

## Microbial Siderophores: A Prospective Tool for Strategic Medical Interventions

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Received: 13.10.2020 | Revised: 20.11.2020 | Accepted: 26.11.2020

### ABSTRACT

*Siderophores are naturally occurring iron-chelating compounds secreted by micro-organisms and plants. They are responsible for enhancing the uptake of iron from the surrounding environment to carry out vital metabolic processes. The production of siderophore is induced under iron-limiting conditions, and accumulation/transport of iron is strictly regulated at the molecular level. A variety of siderophores are identified from micro-organisms and, based on their chemical structure and functional groups, classified into 3 types viz., hydroxamate, catecholate and carboxylate. Modern biotechnological strategies have widened the scope of siderophores for various medical applications by uniquely exploiting the metabolic processes of pathogens against their own survival. This is achieved with the help of “Trojan horse” and similar strategies that has allowed targeted drug delivery for treatment of infections caused by multi-drug resistant pathogens, iron overload disorders as well as metal poisoning. Although siderophores show considerable potential in various environmental fields, the current review selectively explores the medical applications of siderophores.*

**Keywords:** Biotechnology, Iron, Iron overload disorders, Siderophores, Trojan horse.

### INTRODUCTION

In nature, innumerable biochemical reactions occur in perfect balance to aid the life processes of unicellular as well as multicellular organisms. The availability of nutrients is a prerequisite for these unbiased progressions of life. Although ready availability of all nutrients may seem to be an ideal scenario, their regulation is the key to establish control over the complex multitude of interconnected biochemical cycles. Besides, several micronutrients can be toxic if present in higher concentrations than required. These

environmental observations are clearly reflected in the growth cycle of micro-organisms due to the simplicity of their cellular structures. For instance, iron is a vital micronutrient present abundantly in nature, yet its availability is limited for acquisition by microorganisms (Saha et al., 2013). Iron undergoes oxidation from  $Fe^{2+}$  to  $Fe^{3+}$  to form an insoluble ferric oxyhydroxide, under normal environmental conditions (neutral pH and presence of oxygen), which cannot be utilized for any cellular processes by microorganisms.

Cite this article: Alazhar, C., & Aruna, K. (2020). Microbial Siderophores: A Prospective Tool for Strategic Medical Interventions, *Ind. J. Pure App. Biosci.* 8(6), 34-45. doi: <http://dx.doi.org/10.18782/2582-2845.8429>

The uptake of iron is further regulated with several repressor proteins identified in gram negative as well as gram positive bacteria (Visca & Imperi, 2018). These strict regulations are necessary since the free ferric form of iron is toxic and its increased uptake may inhibit microbial growth.

The above perspective leads to logical reasoning that iron requirement for optimal bacterial growth, i.e., approximately  $10^{-6}$  to  $10^{-7}$  M, shall be met by a suitable biochemical tool that functions under another strictly regulated mechanism (Brandel et al., 2012). This requirement is fulfilled with the help of siderophores. They are compounds secreted by bacteria, fungi and plants under iron-starvation conditions, which are capable of scavenging insoluble forms of iron in the environment to form soluble iron complexes. They are low molecular weight compounds with very high affinity for iron, and hence act as natural iron chelators (Jenifer & Sharmili, 2015 & Krewulak & Vogel, 2008). Siderophores use negatively charged oxygen groups as donors to form strong complexes with iron which is transported in the periplasm through unique Outer Membrane Proteins (OMPs) or Siderophore Binding Proteins (SBPs) on the bacterial cell membrane. Many such OMPs and SBPs have been characterized in gram negative and gram positive bacteria respectively (Schalk et al., 2009 & Fukushima et al., 2014).

The importance of siderophores can be understood by discerning the indispensable role of iron in metabolic processes. The microstructure of iron shows properties of plasticity, and exhibit excellent co-ordination and redox potential. These are essential features for binding of molecules to diverse ligands. Iron acquires a central position in the structure of hemoglobin and myoglobin, in eukaryotes. Moreover, all fundamental life processes in prokaryotes as well as eukaryotes viz., respiration, photosynthesis, nitrogen fixation and ATP synthesis, driven by electron transport chain, cofactors and other essential enzymatic reactions require iron containing proteins (Ali & Vidhale, 2013). Over 100 enzymes have been identified and reported in

the literature, which possesses iron containing cofactors (Chincholkar et al., 2000).

