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Siderophore production by some soil Cyanobacteria

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Abstract: *Cyanobacteria are gram negative oxygenic photosynthesizer prokaryotes commonly found in fresh water, marine water and soil. They are considered as an important group of microorganisms capable of fixing atmospheric nitrogen. Cyanobacteria also play a major role in reducing soil erosion because of ability to secrete polysaccharides that bind soil. They also control soil run off and increase soil organic matter content and in producing certain substances which enhance the growth of plants. Due to these important characteristic, the utility of cyanobacteria in agriculture to enhance production is beyond doubt.*
Under conditions of low iron availability, cyanobacteria produce low molecular weight ferric chelators known as siderophores. Siderophores include iron chelators viz. schizokenin and synechobactin, and catecholate siderophores viz. anachelin.
In the present investigation the frequency of Siderophore production by filamentous and coccoidal cyanobacteria viz. Oscillatoria nigra, O. princeps,O. curviceps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum, Calothrix clavata, Aulosora prolifica, Stigonema dendroideum, Nostoc muscorum, Nostoc calcicola, Anabaena oryzae, Scytonema varium, Tolypothrix tenuis, Gloeocapsa calcarea, Microcystis, Synechococcus, Synecocystis, Gloeocapsa and Agmenellum in iron deficient BG11 medium was studied. The results revealed that the iron chelator siderophores and catechole siderophores were prevalent in these cyanobacteria. The presence of catechol siderophores indicated that these iron cheletors may be as important in high affinity iron transport as are the hydroxamic acid- type siderophores.
It can be concluded that the presence of catechol siderophores function as high affinity part of iron uptake by enhancing the re-encounter efficiency and selectivity and therefore successful in scavenging iron. When Iron (III) bound hydroxamate Siderophore comes within proximity of a surface- associated catechol, the catechol's high affinity for the iron molecule would facilitate a transfer or stripping event. This process moves the iron from the hydroxamate chelator to the catechole. Therefore, the iron would be stripped from the hydroxamate-type Siderophore before it was transferred into the cell or lost back to external medium. The iron (III)-catechol complex could then be transported into the cell via a catecholin iron (III) - specific protein.

Key Words: *Cyanobacteria, Siderophore, Anachelin, Synechobactin, Schizokinen*

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I. Introduction

Cyanobacteria are gram negative oxygenic photo synthesizers commonly found in fresh water, marine water and soil. They are considered as an important group of microorganisms capable of fixing atmospheric nitrogen. They have a unique potential to contribute to productivity in a variety of agricultural and ecological situations. Many cyanobacteria fix nitrogen under aerobic conditions in specialized cells called heterocyst which comprise 5-10% of cells in a filament (Gantar, 2000) [1]. Nonheterocystous cyanobacteria are also able to promote plant growth and can also be used as bio fertilizer.

Besides fixing atmospheric nitrogen, cyanobacteria play a major role in reducing soil erosion because of ability to secrete polysaccharides that bind soil (Nayak and Prassana, 2007) [2]. They also control soil run off and increase soil organic matter content and in producing certain substances which enhance the growth of plants (Ordog, 1999) [3]. Due to this important characteristic of nitrogen fixation, the utility of cyanobacteria in agriculture to enhance production is beyond doubt.

Cyanobacteria evolved very early in the history of life, and share some of the characteristics of gliding bacteria on one hand and those of higher plants on the other. Cyanobacteria can photosynthesize and fix nitrogen, and these abilities, together with great adaptability to various soil types, make them ubiquitous. Cyanobacteria also have a unique potential to contribute to productivity in a variety of agricultural and

ecological situations. Cyanobacteria have been reported from a wide range of soils, thriving both on and below the surface. They are often also characteristic features of other types of sub aerial environment and many intermittently wet ones such as rice fields. Most paddy soils have a natural population of cyanobacteria which provides a potential source of nitrogen fixation at no cost. Nitrogen fixation in cyanobacteria is brought about by a high molecular weight, oxygen labile, metalloprotein enzyme known as Nitrogenase. Nitrogenase reduces molecular nitrogen to ammonia in presence of hydrogen.

Many studies have been reported on the use of dried cyanobacteria to inoculate soils as a means of aiding fertility, and the effect of adding cyanobacteria to soil on rice yield was first studied in the 1950s in Japan. The term algalization is now applied to the use of a defined mixture of cyanobacterial species to inoculate soil, and research on algalization is going on in all major rice producing countries. The average of the results from all these studies has shown an increase in grain yield of 15-20% in field experiments. It has been suggested that the cyanobacteria introduced as a result of algalization can establish themselves permanently if inoculation is done consecutively for 3-4 cropping seasons. The basic method of mass production involves a mixture of nitrogen fixing cyanobacteria in shallow trays or polythene lined pits filled with water kept in open air, using clean, sieved farm soil as a carrier material. To each pit 10 kg soil and 250 g single super phosphate is added and water is filled up to a height of 12 15 cm. Starter culture, a mixture of *Anabaena, Nostoc, Aulosira* and *Tolypothrix,* is inoculated in each multiplication unit. Malathion (5 10 ml per tank) or carbofuran (3% granules, 20 g per tank) is also added to prevent insect breeding. In hot summer months, the cyanobacteria form a thick mat over the surface after 10 12 days of growth in open sun. The contents are allowed to dry and the dried flakes are collected, packed and used to inoculate rice fields. The basic advantage of this technology is that farmers after getting the soil based starter culture can produce the biofertilizers on their own with minimum additional inputs. An inoculum of 10 12 kg is considered sufficient to inoculate one hectare of paddy field 3-4 days after transplantation (Upasana Mishra and Sunil Pabhi, 2004) [4].

