Endophytic Bacteria from Salt Marsh Plants as Potential Agents for Heavy Metal Bioremediation: A Preliminary Investigation of Siderophore Production and Metal Removal Efficiency

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Endophytic Bacteria from Salt Marsh Plants as Potential Agents for Heavy Metal Bioremediation: A Preliminary Investigation of Siderophore Production and Metal Removal Efficiency

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Abstract:

Siderophores are low molecular weight compounds widely produced by microorganisms. These molecules exhibit a high affinity for iron, a crucial metal involved in many microbial metabolic activities. In addition to iron, siderophores have also been found to chelate other metal ions such as chromium (Cr), zinc (Zn), cobalt (Co), and cadmium (Cd). In recent years, their potential in the bioremediation of heavy metals has been extensively studied, as they can effectively chelate a variety of metal ions, including toxic heavy metals. Among siderophore producing microbes, those originating from the saline environment remains less explored. Therefore, in the present study, halophilic endophytes from salt marsh plants were studied for its potential to produce siderophores and analysed for its efficacy in remediating heavy metals. From our previous study six endophytes designated as SR, IR, IS, CL, SS, SL from four halophytes of two different saline environments *Rhizophora mucoronata, Ceriops decandra, Ipomea pes-caprae and Spinifex littoreus* were identified as potential siderophore producers. Among them, three isolates SR, IS, IR with highest Siderophore Production Index (SPI) in CAS agar plates were further selected for optimization and remediation studies. Optimization by Response Surface methodology revealed the model fits best for isolate IR, while it showed lack of fit for both SR and IS. Bioremediation studies of these isolates by FTIR revealed that these organisms were capable of removing copper, chromium and lead making it a potential candidate for bioremediation of heavy metals and water treatment.

Key words: Bioremediation, Halophiles, Iron, Response Surface methodology, Siderophore

1 Introduction:

Microorganisms produce metabolites for its growth and survival. These metabolites are classified as primary metabolites, which are specifically utilized by the microbes for their growth, and secondary metabolites, which play a vital role in survival under unforeseen or stressful circumstances (LeBlanc and Wuest 2024). The secondary metabolites, currently recognized as specialized molecules include compounds such as pigments, antibiotics, immunomodulating agents and enzymes These molecules in addition to their role in microbial survival and ecological fitness, have also been identified to possess significant potential for use in healthcare, nutrition and other industrial applications (Monciardini et al. 2014; Fouillaud et al. 2016).

Siderophores, a class of secondary metabolite is synthesized by the microorganisms to sequester iron from the environment. These are low molecular weight molecules that regulates iron uptake by the microbes (Lacava et al. 2008; Passari et al. 2023). The ability of these compounds to chelate iron molecules were found to exert beneficial effects, particularly in agriculture and environmental restoration. It has been observed that most plant endophytes produce siderophores. Plant endophytes are the microorganisms such as bacteria and fungi that resides within plant tissues without causing cellular or structural damage. These endophytic microorganisms exhibit mutualistic relationship with host plants wherein they obtain nutrient from plants for their growth and survival and in turn produce secondary metabolites that supports plant growth and protects against biotic and abiotic stress (Dimpka 2016; Xavier et al. 2022). Siderophores are one such metabolite produced by these plant endophytes contributing to plant growth by enhancing iron availability to host. Several research on plant endophytes and plant growth promoting rhizobacteria showed the role of siderophores in promoting plant growth and its significance to be used in agriculture for promoting growth in agricultural and economically important crops (Timofeeva et al. 2022; Srivastava et al. 2022; Zhang et al. 2023).

The high affinity of siderophores for iron prompted scientist to investigate their capacity to chelate other metal ions other than iron. These studies have revealed that siderophores binds to metals such as Cadmium (Cd), Cobalt (Co), Molybdenum (Mo), Copper (Cu), Manganese (Mn), Tin (Sn), and Zinc (Zn) (Gaonkar and Bhosle 2013; O'Brien et al. 2014; Syed et al. 2023; Majewska et al. 2024). The ability of these siderophores to complex with metals other than iron opened new possibilities for their application in the bioremediation of heavy metals from the environment (Williamson et al. 2024). Siderophores produced by microbes of various sources, particularly plant associated endophytes, have been employed for such purposes. However, plants that thrive in saline environments relatively remain unexplored. In the present study, endophytes isolated from halophytes (plants of saline environments) were screened for siderophore production and evaluated for its efficacy in chelating heavy metals for potential use in bioremediation.

