be specific to marine and freshwater organisms, respectively, barring a few (Luque and Forchhammer, 2008; Ohashi et al., 2011). This may reflect the evolutionary adaptation of *S. platensis* to suit nitrate uptake in both types of environments, considering that there is no such habitat specificity in the other genes *nirA*, *narB*, *glnA* and *gltS*.

Mechanism for nitrate assimilation genes in *S. platensis*: On the basis of accumulating *S. platensis* genomic information, the regulation of nitrate assimilation is summarised in Fig. 2.

Effect of N metabolites on the regulation of nitrate assimilation in *S. platensis*:

Effect of ammonium: ammonium inhibition of N_2 -assimilatory genes: Ammonium showed clear inhibitory effect on the nitrate induction of gene expression of *nrtP*, *narB* and *nirA* as well as the enzyme activities of NR and NiR in *S. platensis* (Lochab et al., 2014). In non- N_2 -fixing cyanobacteria, expression of the nitrate assimilation genes is repressed by the addition of ammonium to the medium and induced simply by de-repression, i.e. removal of ammonium from the medium (Flores and Herrero, 2005; Herrero et al., 2001). In N-depleted cultures, the fall in the activities of the above enzymes is further accentuated by the addition of ammonium ions. Therefore, in addition to corroborating the well known ammonium repression of the above genes/enzymes in other cyanobacteria was also noticed (Muro-Pastor et al., 2001). They may be explained in part by the lack of operon architecture.

Fig. 2 : Regulation of nitrate assimilation in *S. platensis*

The nitrate assimilation genes in *S. platensis* can be regulated by two different mechanisms. Ammonia exerts its inhibitory effect on the genes and enzymes of nitrate assimilation through the repression of *ntcA* gene. In the presence of nitrate the three core nitrate assimilatory genes (*nrtP*, *narB* and *nirA*) are upregulated

Role of transcription factor-NtcA : Characterisation of *ntcA* gene and its binding elements in the promoter regions of *nrtP*, *nirA*, *glnA*, *gltS* of *S. platensis* pave the way for the more detailed studies on the mechanisms of N regulation in this organism. The genes of cyanobacterial N-assimilatory pathway are regulated by a nitrogen control mechanism mediated by *ntcA*, a transcriptional activator belonging to CRP (cAMP receptor protein) family. Being essential for the activation of many genes related to acquisition and assimilation of nitrogen, NtcA is defined as global nitrogen regulator (Herrero et al., 2001). *S. platensis* genome also contains 14 genes encoding cNMP-binding domains. One of them [NIES39-C02730 (NtcA)], encodes the helix-turn-helix DNA-binding motif of the CRP family and it is one of the most conserved gene sequence for a transcriptional regulator (Fujisawa et al., 2010). The promoter is activated by NtcA and has the consensus sequence GTA-N₆-TAC-N_{22/23}-TAN₃T, in which GTA-N₆-TAC represents the NtcA-binding motif and regulates the genes of multiple metabolic and developmental pathways (Herrero et al., 2001). NtcA activates the expression of *nir* operon, as well as of other genes involved in nitrogen assimilation upon ammonium withdrawal, probably responding to the cellular C/N ratio (Luque et al., 2004). This ratio is likely signalled in cyanobacteria by the cellular levels of 2-OG (Muro-Pastor et al., 2001).

Lochab et al. (2014) have reported that *ntcA* induction of nitrate uptake and assimilation operates at the highest level

during N-depletion and falls to intermediate level with nitrate nutrition and to lowest level with ammonium nutrition in *S. platensis*. Thus, *ntcA* plays a key role in sensing the nitrogen status of the cell in terms of both the form and amount of N and regulates the genes of N-assimilatory pathway accordingly. This was further verified by using ammonium-grown cultures and by supplementing them with nitrate or nitrite, when an increase in the *ntcA* gene expression was observed. Similar results were reported earlier in *Synechococcus*-WH7803 (Lindell and Post, 2001; Lindell *et al.*, 2005).