In India, considerable progress has been made in the development of cyanobacteria based biofertilizers technology. It has also been demonstrated that this technology can be a powerful means of enriching the soil fertility and improving rice crop yields. However, the technology needs to be improved further for better exploitation under sustainable agriculture systems. It is important to obtain a much more detailed understanding of cyanobacterial population dynamics over the whole annual cycle in agriculture systems. Extensive field studies aimed at developing region specific high quality inoculums are also needed. Understanding the biology of drought resistant cyanobacteria may be useful in terms of extending this approach to dry crops.

The role of cyanobacteria as biofertilizer has largely been reviewed by Dola Bhowmik *et al*., (2010), Nayak and Prassana (2007) [5], Haroun and Hussein (2003) [6], Lakshmi and Annamalai (2008) [7], Gallab and Salem (2001) [8], (Gayatri and Anand, 2002; Manoj Kumar *et al*., 2013, etc.) [9, 10], Pitchai Palaniappan *et al*., (2010) [11].

Plant growth promoting substances produced by cyanobacterial consortium with crops have largely been reviewed by Karthikeyan *et al*., (2007) [12], Prasanna *et al*., (2008) [13], Fatima and Venkataraman (1999) [14], Anand Mohan and Baidyanath Kumar (2019) [15] etc. Effect of exopolysaccharides (EPS) produced by a consortium of cyanobacteria of three crops, wheat, rice and maize have been studied by Manu Arora *et al*., (2010) [16]. The role of cyanobacterial consortium in the improvement of Maize crop has been studied by Anand Mohan *et al*., (2015) [17]. Plant growth promoting activity of rhizosphere cyanobacteria has recently been investigated by Anand Mohan and Baidyanath Kumar (2019) [15].

Cyanobacteria rely on iron as an essential cofactor for performing oxygenic photosynthesis and therefore have much larger iron requirements than non-photosynthetic organisms (Sholnik *et al*., 2006) [18]. As iron within cyanobacteria is at a concentration that is 4–6 orders of magnitude higher than in the extracellular environment, the acquisition of iron necessitates a large energetic investment (Shaked and Lis, 2012) [19]. While iron is one of the most abundant elements on Earth, bioavailable iron in aquatic environments is severely limited. In aqueous environments, bioavailable Fe2+ is rapidly oxidized to Fe3+ and forms poorly soluble complexes. Fe3+ either precipitates as ferricoxyhydroxides or is bound to a wide variety of organic ligands in a "ligand soup" (Kranzler *et al*., (2013) [20].

Cyanobacteria have developed a number of strategies to survive under iron limitations. The mechanisms involve include remodeling photosynthetic complexes (Gonzalez *et al*., 2018) [21], use of iron-accumulating bacterioferritin and replacing iron-containing enzymes with iron-free analogs (Keren *et al*., 2004) [22]. Iron import systems are also activated, among them a membrane-bound iron reduction system, or an extracellular reduction system (Lis *et al*., (2015) [23] that may involve pili (Lamb *et al*., 2014) [24].

Cyanobacteria excrete siderophores. Siderophores are biologically produced low-molecular-weight (400–1000 kDA) compounds with an extremely high affinity for iron. Increased iron bioavailability can also be achieved by the complex formation of Fe3+ with common metabolites (Gledhill *et al*., 2012) [25] including citric acid (Guerinot *et al*., (1990; Silva *et al*., 2009) [26, 27] and humic acids (Laglera *et al*., (2009) [28]. Typically, siderophores form a strong hexadentate octahedral complex with ferric iron (Fe3+). This complex can

then be transported inside the cell. The reduction of Fe3+ then facilitates its release, as siderophores do not typically chelate ferrous iron (Fe2+) (Hider *et al*., (2010) [29]. Based on the primary oxygen-donating ligands that bind the iron, siderophores are divided into four different types (Khan *et al*., 2018) [30]. These are the hydroxamates, catecholates, and carboxylates, as well as siderophores with mixed types of these functional groups. All cyanobacterial siderophores that have been identified so far are either hydroxamates or catecholates.

Hydroxamate Siderophore: Hydroxamate-type siderophores are the most common siderophores in nature. They consist of hydroxamate-moiety and is made up of hydroxylated and acylated alkylamines. Many cyanobacteria produce hydroxamates siderophores. Two most popular hydroxamate siderophores are schizokinen and synechobactin (Figure-1). Ferric iron chelation for schizokinen and synechobactin is thought to work by the Siderophore forming a hexavalent octahedron with the two oxygens of the two hydroxamate groups, along with the α- hydroxycarboxylate groups of the citrate moiety.