2 Materials and Methods

2.1 Isolation and Characterization of microbes

The bacterial endophytes utilized in the study were isolated from the plant samples of *Ipomea pes-caprae* and *Spinifex littoreus* from Muttukadu Salt marsh, Chennai, Tamilnadu, India and *Rhizophora mucronata* and *Ceriops decandra* from Mangroves of Kalpakkam, Tamilnadu, India. The detailed description on isolation, morphological, biochemical and molecular analysis of the isolates were given in our previous paper (Pradeep Manikandan et al. submitted manuscript under review).

2.2 Screening for Siderophore production

Siderophore production by the isolated endophytes was performed using modified Chrome Azurol S (CAS) method described by Louden et al. (2011). HEPES was used instead of PIPES in the method followed in our study. The CAS agar was inoculated with the bacterial endophytes and incubated at 30 ˚C for 5 days. The formation of halo zone around the colony after incubation indicates the production of siderophore by the endophytes. The isolates with noticeable zones were identified and the siderophore producing index was calculated using the formula

$$\text{Siderophore Producing Index (SPI)} = \frac{\text{Radius of the halo + radius of the colony}}{\text{Radius of the colony}}$$

The isolate showing higher SPI were selected for further studies.

2.3 Optimizing siderophore production by Response Surface Methodology

Optimizing parameters for siderophore production by the selected isolates was performed using Response Surface Methodology (RSM) in Design Expert trial version 13. Four independent variables—temperature, pH, incubation period, and iron concentration—were selected to construct a Central Composite Design (CCD). The optimization study considered the following ranges: temperature (20–40 °C), pH (5–9), incubation period (4–8 days), and iron concentration (25–125 ppm). Iron concentration was used for optimization studies as siderophores has natural tendency to bind with iron. A total of 24 experimental runs were conducted, with each run incubated for 8 days. After analyzing the response data, a model equation was proposed, and terms with a 95% confidence level were considered statistically significant. Graphical analysis was performed to identify the optimum conditions for siderophore production (Raje Nimbalkar et al., 2022).

2.4 Preparation of heavy metal solution

Stock solutions of six metals—copper sulphate, cadmium chloride, lead acetate, potassium dichromate and sodium arsenate—were prepared at a concentration of 1000 ppm using Milli-Q grade deionised water. The stock solutions were stored at 4 °C for future use. (Syed and Chinthala 2015)

2.5 Bioremediation analysis:

The stock solutions were used for bioremediation analysis by adding 2.5 mL of each metal stock solution to 50 mL of sterilized nutrient broth, resulting in a final concentration of 50 ppm. The nutrient broth (50 mL) was first sterilized, followed by the addition of 2.5 mL of the respective stock solution. The medium was then inoculated with selected endophytic isolates and incubated for 3 days in a rotary shaker incubator at 150 rpm and 37 °C. After incubation, optical density (OD) values were measured using UV-Visible spectrophotometer over four subsequent days. Nutrient broth containing heavy metals without inoculation served as the control. In addition, FTIR analysis was performed to identify the preliminary confirmation of bioremediation by the selected isolates. The incubated samples were centrifuged at 8000 rpm for 20 mins and the supernatant was utilized to analyse the effect of bioremediation by these siderophore producing isolates. FTIR was recorded in 4000 cm^{-1} - 450 cm^{-1} region at room temperature by Perkin Elmer Spectrum 100 (Guven et al., 2015).