Effect of nitrate/nitrite : Nitrate/nitrite is known to be a requirement for expression of N-assimilatory genes in N_2 -fixing cyanobacteria. Whether they have any specific role in upregulating the expression of same genes in case of non N_2 -fixing cyanobacteria is not well understood. Perhaps because experiments were conducted in ammonium grown cultures and the role was interrupted in terms of the presence or absence of ammonium (Flores and Herrero, 1994).

Lochab *et al.* (2014) showed for the first time that nitrate upregulates the gene expression of *narB*, *nirA* and *nrtP* in *S. platensis*. Their transcript levels dropped drastically when the cultures were harvested and resuspended in N_2 -free media and recovered when nitrate was reintroduced. Similarly, nitrate induction was also found at the enzyme activity level for NR and NiR. Earlier studies showed that nitrate causes induction of NR activity based on a different strain of *S. platensis* (Jha *et al.*, 2007). It not only confirms it at the transcriptional level in the type strain PCC7345, but also provides the first evidence for nitrate induction of multiple genes of nitrate assimilatory pathway in *S. platensis* or in any other filamentous, non- N_2 -fixing, nitrate-utilizing cyanobacterium. Another group has also shown recently that the activity of NR, NiR, GS and GOGAT increased with increasing concentrations of sodium nitrate (Esen and Urek, 2014). In a similar study they have shown that highest growth, pigment and metabolite levels and enzyme activities of nitrate assimilating enzymes in *Spirulina platensis* were achieved by supplying ammonium nitrate and iron (Esen and Urek, 2015). The presence of carbon in the medium is one of the most important factors for the optimum growth and enzyme activities in this organism (Chauhan *et al.*, 2013).

Effects of downstream N metabolites, nitrite, ammonium and glutamine using N-depleted cultures in the presence or absence of nitrate have also been studied by Lochab *et al.* (2014). Two patterns of response to nitrite emerged, depending on the genes involved. In the case of *nrtP* and *nirA*, nitrite enhanced their gene expression to levels comparable to that of nitrate alone, or a combination of nitrite and nitrate. In case of *narB*, nitrite alone had no inductive effect and instead inhibited nitrate induction of gene expression when provided in combination. Glutamine had no effect at the RNA level while ammonium had significant inhibitory effect on the expression of these genes, either on its own or on

nitrate response. However, at the level of enzyme activity, all three downstream N metabolites had inhibitory effects on NR and NiR activities.

The only nitrite regulation known so far operates through *ntcB*, a lysR-type transcriptional activator that upregulates the *nirA* operon in *S. elongatus* strain PCC 7942 (Aichi *et al.*, 2004). It also revealed that nitrite induces *nrtP* and *nirA* gene expression in a manner similar to nitrate, but inhibits induction of *narB* by nitrate. Upregulation of *nir* operon by nitrite is known in *Synechococcus* sp. strain PCC 7942 and *Plectonema boryanum* (Kikuchi *et al.*, 1996). Nitrite inhibition of nitrate induction is not known in non- N_2 -fixing filamentous cyanobacteria, because nitrate induction itself was not known earlier. However, this is a well-known phenomenon in higher plants like maize (Raghuram and Sopory, 1999) and rice (Ali *et al.*, 2007).

Other factors associated with the regulation of nitrate assimilating genes: non- N_2 metabolites in *S. platensis* : The studies are in progress to understand the mechanism of regulation of gene expression by *ntcA* and other regulators, *ntcB*, PII and LysR (*ntcB*-JX489170, *PII*-JX431864, *lysR*-JX431863) which have been cloned recently from *S. platensis* (Lochab *et al.*, 2014). The role of PII and pipX regulators in enhancing the 2-OG-dependent *ntcA*-mediated upregulation of *nirA* operon has been shown in unicellular non- N_2 -fixing cyanobacterium *S. elongatus* PCC 7942 (Espinosa *et al.*, 2006). However, none of these regulators have been studied at any level in *S. platensis*, and therefore, their role in regulating N-metabolism remains to be understood.