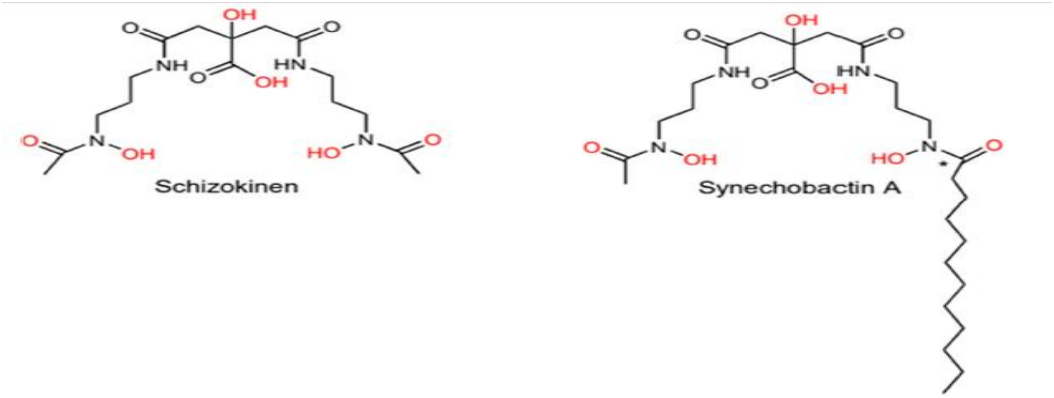

Figure 1. The structures of schizokinen and Synechobactin

Catecholate Siderophores: Catecholates are siderophores characterized by one or more iron-binding catechol moieties: the ortho-isomer of dihydroxybenzene. In Cyanobacteria, catecholate-type siderophores have been structurally characterized as anachelin which occurs in various forms viz. Anachelin H, Anachelin 1(Beiderbeck *et al*., 2000) [31] and Anachelin 2 (Itou *et al*., 2001) [32] (Figure-2). Anachelins have a tetrahydroquinolinium-derived chromophore with a catechol diamine. This unique alkaloid is bound to a polyketide through a tripeptide consisting of l-Thr, d-Ser, and l-Ser. Anachelin H contains a terminal salicylamide in the polyketide-part, while anachelin 1 and 2 are terminated by an oxazoline ring. Anachelin H is the biologically relevant product, while anachelin 1 and 2 are formed under dehydrating conditions (Gademamm *et al*., 2008) [33]. At physiological pH, mono, bis, and tris anachelin-iron complex can also occur (Bethuel *et al*., 2005) [34].

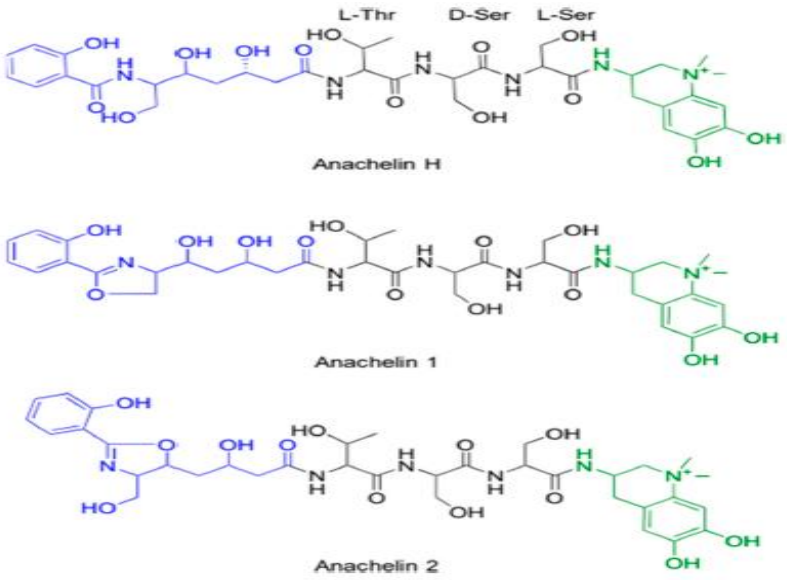

Figure-2: Structures of Catechole-type siderophores, the Anachelin

Amphiphilic siderophore, the Synechobactin: Synechobactin is an amphiphilic siderophore identified from marine cyanobacteria Synechococcus sp. PCC (Armstrong *et al*., 1979) [35]. It is essentially a schizokinen with a fully saturated fatty acid tail on one of the two hydroxamate groups. This siderophore has three forms viz. Synechobactin A, B, and C (Ito *et al*., 2005) [36]. The Fe^{+3}- synechobactin complex is photoreactive. Exposure of Fe+3-synechobactin A to light results in the decarboxylation of the citrate moiety to a 3-α-ketogluterate, while the iron remains bound to the siderophore (Ito *et al*., 2005) [36].

Biosynthesis of Siderophores: The biosynthesis of siderophores involves either a combination of non-ribosomal peptide synthases (NRPS) and polyketide synthase (PKS) or NRPS independent synthases (NIS). Citrate-based siderophores such as schizokinen and synechobactin are produced by the latter system. While the NIS pathways function primarily in the synthesis of polycarboxylate siderophores, the synthesis of hydroxamates and mixed-type siderophores by NIS has also been shown, with the genetic components predominantly found in actinobacteria and proteobacteria (Carrol *et al*., 2018) [37].Typically, NIS biosynthesis pathways of siderophores contain at least one enzyme with conserved N-terminal iron uptake chelate (Iuc A/Iuc C) domains, with a C-terminal domain used for iron metabolism or transport. NIS synthases can be categorized by the formation of a peptide bond between a hydroxamate-amine with a carboxylic acid substrate, Citric acid (type A and A' NIS), α-ketoglutaric acid (type B NIS) or a succinic- and citric acid derivative (type C and C' NIS) (Carrol *et al*., 2018; Oves-Costales *et al*., 2009) [37, 38]. NIS-enzymes are responsible for a single enzymatic reaction, typically the activation of citric acid by adenylation, and nucleophilic capture of alcohol or amine groups, which with the release of AMP produces a citryl intermediate. Many siderophores require more than one NIS-enzyme for biosynthesis (Carrol *et al*., 2018) [37]. NIS-synthases catalyze the peptide bond formation with citric acid (Iuc A), an a-ketoglutaric (Acs A) acid and succinic acid (Iuc C) (Oves-Costales *et al*., 2009) [38].