From an evolutionary point of view, siderophores also played a characteristic role. During early evolution, siderophores were majorly responsible for developing iron homeostasis to prevent Fenton induced radical damage. The stringent requirement of these molecules for survival, pathogenicity and suppression of host defense mechanisms is also well documented (Albelda-Berenguer et al., 2019). Recently there are interesting reports on the ability of bacteria and fungi to utilize siderophores (from other microorganisms) without its production (Butaite et al., 2017). It has also been suggested that they may play a role in cell communication, virulence and oxidative stress (Johnstone & Nolan, 2015).

Siderophores show a wide range of applications in agriculture, bioremediation, conservation of cultural artifacts and medicine. This review, however, limits its scope and focuses on types and mechanism of bacterial siderophores, and their potential application in the field of medicine.

### **Mechanism of siderophores**

The mechanism of iron uptake in bacteria operates with the help of a multi-component system. The detailed mechanism is outlined in Figure 1. These systems include siderophores, OMPs like Fec A, Fep A, transport protein complex like TonB-ExbB-ExbD, periplasmic binding protein and an inner membrane ATP-dependent Fec CDE-Fep CDE protein. Under iron starvation conditions, siderophores are synthesized along with various receptor molecules and secreted in the surrounding environment. These siderophores bind with iron to form siderophore-iron complex which is identified and transported through OMPs in the periplasm. It is then transported through the ABC-transporter systems i.e., Fec C, D, E and Fep C, D, E from ATP-binding cassette into the cytoplasmic membrane. One of these proteins act as a permease and spans the cytoplasmic membrane, whereas the other protein hydrolyses ATP to provide energy for transport of siderophore-iron complex towards the inner membrane of cytoplasm. This

complex is released inside the cytoplasm with the help of membrane protein Ton B. Although the exact mechanism of release of iron from the complex is still unknown, it is suggested to occur as a result of hydrolytic action on siderophore molecule. Another possible

mechanism may be the reduction of  $\text{Fe}^{3+}$  by NAD (P) H linked siderophore reductase to form low affinity  $\text{Fe}^{2+}$  molecules which can be dissociated from the complex (Ali & Vidhale, 2013).

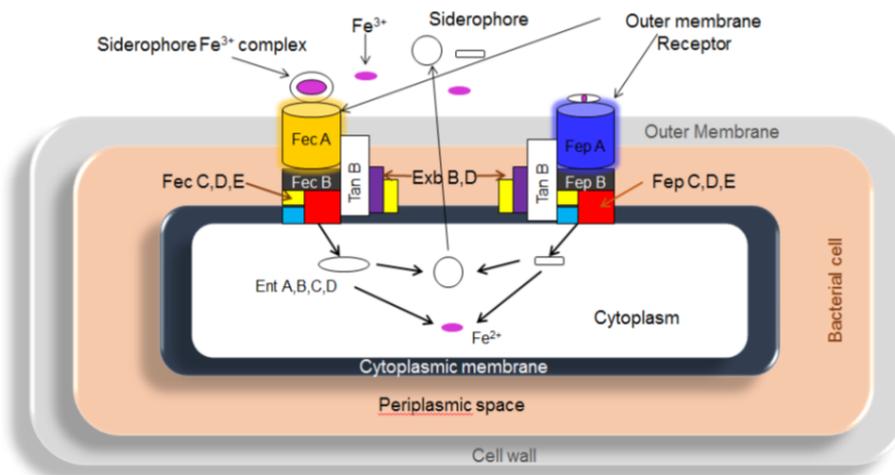
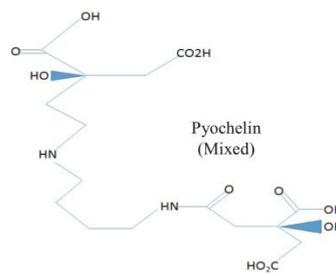
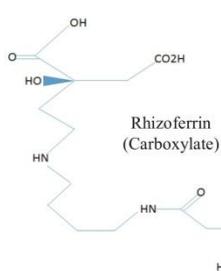
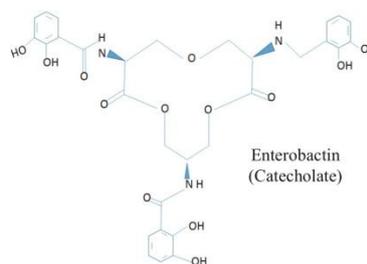
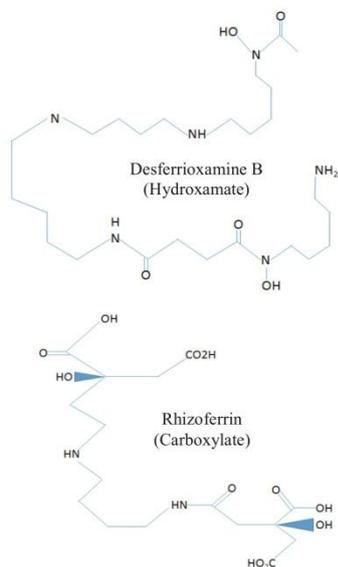