Salient features of nitrate assimilatory enzymes of *S. platensis*: higher specific activities and thermostability : Purified nitrate assimilating enzymes are used for environmental testing (Campbell *et al.*, 2006), nitrate decontamination (Mellor *et al.*, 1992), biosensors (Chen *et al.*, 2008) and other applications (Angeby *et al.*, 2002), while whole organisms are useful for bioremediation (Hu *et al.*, 2000). However, the poor stability and thermosensitivity of these enzymes represents a major limitation in their widespread use. *S. platensis*, whose N-metabolic pathway is similar to higher plants, produces tenfold more protein in comparison to rice (*Oryza sativa*), indicating a higher capacity for nitrogen use efficiency and nitrate utilization/removal.

Ali *et al.* (2008) showed that the N-assimilating enzymes, nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) of *S. platensis* have higher specific activities and stabilities at room temperature than those of rice. They carried out *in vitro* analysis in crude extracts revealing that the higher specific activities (3-6 fold) and half-lives (1.2-4.4 fold) of the NR, NiR and GS in *S. platensis* which likely contribute to the far higher nitrate utilizing capacity of *Spirulina* as compared to that of rice leaves. This is further enhanced by the higher stabilities of NR and GS in *S. platensis* at room temperature, assuming that the *in vitro*

stabilities of these enzymes in crude extracts are a reflection of their longer half-lives *in vivo*.

It has been also shown that *S. platensis* could be an attractive natural source of NR for environmental, diagnostic and other applications (Chuntapa *et al.*, 2003), considering that it has over 3 fold higher specific activity and almost 5 fold higher stability than that of rice, with a half-life of over 22 hrs at room temperature *in vitro*. The specific activity of NiR in *S. platensis* is not only 6 fold higher than that of rice, but the fold difference between NiR and NR is far higher in *S. platensis* (9 fold) than in rice (5 fold) (Ali *et al.*, 2008). This may reflect the ability of *S. platensis* to effectively prevent the accumulation of nitrite, which is toxic to the cell.

Lochab *et al.* (2009) have assessed the thermotolerance of NR, NiR, and GS of *S. platensis* compared with those of rice in crude extracts *in vitro*, since higher plants are traditional sources for these enzymes. When the extracts were pre-exposed to 80°C for 1 hr, the three *S. platensis* enzymes retained higher activity by 3.4, 1.7 and 3.7 fold, respectively than corresponding enzymes in rice. These results revealed that *S. platensis* enzymes have relatively higher thermotolerance which renders *S. platensis* an attractive natural source of sturdy enzymes for various applications.

Bioremediation : Environmental contamination by toxic metals is a serious problem worldwide. Toxic metals are often discharged by a number of industrial processes and this in turn to the contamination of soil, freshwater and marine environment. Due to their non-biodegradability and persistence these metals can accumulate in the environment elements such as food chain, and may thus pose a significant danger to human health. Decontamination of heavy metals in the soil and water has been a challenge for a long time. Number of efficient methods such as evaporation, ion exchange, electroplating, membrane processes or precipitation etc. has been developed for the removal of heavy metals particularly from liquid wastes.

Microorganisms have evolved various processes such as transport across the cell membrane, biosorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions to protect themselves from the harmful effects of heavy metals present in environment. It has been proved that they are capable of adsorbing heavy metals from aqueous solutions, especially for the metal concentration below 50 mg l⁻¹ (Lu and Wilkins, 1995). The metal-binding capacities of several biological materials have been identified to be very high, including marine algae, fungi and yeasts. Microorganisms can also accumulate a wide range of metal species.

Metal ions can bind to the biomass by either non-living (biosorption) or living (bioaccumulation) technique. Biosorption is mostly useful for the waste water treatment and bioaccumulation finds the application of microalgae. Selection of biomass

(microalgae), pre-treatment, immobilization etc are some of the procedure involved in the metal recovery by biosorption. The biological materials can accumulate heavy metals from waste water either metabolically or by physicochemical pathway. The process of biosorption utilizes the above mentioned capacity of accumulating heavy metals (Fourest and Roux, 1992). Several studies have been done to look for the more efficient and economical metal-removal biosorbents from the microbial sources (Chang *et al.*, 1997; King *et al.*, 2007). Microalgal species have become material of choice because of their greater ability to bind metals by virtue of the presence of some functional groups in the cell wall constituents (Yu *et al.*, 1999; Schiewer and Volesky, 2000).