Anachelin biosynthesis: Anachelin is produced by non-ribosomal peptide synthase combining NRPS and polyketide synthases (PKS) (Barry *et al*., 2009; Kehr *et al*., 2011; Calteau *et al*., 2014; Dittmann *et al*., 2015) [39, 40, 41, 42]. The NRPS pathways are typically composed of multi-domain peptide synthases, where a peptide bond formation between the amino acids is catalyzed by consequent modules (Weissman, 2015) [43]. Each amino acid bound to the chain requires a separate module, which is composed of three domains: The amino acids are activated by the adenylation domain (A), while monomers are transferred by the peptidyl carrier domain (PCP). A condensation (C) domain then forms the peptide bonds between the amino acids. In the end, the mature oligopeptide is released by a thioesterase (Te) domain (Figure-3). NRPS is often coupled with the activity of polyketide synthases (PKS) to create more complex structures (Sussmuth *et al*., 2017) [44].

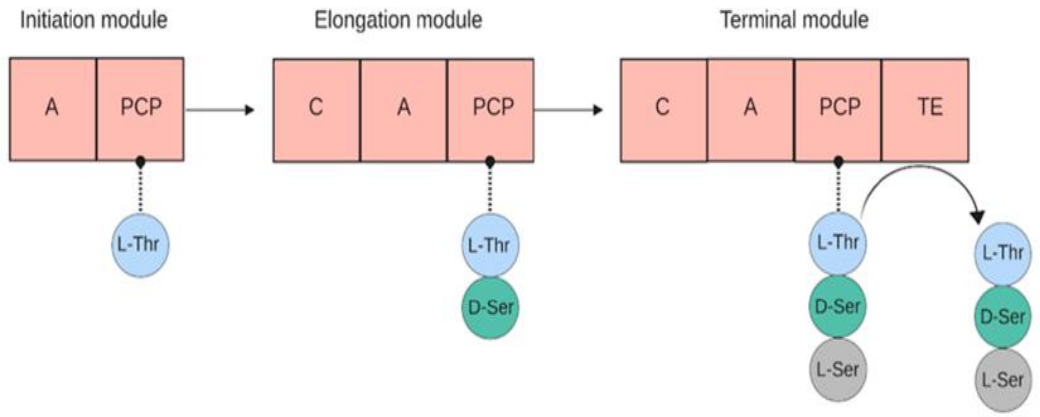

Figure-3: Synthesis of peptide chains by non-ribosomal peptide synthases (NRPS) by a sequence module. Amino-acids are activated by adenylation domain (A), transferred by the peptidyl carrier domain (PCP) and bound to each other by condensation domain (C). A final thioesterase (TE) domain releases the peptide.

Binding of Hydroxamate siderophores with other Metals: Hydroxamate siderophores have the capability of complexing with metals other than iron. This feature can be used by microorganisms either for the uptake of essential metals like manganese or zinc, or it can sequester toxic metals outside of the cell. In general, the metal binding of siderophores is governed by the hard-soft acid-base theory. In these terms, the hard oxygen atom donors of siderophores are not necessarily well suited to bind to softer metals like Cu(II) or Zn(II), leading to

lower affinities (Johnstone *et al*., 2015) [45]. Nevertheless, cyanobacterial siderophores have been shown to interact with other metals in biologically and potentially technologically meaningful ways.

In cyanobacteria, hydroxamate siderophores have been shown to bind to copper, and siderophore cycling may have a role in preventing copper toxicity (Clarke *et al*., 1987) [46]. Under conditions of high copper but low iron, schizokinen functions as a copper chelator in Anabaena sp PCC 7120, reducing the toxicity of the metal, while a siderophore-independent system appears to be largely responsible for iron uptake. Schizokinen thus appears to have a double functionality in this organism (Nicolaisen *et al*., 2010) [47]. In another case, the chelation of copper appears to play a role in microbial competition: the hydroxamate siderophores from Anabaena flos-aquae have been shown to inhibit the growth of the alga Chlamydomonas reinhardtii (Matz *et al*., 2004) [48]. The effect is independent of the iron level available to the algae, instead of appearing to rely on siderophoric chelation of cobalt and copper.

Complexation of siderophores with metals other than iron has raised interest in the use of siderophores for heavy-metal sequestration. This can be done by combining siderophore-producing bacteria with the growth of metal-resistant plants (Rajkumar *et al*., 2010) [49]. Hydroxamate siderophores from Synechococcus elongatus BDU 130911 have been shown to complex with uranium (Rashmi *et al*., 2013) [50]. Interestingly, as long as uranium is present, siderophore production is maintained in this organism even under iron-replete conditions, indicating some biological role of the siderophore in the detoxification of heavy metals. Similarly, the rice paddy-field cyanobacterium Anabaena oryzae produces a hydroxamate-type siderophore that can chelate Cd2+ even under iron-replete conditions (Singh *et al*., 2016) [51].

Siderophore Cycling:

Siderophore cycling has two components: (1) the export of siderophores and (2) the import of the iron-loaded Siderophore.The siderophores are recycled and used multiple times by the producing organism or community. In the case of photoreactive siderophores such as synechobactin, the chemical change induced by light permanently changes the siderophore, making its recycling difficult to envisage. In the case of more complex siderophores such as anachelin, the energetic investment per molecule makes recycling more likely. The overall cycling of siderophores is illustrated diagrametically (Figure-4).