Fig. 1: Mechanism of siderophore mediated iron transport in bacteria (Adapted from Ali and Vidhale 2013)

### Types of Siderophores

Siderophores are characterized, based on their functional groups and oxygen ligands for Fe (III) coordination, into three main categories i.e., hydroxamates, catecholates and carboxylates. However, certain bacteria produce siderophores containing mixed functional groups. Figure 2 represents the structures of common siderophores from these categories. Over 500 natural iron chelating compounds are identified till date and over 270 have been structurally characterized (Ahmed & Holmstrom, 2014). Few examples of diverse siderophores produced by micro-

organisms are indicated in Table 1. Among the different types of siderophores, the occurrence of catecholates is more common in bacteria as compared to carboxylates and hydroxamates. Fungi, on the other hand, predominantly produce hydroxamate type of siderophores (Stintzi & Raymond, 2000). Their structure may possess bidentate to hexadentate chelation sites and the increase in this denticity is associated with higher affinity for iron. Factors like cyclization of di-, tetra- and hexadentate siderophores further enhance the chemical stability, and improve resistance to degrading enzymes (Ahmed & Holmstrom, 2014).



### Hydroxamate siderophores

Hydroxamate siderophores have a molecular formula of C (=O) -N-(OH) R, where R is either an amino acid or its derivative. They show a higher affinity for ferric iron and thus protect the siderophore-iron complex against hydrolysis and enzymatic degradation in the environment (Winkelmann, 2007). Several

methods of detecting hydroxamate siderophores include Neilands spectrophotometric assay, electrospray ionization mass spectrometry (ESI-MS), Modified overlaid chrome azurol S (O-CAS) assay and Csaky's assay (Neilands, 1981; Gledhill, 2001; Perez-Miranda et al., 2007; & Pal & Gokarn, 2010).

**Table 1: Siderophores produced by micro-organisms**

Type	Siderophore producer	Example	Reference
Catecholates	<i>Escherichia coli</i> and <i>Pseudomonas aeruginosa</i>	Enterobactin	Poole et al. 1990
	<i>Salmonella</i> sp.	Salmochelin (glycosylated-derivative)	Hantke et al. 2003
	<i>Bacillus</i> sp.	Bacillibactin	Miethke et al. 2006
Carboxylates	<i>Rhizobium</i> sp.	Rhizobactin	Smith et al. 1985
	<i>Rhizopus microsporus</i>	Rhizoferrin	Drechsel et al. 1995
	<i>Staphylococcus hyicus</i> DSM 20459	Staphyloferrin A (derivative of citric acid)	Meiwes et al. 1990
Hydroxamates	<i>Actinobacter</i> sp.	Ferrioxamine B	Ahmed and Holmstrom 2015
	<i>Actinobacter</i> sp.	Ferribactin	Ahmed and Holmstrom 2015
	<i>Trichoderma</i> sp.	Coprogens	Maurer and Keller-Schierlein 1968
	<i>Fusarium</i> sp.	Fusigen	Zahner et al. 1963
	<i>Streptomyces</i> sp.	Desferrioxamine	Diekmann and Zahner 1967
Mixed (phenolate, thiazole, oxazoline and carboxylate)	<i>Yersinia pestis</i>	Yersiniabactin	Sebbane et al. 2010
Mixed (oxazoline rings and catechol groups)	<i>Paracoccus denitrificans</i>	Parabactin	Bergeron and Weimar 1990
Mixed (hydroxamate and carboxylate moieties)	<i>Mycobacterium tuberculosis</i>	Mycobactin	Mitra et al. 2019
	<i>Pseudomonas</i> sp.	Pyoverdine	Leong and Neilands 1982
	<i>Escherichia coli</i>	Aerobactin	Gao et al. 2012
	<i>Erwinia carotovora</i>	Aerobactin	Ishimaru and Loper 1992
Mixed (catechol and carboxylate moieties)	<i>Marinobacter hydrocarbonoclasticus</i>	Petrobactin	Hickford et al. 2004
Mixed (hydroxyphenyl-thiazolinyl-carboxylate)	<i>Pseudomonas aeruginosa</i>	Pyochelin	Brandel et al. 2012
Cyclic	<i>Escherichia coli</i> , <i>Salmonella typhimurium</i> , and <i>Klebsiella pneumoniae</i>	Enterochelin	Dertz et al. 2006
	<i>Streptomyces pilosus</i>	Ferrioxamine E and Rhodotorulic acid	Muller et al. 1984