3 Results and Discussion

3.1 Screening foe siderophore production

Endophytes are organisms that associates with plant tissues without causing any harm to the host (Ryan et al 2008; Dudeja et al. 2012). They possess several beneficial characteristics such as IAA production (Khianngam et al. 2023), nitrogen fixation (Renugadevi et al. 2024), phosphorous solubilization (Prasad et al. 2022), ammonia production (Renugadevi et al. 2024), antagonistic activity against plant pathogens (Zhang et al. 2023), enzyme production (Tsipinana et al. 2024) and essential metal sequestration such as iron through siderophores (García-Latorre et al. 2024), all of which support plant growth. Among many plant growth–promoting factors, siderophores have gained particular attention not only for their role in agriculture but also for their applications in bioremediation, especially in the treatment of heavy metals. Siderophores from various sources have been studied to remediated different kinds of heavy metals (Li et al. 2023; Xie et al. 2024). However, those produced by the endophytes of halophilic plants are less explored (Cen et al. 2024). In the present study, siderophores produced by the halophilic plants *Ipomea pes-caprae* and *Spinifex littoreus* from Muttukadu Salt marsh, Chennai, Tamilnadu, India and *Rhizophora mucronata* and *Ceriops decandra* from Mangroves of Kalpakkam, Tamilnadu, India was utilized for isolation of endophytes, their ability to produce siderophores and bioremediation of heavy metals.

The isolates were screened for the production of siderophores by CAS agar plating method (Fig 1a-c and Fig S1a-d). The isolates SR - *Stutzerimonas stutzeri*, IR - *Bacillus safensis*, IS - *Proteus mirabilis*, SS – yet to be identified, CL - *Cytobacillus kochii*, IL - *Shouchella clausii* and SL - *Bacillus velezensis* were found to have halos around them indicating they are positive for siderophore production. Among them, the strains IR, IS and SR which showed highest Siderophore Producing Index (SPI) were selected for further studies (Table 1). Though the strain SL showed higher SPI, there was no growth or halo development on further incubation, therefore, the strain SL was not selected for further studies. The Siderophore Production Index of greater than 1 is considered positive for production of siderophore (Yamaji et al. 2016). The organisms IR, IS, SR and SL were found to have Siderophore Production Index of greater than 1. It was identified that some endophytes show SPI higher than 2. However majority of the organisms show SPI between 1 and 2. The SPI between the range 1 and 2 was reported to be observed in endophytes isolated from *Zea mays, Brassica juncea, Brassica carrinata* and several other endophytes such as *Pseudomonas putida P57, Stenotrophomonas maltophilia P77, Pseudomonas lurida P88, Pseudomonas lurida P10-1, Phyllobacterium salinisoli, Phyllobacterium ifriqiyense, Xanthomonas translucens,* and *Cupriavidus respiraculi.* These studies supports the fact that SPI greater than 1 can be considered potential for several applications (Abirammi et al. 2018; Abhinandana et al. 2023; Ben Gaied et al. 2024; Shalovylo et al. 2024).

3.2 Optimizing siderophore production by Response Surface Methodology

Optimization of siderophore production by central composite method using RSM software showed optimal conditions of pH 7, temperature 30 °C, incubation period of 6 days and iron concentration of 75 ppm with a maximum siderophore production of 99.6 % (#Run 15) for SR - *Stutzerimonas stutzeri*, optimal conditions of pH 7, temperature 20 °C, incubation period of 4 days and iron concentration of 125 ppm with a maximum siderophore production of 99.1 % (#Run 1) for IS - *Proteus mirabilis*, optimal conditions of pH 9, temperature 30 °C, incubation period of 6 days and iron concentration of 75 ppm with a maximum siderophore production of 99.7 % (#Run 21) for IR - *Bacillus safensis*.

Figure 2a, 2b and 2c shows the highest siderophore production at different temperature, incubation time, iron utilization and pH respectively. ANOVA showed that there were significant fit and lack of fit values of strain IS. The ANOVA analysis for the suggested quadratic model shows that the model is significant with p value of 0.0141 and the lack of fit was also found to be significant with the p value of 0.0276 indicating that the level of fit is not satisfactory. The results indicate that the model fits with the experimental design but there is also some variation in the design point of predicted values and the mean values of the replicates. Figure 3a, 3b and 3c shows the highest siderophore production at different temperature, incubation time, siderophore production and pH respectively. ANOVA showed that there was significant fit and lack of fit values of strain IR. The ANOVA analysis for the suggested 2FI shows that the model is significant with p value of 0.0347 and the lack of fit was found to be not significant with the p value of 0.1311 indicating that the level of fit is satisfactory. The results indicate that the model fits perfectly with the experimental design.