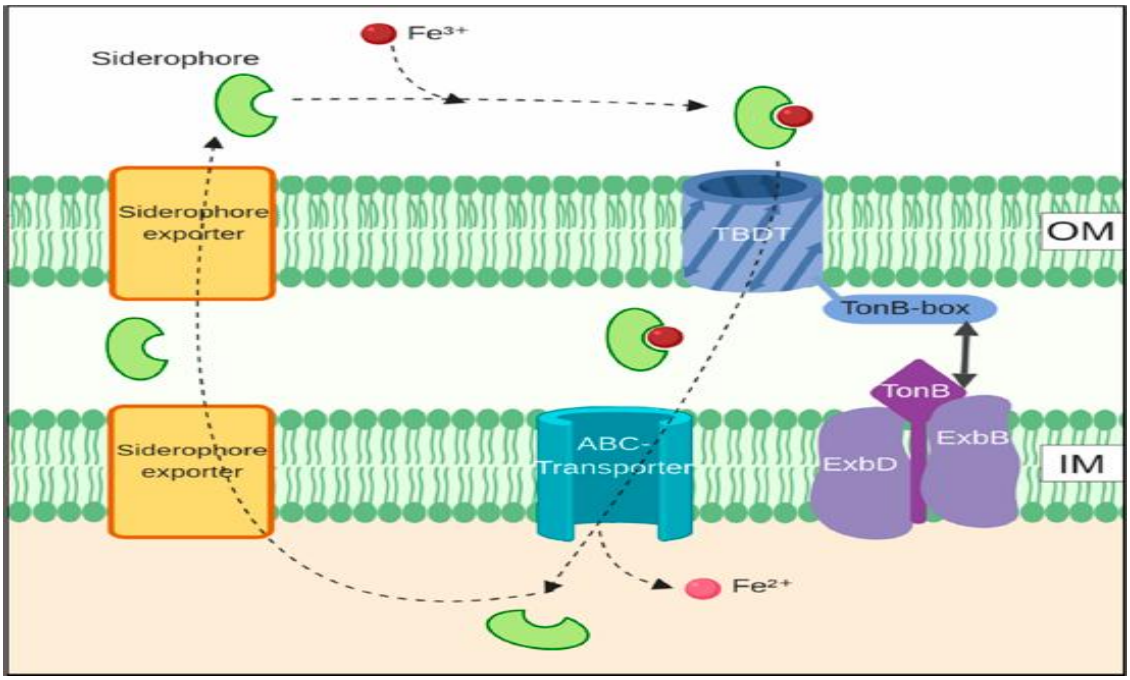

Figure-4: An overview of Siderophore cycling in cyanobacteria. Non-loaded siderophores are imported through TonB-dependent transporters (TBDT) in the outer membrane, which power supplied by a TonB-ExbB-ExbD-complex in the inner membrane. Further transport is done by ABC-transporters. Export of siderophores can happen by a variety of transporter types.

Siderophore export and import: Three different protein types are involved in the siderophore export process in cyanobacteria viz. the major facilitator superfamily (MFS), resistance nodulation and cell division (RND), and the ATP-binding cassette (ABC) superfamily (Mietthke *et al*., 2007) [52]. The only characterized siderophore export system in cyanobacteria is that of *Anabaena* sp. PCC7120. In this organism, the inner

membrane MFS proptei SchE (*all4025*) is required for the secretion of schizokinen, along with the TolC-like outer-membrane protein hgdD (*alr2887*), with the two proteins likely working in tandem (Nicolaisen *et al*., 2010) [53].

The import of iron-loaded siderophore is similar in bacteria and cyanobacteria, and illustrated in Figure-4. The transport of incoming siderophore-iron complexes into the periplasmic space is enabled by receptor proteins, TonB-dependent transporters (TBDTs) located in the outer membrane.

TBDTs consist of a β-barrel domain and a "plug" domain in the barrel interior that acts in concert with a TonB-box on the periplasmic side. Energy for transport is derived from the proton-motive force and is mediated through the association of TonB with inner membrane proteins ExbB and ExbD, forming a TonB-ExbB-ExbD-complex. In the periplasm, Fe^{3+}-loaded siderophores are transported across the inner membrane by ABC-transporters (Noinaj et al., 2010) [54]. Inside the cell, iron is removed from the siderophore. This can happen either through the reduction of iron from Fe3+ to Fe2+ by ferric siderophore reductases or by ferric-siderophore hydrolases (Mietthke *et al*., 2007) [52] or by the photolysis of iron-bound siderophores. In *Anabaena* sp. PCC 7120, the import of iron complexed schizokinen across the outer membrane is accomplished by two TBDTs, named the schizokinen transporter (alr3097, SchT) (Nicolaisen *et al*., 2008) [55] and IutA2 (alr2581).

The TBDTs are dependent on the inner membrane complex TonB3-ExbB3-ExbD3 (all2585-all5047-all4056) for energy. The genes are upregulated under an iron limitation, while similar genes show distinct regulation pattern under other conditions. Additionally, deletion mutations of the genes show an iron starvation phenotype (Stevanovic *et al*., 2011) [56]. In the periplasm, Fe-schizokinen is recognized and transported to the cytoplasm via the hydroxamate system FhuBCD (all0387, all0388, and all0389, respectively). These genes are similar to FhuBCD in E. coli, where FhuD functions as a periplasmic binding protein, FhuB is a membrane-embedded transporter and FhuC is an ATP-binding protein. Interestingly, while all three genes are upregulated under an iron limitation, they are regulated independently, as fhuC responds differently than fhuB/D to changing copper and citric acid levels (Rudolf *et al*., 2016; Stevanovic *et al*., 2011) [57, 56].

The release mechanism for ferric iron bound to schizokinen has been found to involve a ferric siderophore reductase in other bacteria (Miethke *et al*., 2011) [58]. TBDTs are unevenly distributed in cyanobacteria. In general, TBDTs seem to outnumber the amount of TonB-proteins, indicating some functional flexibility in TonB-complexes. The genome of *Anabaena* sp PCC 7120 contains 22 TBDTs. Synechococcus sp PCC 7002 contains 6 TBDTs, with two found by sequence similarity to resemble known schizokinen transporters, and two found to resemble hydroxamate transporters (Mirus *et al*., 2009) [59].