### Catecholate (phenolate) siderophores

As compared to hydroxamates, catecholates are produced by lesser number of bacteria (Dave et al., 2006). They show 2, 3 dihydroxy benzoyl units attached via an amide linkage in their molecular structure. This backbone structure can be polyamine, a peptide or a macrocyclic lactone. Siderophores with one, two or three catecholate or phenolate chelating groups attached to a peptide backbone have been reported. Enterochelin siderophore have been reported to show very high affinity to ferric ion and can detect minimalistic amounts of iron present in the environment (Dertz et al., 2006). In general, catecholates show highest affinity to ferric iron due to the formation of five-membered chelate rings. Similar to hydroxamates, Neilands spectrophotometric assay can also be used to detect catecholates by observing wine red coloration after binding to FeCl<sub>3</sub> during the assay and absorbance at 495nm (Neilands, 1981). Other methods like

HPLC coupled with Diode Array Detection (DAD) and Electrospray Ionization Mass Spectroscopy (ESI-MS) are also reported to be helpful in detecting catecholate siderophores (Saha et al., 2016).

### Carboxylate siderophore

This class of siderophore is largely produced by bacteria like *Rhizobium* sp., *Staphylococcus* sp. and fungi like *Mucorales* sp. Very few siderophores are characterized and reported to have an iron chelating moiety composed of only carboxylate and  $\alpha$ -hydroxy donor groups (Hickford et al., 2004). One of the best characterized carboxylate siderophore is Rhizobactin produced by species *Rhizobium meliloti* strain DM4 (Smith & Neilands, 1984). They are considered to be less efficient than other siderophores, however, they are tolerant to acidic pH and hence useful for microbes living in acidic environments. There are various methods for detecting carboxylate siderophores. One such method is a

spectrophotometric assay to detect absorbance of siderophore copper complex between 190-280nm (Shenker et al., 1992). Similarly O-CAS assay, or Vogels chemical test can also be used for the detection of carboxylate siderophores (Perez-Miranda et al., 2007; Saha et al., 2016 & Vogel, 1987).

### Medical applications of siderophores

Exploiting the metabolic activities of pathogens against their own propagation is one of the most intelligent applications of biotechnology. Siderophores and their substituted derivatives have been modified by using bactericidal conjugates and used as a drug delivery system to treat life threatening diseases. Few examples of such diseases include malaria, tuberculosis and cancer. It is also helpful in treating infections caused by Multi-Drug Resistant (MDR) bacteria, and diseases caused due to overload of iron in the body. Figure 3 represents the potential of siderophores in treating these infections. Three strategies are introduced to circumvent the iron dependence in pathogens with the help of siderophores. These include

- 1) Trojan horse strategies to facilitate cellular uptake of antibiotics
- 2) Artificial iron starvation by using antagonists of ferric ions
- 3) Inhibition of iron metabolic pathways

### *Siderophore mediated drug delivery for treatment of MDR bacteria*

Of lately, one of the most critical challenges faced by the pharmaceutical industry is the increasing prevalence of antibiotic resistance among pathogens and the relatively limited discovery of new antibiotics. This challenge has been met by exploring the metabolic requirements of pathogens. Since the iron uptake is essential for the survival of pathogens, the iron-siderophore system has been exploited to deliver drugs inside the cells, instead of or along with iron (Saha et al., 2013).