The second technique, bioaccumulation, can be defined as the transfer of organic or inorganic pollutants into the interior of living cells (Barron, 1995). As a result of this transfer there can be an accumulation of toxic elements in the organism. This process can be used to remove the nutrients from the treated effluents (nitrates, phosphates, sulfates, organic and inorganic carbon compounds). Ehrlich (1986) has reported the use of artificial ponds containing photosynthetic microorganisms to treat mining leachates. Bioaccumulation occurs when an organism takes up a toxic substance from the environment at a rate higher than that of the loss of the substance. This may pose a greater risk of poisoning if the substance has a longer half-life (Bryan *et al.*, 1979).

Bioremediation of heavy metal by *S. platensis* : The industrial development and urbanization have caused pollution of water resources. One of the major pollutant in the water bodies is the presence of heavy metals. The presence, accumulation and magnification of toxic metals like Cu, Zn, Cd, Cr, Pb and Ni etc. has imposed a greater threat to the ecology by causing imbalances in aquatic ecosystems (Pergent and Pergent-Martini, 1999). Biological materials have been proved to be capable of adsorbing heavy metals from aqueous solutions, especially for the metal concentration below 1 mg l⁻¹.

S. platensis is currently being investigated as a potential bioremediation agent for the removal of heavy metals from waste water, mining and other industrial effluents (Chen and Pan, 2005). When the toxic effects of metals were investigated, *Spirulina* was found to have a threshold level of about 30 µM for Cu, Zn and Pb. Cu and Zn appeared to have a direct effect on the photosynthetic pathway, thereby causing a rapid decline in cell growth. Lead on the other hand seemed to affect surface properties and hence took longer to cause deterioration in growth. However, these studies have indicated that *S. platensis* has a very low capacity for binding metal ions, as well as a very low tolerance for heavy metals. *S. platensis* does however show considerable potential as a precipitation agent as it is able to maintain a high pH in the surrounding medium, possibly through the enzyme carbonic anhydrase. The idea of the application of microalgae in

bioaccumulation of heavy metal ions has gained attention recently (Doshi *et al.*, 2007). Some algal polysaccharides (carrageenan) also played a key role in accumulation of metals and are considered to be correlated with the ability of the polysaccharides to form gels (Pengfu *et al.*, 2001). Further, it has shown that the green algae appeared to be more tolerant to metals such as Zn, Pb and Cu than blue green algae and diatoms. Chojnacka *et al.* (2005) studied heavy metal biosorption equilibrium of *S. platensis* in the absence of metabolic processes. Rangsayatorn *et al.* (2004) reported that metal adsorption rate was rapid in *S. platensis*. Bioremediation potential of *S. platensis* was also studied by various researchers with almost all heavy metal ions and other toxic compounds (Babu *et al.*, 2013; Chen and Pan, 2005; Seker *et al.*, 2008). Many of these heavy metals show their adverse effect by increasing the generation of free radicals in the cells. *Spirulina* has been shown to possess a very strong antioxidant defense mechanism making it a good system for bioremediation of heavy metals (Shilpi *et al.*, 2015). Very few studies are reported using *S. maxima* and *S. indica*. Most of these biosorption and bioaccumulation studies on *S. platensis* or *S. maxima* are done in shake flask at restricted conditions. Commercial production of *S. platensis* biomass is done using large raceway ponds.

Most of the studies dealing with biological removal of metals used dead biomass. *Spirulina*'s rapid lead adsorption rate and high lead adsorption capacity made them well suited for the removal of lead in wastewater. Chen and Pan (2005) examined the possibility of using live *Spirulina* to biologically remove the low concentration (below 50 mg l⁻¹) of Pb from wastewater. They observed that the adsorption rate was rapid at the initial stage (0-12 min) and the maximum biosorption capacity of live *S. platensis* was estimated to be 0.62 mg lead per 105 alga cells. Living cells of *S. platensis* were found to have high tolerance to Pb and can be regarded as an attractive adsorbate option for the biosorption of heavy metal contaminant. However the system of treating wastewater by living algae has still not developed completely and needs some more work to clear the uncertainties. In the recent times the biosorption of Cd by live and immobilized cells of *S. platensis* has been reported by Murugesan *et al.* (2008).