Anabaena sp PCC 7120 has been shown to make use of siderophores such as aerobactin (Goldman *et al*., 1983) [60] and the tris-hydroxamate desferroxamine B (DFB). Aerobactin is likely taken up by the same TBDTs as schizokinen, but DFB is more dissimilar to schizokinen and is transported across the outer membrane by an unidentified TBDT. The import of differerent siderophores appears to converge on the use of FhuBCD across the inner membrane (Rudolf *et al*., 2016; Rudolf *et al*., 2015) [57, 61].

Distribution of Siderophores in Cyanobacteria: Siderophores are widely distributed in Cyanobacteria viz. *Anabaena catenula* (UTEX 375), *Anabaena cylindrica* NIES-19, *Anabaena cylindrical* CCAP 1403/2A, *Anabaena cylindrica* Lemm. 1611, *Anabaena cylindrica* Lemm., *Anabaena flos-aqua*, *Anabaena oryzae*, *Anabaena* sp. PCC 6411, *Anabaena* sp. PCC 7120, *Anabaena variabilis, Anacystis nidulans, Gloeocoapsa alpicola, Microcystis aeruginosa, Microcystis aeruginosa* NPCD-1, *Microcystis* sp. SPC804, *Nostoc calcicola* BDU 4030, *Nostoc* sp. CENA21, *Oscillatoria tenius, Oscillatoria boryana* BDU 140791,*Oscillatoria quadripunctulata* NPRG-1, *Phormidium autumnale* UTEX1580, *Phormidium valderianum* BDU 140081, *Synechococcus elongatus* BDU 130911, *Synechococcus* sp. PCC 6031, *Synechococcus* sp. PCC 6908, *Synechococcus* sp. PCC 7002, *Synechococcus* sp. PCC 7942, *Synechococcus* sp. WH 8101 etc.

The present investigation is aimed to evaluate the siderophore production by soil cyanobacterial isolates viz. *Oscillatoria nigra, O. princeps,O. curviceps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum, Calothrix clavata, Aulosora prolifica, Stigonema dendroideum, Nostoc muscorum, Nostoc calcicola, Anabaena oryzae, Scytonema varium, Tolypothrix tenuis, Gloeocapsa calcarea, Microcystis* sp., *Synechococcus* sp., *Synecocystis* sp., *Gloeocapsa* sp. and *Agmenellum* sp.

II. Materials and Methods

In the present investigation twenty soil cyanobacterial isolates viz. *Oscillatoria nigra, O. princeps,O. curviceps, Schizothrix vaginata, Lyngbya gracilis, Phormidium dimorphum, Calothrix clavata, Aulosora prolifica, Stigonema dendroideum, Nostoc muscorum, Nostoc calcicola, Anabaena oryzae, Scytonema varium, Tolypothrix tenuis, Gloeocapsa calcarea, Microcystis* sp., *Synechococcus* sp., *Synecocystis* sp., *Gloeocapsa* sp. and *Agmenellum* sp. were examined for their ability to produce siderophores. Cyanobacteria were cultured

under iron limiting (5.1 X 10^9 M $FeCl_3$) conditions in BG11 medium. All waters and nutrient stocks were pretreated with Chelax-100 to reduce potential iron contamination (Price *et al*., 1989) [62]. In late logarithmic phase, the cells were removed from the culture media by centrifugation at 8000g, and the supernatant was passed through glass microfiber filters to ensure cell removal. The spent culture medium was acidified to pH3.0 with 12NHCl, and the organic materials in the medium were collected on a column of XAD-16.

Acidification of the medium allows for protonation of any ferrisiderophore complexes and the subsequent release of the iron molecules; it also provides for the retrieval of siderophores in a deferrated state. XAD-16 is a polymeric, non-ionic absorbent that is less selective than other XAD resins that have been used for siderophore isolation and thus retains most of the organic material from the medium. After acidification, the column was washed several times with Chelax-100-treated water binding components from the system. Any organic materials that retained on the column were then eluted with chloroform: methanol (1:1) solvent system. When apparent fractionation occurred upon elution, fractions were collected and analyzed separately. Excessive solvent was removed by rotary evaporation, and the final concentration of 1.5 ml was kept into micro tubes by means of a centrifugal vacuum concentrator. The iron-binding ligands in the extracellular supernatants were determined by the CAS assay of Schwyn and Neilands (1987) [63] (modified by replacing the anhydrous piperazine with an equimolar amount of 4-Morpholinethanesulfonic acid). Further verification of iron-chelating ability and the number of iron chelators per sample was obtained by thin-layer chromatography (TLC) of the sample in one of several solvent systems. Iron chelators were resolved by spraying the dried plate with 1.0% $FeCl_3$, in ethanol (Neilands 1982) [64].

In order to determine the chemical nature of the iron chelators isolated from the cyanobacteria, the procedures of Czaky (Gilliam *et al*., 1981) [65] were followed, and Arnow (1937) [66] method was used to test for respective hydroxamate- and catechol-type siderophores. The samples were resolved by TLC in various solvent systems and the Rf values were determined to examine the diversity of the siderophores produced.

The effects of siderophores on iron uptake in cyanobacteria were assayed by maintaining the cultures in continuous culture chemostats (D = 0.6 d-l at 85 PEinst m^{-2} s^{-l} and 37°C) under iron-replete (4.2 x lO^{-5} M $FeCl_3$, providing $10^{-17.1}$ M Fe3+ to a nitrate limited culture) and iron-deficient conditions. The cultures were monitored for iron uptake and iron-surface associations. Crude extracts of extracellular organic materials from each of the culture were isolated by XAD-16 and quantified with the CAS assay. Fractions with an iron-chelating equivalent of 300 pg rhodotorulic acid (used as a semiquantitative standard) were taken and used for iron-uptake analysis.