In the Trojan horse strategy (Figure 4), siderophore-antibiotic conjugates can be produced synthetically with improved cell permeability and reduced susceptibility to resistance mechanisms. Antibiotics such as  $\beta$ -

lactams, erythromycin, sulphonamides, spiramycin, vancomycin, nalidixic acid, norfloxacin, and antifungal agents like 5-fluorocytosine and neoenactins can be used in conjugation against pathogens. Once such conjugates are synthesized, the iron transport mechanisms can be directed to import the drugs into the cell. This strategy results in a remarkably effective drug delivery system since pathogens recognize the siderophore component as an iron delivery agent and assimilate the modified conjugate (with antibiotic). With an appropriate lethal dose of drugs, it immediately results in cell death (Budzikiewicz, 2001). Since the pathogenic cells do not survive, there is no way to design a defense mechanism, by pathogens, against this strategy.  $\beta$ -lactam drugs, as siderophore conjugates, have been used successfully to treat drug resistant infections (Carvalho et al., 2014). Similarly, gallium (a conjugate of iron) was successful in inhibiting biofilm formation by antibiotic resistant strains of *P. aeruginosa* (Reedy et al., 2007). This strategy is particularly effective in treatment of infections caused by *P. aeruginosa*, since they are often complex and associated with drug resistance. They show intrinsic mechanisms to resist many antibiotics. Moreover, they acquire drug resistance comparatively easily due to their narrower porin channels that act more effectively as an efflux pump for antibiotics (Tomaras et al., 2013). The use of the appropriate pyoverdine, as a transport vehicle, has also been advantageous for a species specific attack when conjugated with 2, 3-dihydroxybenzoic acid and 5-hydroxypyrid-4-one-3-carboxylic acid for drug resistant *P. aeruginosa* (Budzikiewicz, 2001). Sideromycins are another group of antibiotics employed in “Trojan horse” drug delivery strategy to enhance the therapeutic efficiency and overcome antibiotic resistance. Albomycin too displays a broad-spectrum antimicrobial activity (Pramanik et al., 2007).

### *Antimalarial activity of siderophores*

Some siderophores exhibit antimalarial activity by showing a lethal effect on *Plasmodium falciparum* (Tsafack et al., 1996).

Few examples include, siderophores produced by *K. pneumoniae* and *Streptomyces pilosus* (Gysin et al., 1991 & Muller et al., 1984). Both in vivo and in vitro studies have indicated the efficacy of Desferrioxamine B in treating malaria by depleting the intracellular iron required for metabolic activity as well as

pathogenicity of the parasite (Scott et al., 1990). Later this drug therapy was improved by conjugating Desferrioxamine B with Nalidixic acid to cause iron-catalyzed oxidative DNA damage and, in turn, treat malaria caused by MDR *P. falciparum* (Ghosh et al., 1995).

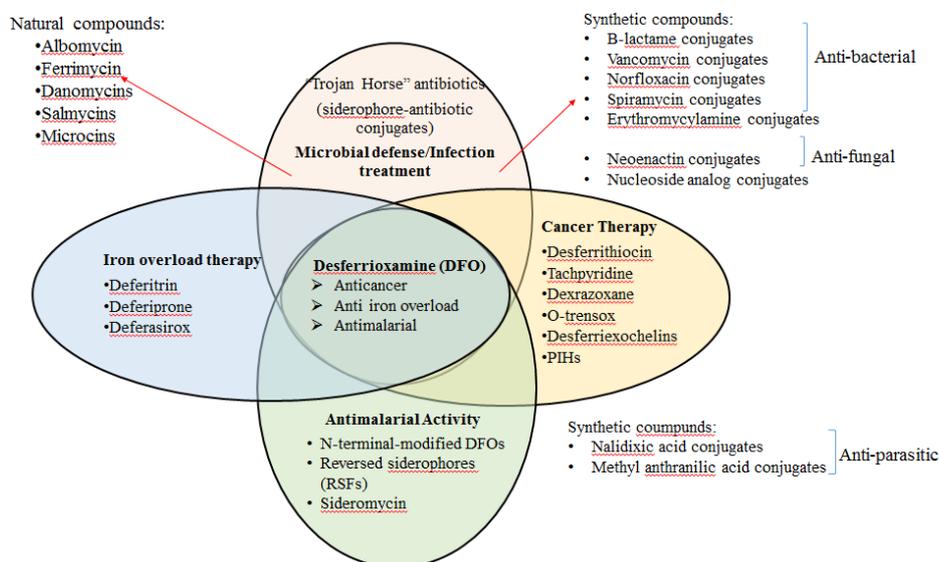


Fig. 3: Representative iron-chelating compounds involved in microbial defense and considered as potential therapeutics for treatment of infection, iron overload, and tumorigenesis (Adapted from Nagoba and Vedpathak, 2011)