Stutzerimonas stutzeri strain SR, was formerly called as *Pseudomonas stutzeri* (Gomila et al. 2023). The organism was found to grow at variable temperature but found to have maximum growth at 40 °C (Palleroni et al. 1970). With respect to pH, it was reported that organism can produce siderophores at pH 8 as solubility of iron is highly affected at this pH thereby inducing the organism to produce siderophores (Lewis et al. 2004). However, from the optimization study, the organism *Stutzerimonas stutzeri* (strain SR) was found to have maximum production at 30 °C and at pH 7. In addition, it took a longer incubation period of 6 days for the production of siderophore at iron concentration of 75 ppm. Also, the optimization study showed a significant lack of fit and non-significant model fit which means the model is inadequate (Fig S2 – S4). This can be attributed to the limiting ranges and runs utilized in the study. Similarly, the organism *Proteus mirabilis* was found to have varying optimal temperature for its growth (Bacdive, 2024). It grows under neutral pH with an incubation period of 1 to 2 days. Even in the present study optimal conditions for production of sideophore was found to be pH 7, temperature 20 °C, incubation period of 4 days and iron concentration of 125 ppm. However, the optimization showed significant model fit and lack of fit. This shows that though, the model is statistically meaningful, it misses structure due to less data points and repetitions.

The organism *Bacillus safensis* was identified to grow in temperature ranging between 10 and 50 and also in pH range of 4 and 8 (Satomi et al. 2006; Kothari et al. 2013). But the optimization study revealed pH 9, temperature 30 °C, incubation period of 6 days for highest siderophore production by the isolate IR - *Bacillus safensis.* In addition, the study showed significant model fit and non-significant lack of fit showing that the model fits and adequate. Though the experimental design remains the same, there were difference noted for each organism. It can be due to the fact that, strain IR, *Bacillus safensis* exhibits adequate model fit, indicating a well-captured response surface within the tested ranges. In contrast, strain IS, *Proteus mirabilis* show significant lack of fit, meaning the model captured part of the variation but failed to represent the full complexity of its response. In addition, strain SR, *Stutzerimonas stutzeri,* show non-significant model fit and significant lack of fit, indicating that the quadratic model was both weak and inadequate. These change in significance for each organism can be attributed to their different behaviour and interaction with the system and the multiple data points required to give a complete structure.

3.3 Bioremediation of heavy metal:

Bioremediation of heavy metals by siderophores has gained significant importance and is now being extensively explored worldwide. In the present study, the bioremediation potential of isolates IS and IR was evaluated against Cadmium, Chromium, Copper, Lead, and Arsenic. Since optimization experiments did not yield promising results, the strain SR was excluded from further analysis. Figures 4a–4c illustrate the bioremediation of Chromium, Copper, and Lead by isolates IS and IR. Chromium removal was most efficient on Day 1 by isolate IR (85.9%), whereas isolate IS achieved a higher removal efficiency of 95.2% on Day 2. Copper removal was observed only on Day 2, with IS and IR achieving 92.4% and 96.9%, respectively. Lead removal was also recorded on Day 2, with IR and IS showing efficiencies of 80.7% and

73.3%, respectively. No significant change in optical density (OD) was observed for Cadmium and Arsenic, indicating the absence of remediation by either isolate after two days of incubation.

The FTIR analysis of the bioremediated sample shows that there are no residues of the heavy metals in the culture supernatant except for cadmium and arsenic. This suggests that the screened bacterial endophytes are capable of removing some major heavy metals. Figure 5a – 5f shows the FTIR analysis of bioremediated samples by the isolates. Table 2 presents the FTIR peaks associated with the bioremediation of metals by strains IR and IS, compared with the reference FTIR spectra of the corresponding heavy metals (based on sources such as the NIST library, ChemicalBook, and SpectraBase, reflecting the possible metal composition in the experiment). In agreement with the UV-Vis spectroscopic analysis, the FTIR results confirmed that chromium, copper, and lead were efficiently removed by the isolates IR and IS.

Phytoremediation is considered one of the most preferable methods for removing heavy metals from the environment, as it is cost-effective and environmentally safe (Luo et al. 2016). However, like all techniques, phytoremediation has certain limitations, the most notable being the longer duration required for effective removal, since plants need time to grow and establish (Cao et al. 2012). To overcome this constraint, endophyte-assisted phytoremediation has gained increasing importance. Endophytes are microorganisms that inhabit plant tissues without causing harm to their host (Dudeja et al. 2012). They enhance plant growth by providing various beneficial factors, among which the production of siderophores is particularly significant (Renugadevi et al. 2024). Siderophores, known for their ability to chelate iron, have attracted research interest for their potential to bind other toxic heavy metals as well. This property has led to the development of endophyte-assisted phytoremediation as a promising strategy for efficient heavy metal removal from contaminated environments.