The cultures were harvested from chemostat and resuspended in 40 ml of fresh, iron-free medium and incubated for 1 h at 85 PEinst m^{-2} s^{-1} at 37°C. Cyanobacteria were then maintained for 30 min in the dark, and the siderophore fraction was added. After another 30 min, 3.47 pg of $^{55}FeCl_3$ were added, and incorporation into the cell was monitored with titanium (III)-citrate-EDTA rinse before scintillation counting. The titanium wash provides for the rapid reduction and removal of any surface-bound or superficially associated iron, thereby giving an accurate account of the iron incorporation into the cell (Hudson and Morel 1989) [67]. Incorporation was monitored for 30 minutes in dark followed by 1 h at 85 PEinst m^{-2} s^{-1}. The results obtained have been presented in Table-1, 2 and 3.

Table-1: Resolution of iron chelators by TLC. Rf values of iron chelators in two different solvents

Cyanobacterial isolates	R_f values in solvent Prop:But:H_2O (1:1:1)	R_f values in solvent Isopropyl alcohol:H_2O (7:3)
Oscillatoria nigra	**0.56**	**0.87**
O. princeps	**0.77**	**0.10**
O. curviceps	**0.81**	**0.26**
Schizothrix vaginata	**0.80**	**0.81**
Lyngbya gracilis	**0.79**	**0.80**
Pformidium dimorphum	**0.75**	**0.85**
Calothrix clavata	**0.65**	**0.89**
Aulosira prolifica	**0.75**	**0.11**
Stigonema dendroideum	**0.85**	**0.83**
Nostoc muscurum	**0.89**	**0.92**
N. calcecola	**0.16**	**0.87**
Anabaena oryzae	**0.26**	**0.88**
Scytonema varium	**0.21**	**0.79**
Tolypothrix tenuis	**0.23**	**0.90**
Gloeocapsa calcarea	**0.24**	**0.91**
***Microcystis* sp.**	**0.53**	**0.85**
***Synechococcus* sp.**	**0.45**	**0.97**
***Synecocystis* sp.**	**0.42**	**0.67**
***Gloeocapsa* sp.**	**0.43**	**0.65**
***Agmenellum* sp.**	**0.44**	**0.73**

Table-2: Production of extracellular iron chelators by cyanobacterial isolates under condition of iron limitation

Isolate number	Cyanobacterial isolates	Siderophores	Hydroxamate-type	Catechol- type
1	*Oscillatoria nigra*	4	1	3
2	*O. princeps*	4	1	3
3	*O. curviceps*	4	1	3
4	*Schizothrix vaginata*	4	1	3
5	*Lyngbya gracilis*	4	1	3
6	*Phormidium dimorphum*	4	1	3
7	*Calothrix clavata*	4	1	3
8	*Aulosira prolifica*	4	2	2
9	*Stigonema dendroideum*	4	2	2
10	*Nostoc muscorum*	5	1	4
11	*N. calcicola*	5	1	4
12	*Anabaena oryzae*	4	1	3
13	*Scytonema varium*	3	1	2
14	*Tolypothrix tenuis*	3	1	2
15	*Gloeocapsa calcarea*	4	3	1
16	*Microcystis* sp.	2	1	1
17	*Synechococcus* sp.	3	2	1
18	*Synechocystis* sp.	2	1	1
19	*Gloeocapsa* sp.	3	1	2
20	*Agmenellum* sp.	3	2	1

Table-3: Effect of crude siderophor extract of five cyanobacterial isolates on iron uptake

Cyanobacterial isolates	Uptake of ^{55}Fe (µg/cell/minute)			Surface association (µg/cell)		
		Iron-replete	Iron deficient		Iron replete	Iron deficient
Oscillatoria nigra	Control (with no added siderophore	$3.05X10^{-12}$	$2.50X10^{-12}$	Control (with no added siderophore	$1.45X10^{-9}$	$1.83X10^{-9}$
	With siderophore	$3.75X10^{-13}$	$1.85X10^{-13}$	With siderophore	$8.21X10^{-9}$	$2.75X10^{-9}$
Nostoc muscurum	Control (with no added siderophore	$3.07X10^{-12}$	$2.55X10^{-12}$	Control (with no added siderophore	$1.47X10^{-9}$	$1.85X10^{-9}$
	With siderophore	$3.65X10^{-13}$	$1.81X10^{-13}$	With siderophore	$8.19X10^{-9}$	$2.76X10^{-9}$
Anabaena oryzae	Control (with no added siderophore	$3.08X10^{-12}$	$2.60X10^{-12}$	Control (with no added siderophore	$1.55X10^{-9}$	$2.15X10^{-9}$
	With siderophore	$3.55X10^{-13}$	$1.81X10^{-13}$	With siderophore	$9.15X10^{-9}$	$2.75X10^{-9}$
Gloeocapsa calcarea	Control (with no added siderophore	$3.10X10^{-12}$	$3.45X10^{-12}$	Control (with no added siderophore	$1.61X10^{-9}$	$2.25X10^{-9}$
	With siderophore	$3.61X10^{-13}$	$1.86X10^{-13}$	With siderophore	$9.21X10^{-9}$	$3.15X10^{-9}$
Synecococcus sp.	Control (with no added siderophore	$3.01X10^{-12}$	$2.75X10^{-12}$	Control (with no added siderophore	$1.63X10^{-9}$	$2.27X10^{-9}$
	With siderophore	$3.65X10^{-13}$	$1.83X10^{-13}$	With siderophore	$9.23X10^{-9}$	$3.21X10^{-9}$

III. Results

In the present investigation the iron chelators produced by cyanobacterial isolates were resolved by TLC. The samples were resolved in two different solvents viz. Propanol: Butanol: Water (1:1:1) and Isopropyl alcohol: water (7:3), and iron chelators were visualized with 1.0% $FeCl_3$ in ethanol. The relative mobility factor (R_f) were determined as the migration distance of the siderophore with respect to the solvent front. This demonstrated that the siderophores produced by each of the twenty cyanobacterial isolates are unique (Table-1).