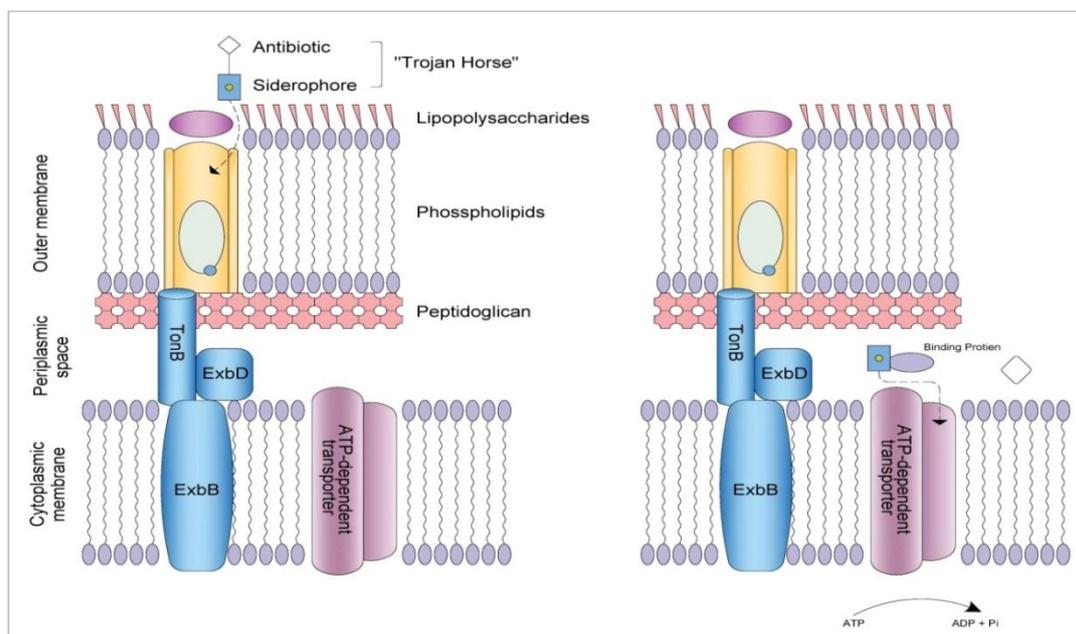


Fig. 4: Schematic representation of the “Trojan horse” strategy (Adapted from Miethke and Marahiel, 2007)

**Iron overload therapy using siderophores**

As indicated earlier in this review, the presence of iron above the optimal concentration can severely restrict the growth of an organism. Same is true for humans. In

addition, several metabolic disorders can lead to overdose of iron in the circulatory system as well as organs, leading to deleterious effect of redox cycle by production of free radicals. Sickle cell disease and Thalassemia major are

genetic disorders characterized by sickle shaped red blood cells and haemoglobin abnormality respectively. The patients often require whole blood transfusion resulting in steady buildup of iron in the body. This is because there is no physiologically specific mechanism for the excretion of iron in humans. Other diseases like hemochromatosis causes a progressive increase in the iron content of the body and deposition in the liver, heart or pancreas. Haemosiderosis is secondary haemochromatosis caused by excessive blood transfusions, severe chronic haemolysis or iron intoxication. Collectively all these disorders are termed as iron overload disorders and require removal of iron from the body (Kontoghiorghes et al., 2005).

Siderophores are used for treatment of these disorders by employing a reverse strategy, where they are used as chelating agents to bind with iron in the circulatory system to form ferrioxamine. This enhances its elimination via urine. Hence acute iron intoxication and chronic iron overload disorders can be managed effectively. Interestingly, Desferrioxamine B has proved useful in removal of vanadium, toxic metals in dialyzed patients, and in iron overload therapy by using conjugates such as deferiprone (Ferriprox), deferasirox (Exjade) and deferitricin (Kontoghiorghes et al., 2005; Trivedi, 2016 & Ali & Vidhale, 2013).