Rangsayatorn *et al.* (2002) demonstrated the possibility of using *S. platensis* TISTR 8217 to remove low concentrations of Cd (less than 100 mg l⁻¹) from wastewater. They estimated inhibiting concentration (IC₅₀) after the determination of growth rate using an optical density at 560 nm of cyanobacteria exposed to six different Cd concentrations for 96 hr. Cellular damage was studied under a light microscope and a transmission electron microscope and found that cell injury increased with increasing concentrations of Cd. Swollen cells and fragmented filaments were observed. Ultrastructural changes were observed in the algae exposed to Cd concentrations both close to IC₅₀ (14.68 mg l⁻¹) and at IC₅₀ (18.35 mg l⁻¹). The alterations induced by Cd were disintegration and disorganization of thylakoid membranes, presence of large

intrathylakoidal space, increase of polyphosphate bodies, and cell lysis. In addition, the cadmium adsorption by the algal cells was studied. Environmental factors were found to have an effect on biosorption. The uptake of Cd was not affected by the temperature of the solution, but the sorption was pH dependent. The optimum pH for biosorption of algal cells was 7. The Cd uptake process was rapid, with 78% of metal sorption completed within 5 min. The sorption data fit well to the Langmuir isotherm and the maximum adsorption capacity for *S. platensis* was found to be 98.04 mg Cd g⁻¹ biomass. These findings have immense potential for harnessing *S. platensis* in bioremediation of polluted ecosystems (Rangsayatorn *et al.*, 2002).

Siva Kiran *et al.*, (2012) discussed the toxic effects and bioaccumulation of Cd (II) ions by *S. indica* and led to the conclusion that bioaccumulation of heavy metal Cd (II) follows Monod kinetics with competitive inhibition mechanism at low concentrations (<1 mg l⁻¹). *Spirulina indica* had low (3.21 mg l⁻¹ Cd (II) at 7 days) lethal concentration and time (LC₅₀) indicating the toxicity of Cd (II) ions at high concentrations (>3.21 mg l⁻¹). In addition, living cells were found highly tolerant to Cd (II) at low concentration and can be regarded as an attractive adsorbate option for the biosorption of Cd contaminant.

Removal of nutrients by *S. platensis* : Nitrate and phosphorus in wastewater contribute to health and environmental threats as they are linked to diseases as well as ecosystem disruption via algal blooms in contaminated water bodies. Contamination of water by nitrates is caused by increased use of artificial fertilizers and land cultivation and it means that higher levels of nitrates are being washed from the soil into rivers, lakes, and aquifers. There they cause an excessive enrichment of the water (eutrophication) leading to a rapid growth of algae, which in turn darkens the water and reduces its oxygen content. The water is expensive to purify and many plants and animals die. High levels of nitrates are now found in drinking water in arable areas. Nitrate in wastewater is generally produced as an intermediate of nitrogen metabolism by microorganisms, beginning with ammonification of proteins or other nitrogen-containing compounds, followed by nitrification of ammonia into nitrite, and later, oxidation of nitrite into nitrate. Based on the understanding nitrate accumulation has become a concern in water quality management (Jalal *et al.*, 2011). Contrary to nitrate, the significant phosphorus content in wastewater is largely due to use of detergents. A concentration of phosphorus, 10 to 20 mg l⁻¹ was reported more recently, compared to the 3 to 4 mg l⁻¹ which was found in treated municipal wastewater (Jalal *et al.*, 2011). Phosphorus levels compared to nitrate do not fluctuate as much, with this nutrient persisting alongside other forms of phosphorous compounds in the wastewater production line.