From the results (Table-2) it is evident that *Oscillatoria nigra*, *O. princeps*, *O. curviceps*, *Schizothrix vaginata*, *Lyngbya gracilis*, *Phormidium dimorphum* and *Calothrix clavata* produced four types of siderophores, one hydroxamate and three catechole-type. *Aulosira prolifica* and *Stigonema dendroidium* produced four types of siderophores, two hydroxamate and two catechole-type. *Nostoc muscorum* and *N. calcicola* produced five types of siderophores, one hydroxamate and four catechole-type. *Anabaena oryzae* produced four types of siderophores, one hydroxamate and three catechole-type. *Scytonema varium* and *Tolypothrix tenuis* produced three types of siderophores one hydroxamete and two catechole-type. *Gloeocapsa calcarea* produced four types of siderophores, three hydroxamate and one catechole-type. *Microcystis* sp. and *Synechocystissp.* produced only

two types of siderophores, one hydroxamate type ond one catechol-type. *Synechocystis* sp. and *Agmenellus* sp. produced three types of siderophores (two hydroxamate-type and one catechol type). Similarly, *Gloeocapsa* sp. ptoduced three types of siderophores, of which two belonged to hydroxamate-type and one of catechol type. From the results it was observed that the filamentous cyanobacteria mostly produced catechole-type of siderophores, whereas coccoidal cynobacteria produced hydroxamate siderophores (Table-2).

The effect of isolated crude siderophore extract on iron uptake by fife cyanobacterial isolates viz. *Oscillatoria nigra, Nostoc muscurum, Anabaena oryzae, Gloeocapsa calcarea* and *Synecococcus* sp. is illustrated in Table-3. A crude extract of siderophores produced by five cyanobacterial isolates was used to test the effect of siderophores on iron uptake in chemostat-grown cyanobacterial populations with different iron growth histories. The marked increase in iron uptake in iron-deficient cells suggests an active high-affinity transport system. Surface-associated iron was also monitored by exposing cells to ^{55}Fe for 5 minutes and then rinsing with 85 µM EDTA-l mM Tris (pH 7.8) in nutrient-free growth medium. The presence of siderophores decreases the affinity of iron-replete grown cells for iron while increasing the surface association of iron with iron-deficient-grown cells (Table-3).

IV. Discussion

In the present investigation all the twenty cyanobacterial isolates show the positive activity against CAS assay. All the cyanobacterial isolates produced at least one iron-chelating compound. Most of the cyanobacterial isolates produced multiple iron chelators, supporting the notion that high-affinity iron acquisition by cyanobacteria may be a general and not an isolated mechanism for iron acquisition during periods of iron-limited growth.

Iron chelators isolated from Cyanobacterial isolates demonstrated that the diverse siderophores are produced with these species. Although the precise chemical nature of these siderophores remains unknown, it is apparent that a variety of chelators of different types (hydroxamate, catechol, and atypical) are produced by present cyanobacterial species. The present findings gain support from the work of Kerry *et al*., (1988) [68] and (Brown and Trick, 1992) [69].

It was observed that the light driven iron uptake was enhanced for both iron-replete and iron deficient populations compared to a control (Table 3). The increase in iron uptake for iron replete cells may result from increased solubility of iron in the extracellular environment around the cell. The higher increase in the uptake of iron by the iron-deficient cultures in the presence of excess siderophore suggests an active high-affinity transport system. The change in iron binding indicates that siderophores increase the association of iron with the surface of cells from iron-deficient with the surface of iron-replete-grown cells.

If a high-affinity system is active in a cell, one would predict that outer membrane associated proteins, specific for ferrisiderophore complexes, would be present on the surface of the cell. Low iron-induced proteins have been identified in many cyanobacteria (Wilhelm, 1994; Scanlan *et al*., 1989) [70, 71] but the relative iron-binding abilities have not been fully elucidated. However, these proteins could increase the amount of iron that would associate with the cell. If, however, siderophores were added to a system where these proteins did not exist, one would expect that the amount of iron associated with the cell would decrease compared to control (no added siderophore) conditions. The decrease would be a result of the siderophores chelating the iron away from the cell. Therefore, it appears that the iron-deficient cells do indeed express ferrisiderophore specific proteins. Since these proteins are not expressed in iron-replete cells, the siderophores function to chelate iron away from the cell surface. The results are in accordance with the work of Brown and Trick, 1992; Kerry *et al*., 1988 [69, 68].

V. Conclusions

Considering the ecological significance of iron uptake, it is surprising that only a few cyanobacterial siderophores have been characterized. In recent years, cyanobacterial siderophores have been identified in studies with a focus on "proof-of-technological-concept", rather than an interest in the biosynthesis and physiological role of the molecule as a target itself. Application of these technologies to non-model organism cyanobacteria will surely lead to novel compounds with ecological as well as technological significance. The unique structure of anachelin and the nascent applications based on this molecule demonstrate that the potential of cyanobacterial siderophores matches their ecological importance.

It can be conclude that the presence of the catechol-type siderophores and hydroxamate-type siderophores may be integral in determining the true high-affinity nature of iron transport in cyanobacteria. The chemical characterization of cyanobacterial siderophores at molecular level requires further investigation at scientific level.

Conflict of interest: Authors declare no conflict of interest directly or indirectly.

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