Siderophores have been studied for their efficiency in binding heavy metals such as Aluminium (Al), Manganese (Mn), Copper (Cu), Zinc (Zn), Cadmium (Cd), Mercury (Hg), Cobalt (Co), and Tin (Sn), and have been identified as effective metal chelators (Majewska et al., 2024). Several endophytes, including *Serratia proteamaculans*, *Bacillus cereus*, *Pseudomonas veronii*, *Pseudomonas aeruginosa* ZGKD3, *Jeotgalicoccus huakuii*, and *Bacillus amyloliquefaciens*, have been reported to tolerate and produce siderophores in the presence of a wide range of heavy metals such as Lead (Pb), Manganese (Mn), Nickel (Ni), Copper (Cu), Zinc (Zn), Cadmium (Cd), Arsenic (As), and Mercury (Hg) (Govarthan et al., 2016; Shi et al., 2017; Yongpisanphop and Babel, 2020; Ustiatik et al., 2021). Furthermore, endophytes producing siderophores have been inoculated into plants and studied for endophyte-assisted phytoremediation, showing promising results in the bioremediation of heavy metals such as Cadmium and Iron (Huo et al., 2021; Li et al., 2023; Syed et al., 2023). Most studies have focused primarily on evaluating the chelation efficiency of siderophores with heavy metals, the role of endophyte-assisted phytoremediation, and the promotion of plant growth under heavy metal stress. However, relatively few investigations have concentrated on the direct removal of heavy metals by siderophore-producing endophytes. Among these, one study reported that *Pseudomonas* sp. achieved 85.6% removal of Lead (Surti and Pius, 2023).

Given the limited number of studies addressing heavy metal removal by siderophore-producing endophytes, the present work is significant as it demonstrates that isolates IR and IS play a promising role in bioremediation. These strains were also identified to be efficient in removing Chromium, Copper, and Lead from the environment. In addition, they can be further explored for broader applications, including the removal of other heavy metals.

4 Limitations and Future perspectives

The main limitation of the present work lies in the optimization study, as additional data points and repeated trials are required to achieve more reliable optimization. Moreover, since this is a preliminary investigation, advanced analytical techniques to confirm bioremediation were not employed, which represents another limitation of the study.

The future progress will involve optimizing the culture conditions with larger data sets for better understanding, performing additional analytical techniques, and conducting application-oriented studies. Further investigations will also focus on scaling up the process and exploring the potential of these strains for the remediation of a wider range of heavy metals under diverse environmental conditions.

5 Conclusion

Heavy metals pose a serious threat to the environment, causing metal poisoning and adversely impacting living organisms in both terrestrial and aquatic ecosystems. Their tendency to undergo bioaccumulation and biomagnification further intensifies these harmful effects. Although several methods are available for the treatment of heavy metals, with phytoremediation being the most widely employed, it is essential to explore alternative and more effective strategies. Siderophores, as natural metal chelators, offer significant potential for the efficient removal of heavy metals from contaminated environments. Continued research into siderophore-mediated bioremediation may lead to the identification of additional promising organisms—such as *Stutzerimonas stutzeri*, *Bacillus safensis*, and *Proteus mirabilis*—that can provide enhanced options for sustainable heavy metal remediation.

Ethics approval and consent to participate

Not applicable

Clinical trial number

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that there is no competing interest.