Biological treatment of nitrate and phosphorus in wastewater have been widely studied, with various organisms namely bacteria, fungi, protozoa and microalgae identified as potent agents for their uptake. Microalgae are interesting for

$\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ and varying the initial biomass concentration from 0.25 to 0.86 g dm^{-3} . These new findings predicted that an increase in the inoculum level up to 0.75 g dm^{-3} enhanced both NO_3^- and PO_4^{3-} removal, verifying a strict dependence of these processes on biomass activity. Moreover, the larger surface area of the vessel and the higher light intensity improved removal yields and kinetics compared to the flasks, especially concerning with phosphorus.

A study based on the above perspectives was also carried out to evaluate nitrate and phosphate reduction in wastewater sludge used as a growth medium for the microalgal strains *S. platensis* (Kim et al., 2000). Algae performance in removing nitrate and phosphorus was evaluated by measuring nitrate and phosphorus content of waste water sludge incubated with the strains for 7 days. *S. platensis* was shown as the most efficient microalgae to reduce nitrate in waste water sludge and was found to be the best-growing strains. Thus *S. platensis* could be potential candidate by showing their intrinsic merit for the reduction of phosphate and nitrate in wastewater treatment. Thus, it may be concluded that *S. platensis* showed better efficiency than those of others microalgae in nitrate removal and overall growth. The exceptional performance exhibited by *S. platensis* in nitrate uptake was most likely contributed by the shared function of the aggregated cells and more robust growth (Lodi et al., 2003). *S. platensis* has certainly proven to be leading species in basic nutrient treatment of wastewater, and the health, environmental and commercial benefits cannot simply be ignored. Furthermore, it could be instrumental for the conversion of 'Waste to Wealth' in our environment.

Conclusion : *S. platensis* is by far the most researched non-heterocystous, filamentous cyanobacterium due to its importance for food and can be used as a potential chemical factory for a variety of useful products if effective genetic engineering can be accomplished. Nitrate assimilation in *S. platensis* is not well characterized, despite its direct link with its high protein content. There is a growing need for investigation of the basic aspects of nitrate assimilation in the cyanobacteria *S. platensis*. The basic structural components of nitrate assimilation system in *S. platensis* are the nitrate/nitrite permease, which can be either an ABC-type uptake transporter or an MFS permease that concentrates the substrates inside the cell, and the nitrate and nitrite reductases, which make use of photosynthetically reduced ferredoxin as an electron donor in what represents one of the simplest examples of photosynthesis. Genes encoding a nitrate/nitrite permease and both reductases are commonly found in a gene cluster that behaves as an operon. The NR activity in *S. platensis* is diminished upon withdrawal of nitrate from the medium, and is fully restored within two hours of resupplying nitrate, indicating substrate induction. Nitrite and ammonium, the downstream metabolites of nitrate assimilation, significantly inhibited nitrate-induced NR activity in a concentration dependent manner. These results in *S. platensis* indicate the need to

reassess the role of nitrate in the expression of NR in this and other non- N_2 -fixing, nitrate-assimilating cyanobacteria. Additionally, closely linked to the nitrate assimilation (*nir*) operon, a set of genes is usually found that encode proteins which influence *nir* operon expression or nitrate assimilation activity. This includes two general N-control proteins, the NtcA and NtcB, transcription factors, play essential roles in nitrate assimilation in *S. platensis*. Physiological and biochemical research and more recent molecular genetics investigations have converged to offer an integrated picture of the *S. platensis* nitrate assimilation system, which has also benefited from recent genomic analysis that has added information on the genetic distribution of nitrate assimilation capabilities in cyanobacteria. In spite of significant advance in understanding the regulation of nitrate assimilation in cyanobacteria, further post-transcriptional regulatory mechanisms would also constitute an important research topic for the future. *S. platensis* also have been proven efficient biological vectors for heavy metal uptake. The biosorption processes have been studied extensively using microbial biomass as biosorbant for heavy metal ions removal. In recent years, the biosorption potential of *S. platensis* strain has been studied under different initial metal concentrations. Biomass of *S. platensis* proves efficient towards removal of heavy metal ions, to extents comparable to those reported for other algae, thus unfolding the potential of those strains for water and wastewater treatment processes. However, there are still many uncertainties associated with the development of treating wastewater by living algae and more future work is necessary.

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