#### **Anticancer treatment using siderophores**

The rapid cell division and metabolism in cancer cells increases their iron requirements. Consequently, the uptake and storage of iron in cancer cells are also higher. Moreover, the involvement of iron is observed in the microenvironment of tumor formation as well as metastasis. This implies the critical role of iron in tumor initiation and growth (Saha et al., 2016). These informations has been used to design siderophore mediated cancer therapy by artificial reprogramming of iron metabolism to disrupt its acquisition, efflux and storage (Torti & Torti, 2013). Derivatives of siderophores are used in cancer therapy coupled with synthetic iron chelators such as dexrazoxane and isonicotinoyl hydrazine in

addition to Desferrioxamine B and mycobacterial desferriexochelins (Chong et al., 2002 & Jayasinghe et al., 2015). Desferrioxamines was reported to significantly decrease the growth of aggressive tumors in patients with neuroblastoma (NB) and leukemia by inhibiting DNA replication (Buss et al., 2003). Several other siderophores such as dexrazoxane, O-trensox, desferriexochelins, desferrithiocin and tachpyridine act as iron chelators in cancer therapy (Miethke & Marahiel, 2007). However, some siderophores are also useful in the clearance of non-transferrin-bound iron present in the serum which is seen in cancer therapy as a result of exposure to chemotherapy (Saha et al., 2016).

#### **Anti-tuberculosis treatment using siderophores**

*Mycobacterium tuberculosis* is a pervasive human pathogen which kills around 1.8 million people every year. The emergence of MDR and extensively drug-resistant strains of *M. tuberculosis* have been increasingly reported, and hence confounded attempts for immediate actions of disease control. Like other pathogens, iron acquisition is vital for the pathogenicity of *M. tuberculosis*. They acquire iron from the host using several strategies like secreting reductants, taking citrate as a carrier, utilizing a hemoglobin-haptoglobin complex and synthesizing hemolysins (Litwin & Calderwood, 1993). However, the role of siderophores remains indispensable for their virulence and proliferation. Hence, they produce siderophores called mycobactins and carboxymycobactins (McBride, 2012). A study supporting the requirement of siderophores for virulence characteristic of pathogens, described the growth impairment of *Mycobacterium* sp. in absence of mycobactin. These findings further indicated the necessity of these compounds for maintaining iron homeostasis and pathogenesis (Kobylarz, 2016). Thus these compounds can be a promising target for novel antibiotics. Mycobactin analogs and heterologous compounds, designed to overcome the impervious cell wall barrier which is

characteristic of *Mycobacterium* sp., can be utilized by *Mycobacterium aurum*. Hence they may aid in formulating novel drug vectors. Approaches have also been made for obtaining naturally occurring antibiotic conjugated siderophores, known as sideromycins. These biomimetic siderophores have shown considerable activity against *M. tuberculosis* (Wells et al., 2013). Similarly, synthetic approaches in another study illustrated the use of mycobactin-artemisinin conjugate, a synthetic analogue of mycobactin T which provides access to the pathogen's cell, while artemisinin serves as the antibiotic pharmacophore (Kurth et al., 2016).

#### ***Other medical applications of siderophores***

Siderophores are efficient metal chelators and hence are capable of mobilizing toxins like aluminum, gallium, uranium, vanadium, chromium, copper, lead, cadmium and manganese. Hence they are used for removal of transuranic elements from the body. Aluminum intoxication occurs in dialysis encephalopathy (Nagoba & Vedpathak, 2011). Similarly, the overload of other metals may occur in the human body as a result of intoxication, over-exposure, accidental ingestion etc. In such cases siderophores provide a safe treatment option by excretion of these metals in soluble forms from the body. Another interesting application of siderophores is clinical and translational imaging. This technique replaces the iron in siderophores with radionuclides like Gallium-68 to obtain live images of pathogenesis (Petrik et al., 2017). A recent study further reported the promising application of Zirconium-89, as a biomarker for radiotracer design and in vivo delivery, for clinical investigations of prostate cancer. They further indicated the use of metallic isotopes for nuclear imaging techniques like positron emission tomography may be effective for detection of cancers in general (Cortezon-Tamarit et al., 2019).

#### **CONCLUSION**

The availability of iron is of paramount importance for the metabolic processes of micro-organisms. Their fundamental role of

extracellular solubilization of iron from minerals or organic substances has been carefully maneuvered with the help of biotechnology to design strategies for the treatment of complicated diseases. They exhibit excellent potential for sustainable approaches in the field of medicine. Moreover, their scope is not limited and they find suitable application in ecological and environmental biotechnology, agriculture, metal recovery from ores and many more areas. Hence it is necessary to upgrade our literary cognition with more specialized studies to explore the currently unidentified microbial siderophores and the potential they offer.

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