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C.K. - Execution of work, Planning, Manuscript preparation; S.V. - Validation; A.R. - Planning, Validation, Manuscript correction. All authors reviewed the manuscript

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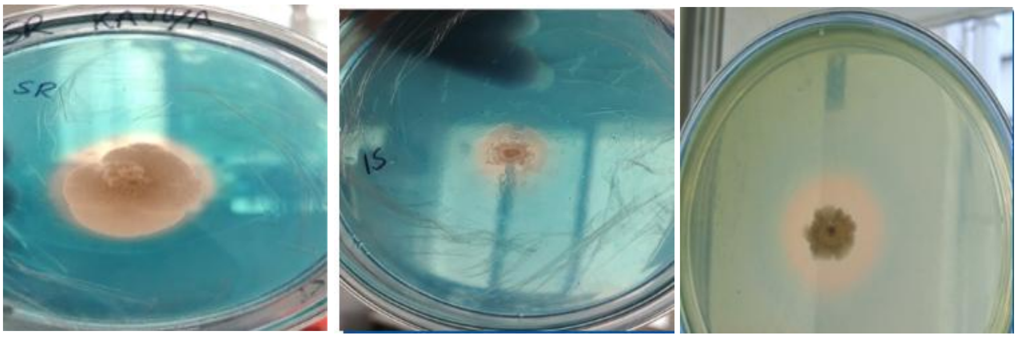
Figures



a) SR b) IS c) IR

Figure 1: Appearance of halos around the colonies indicating the production of siderophores by the isolates SR, IS and IR

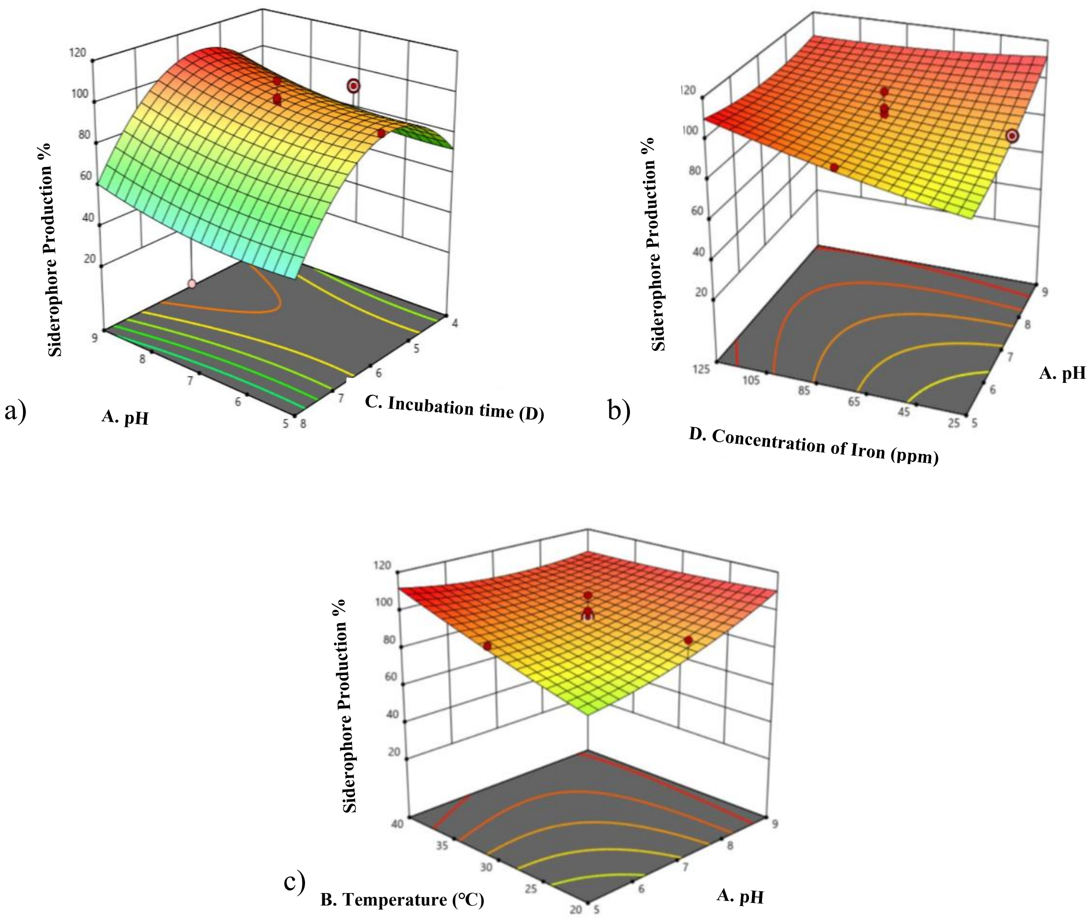


Figure 2a-c: Optimization of Culture conditions for the production of siderophores by strain IS

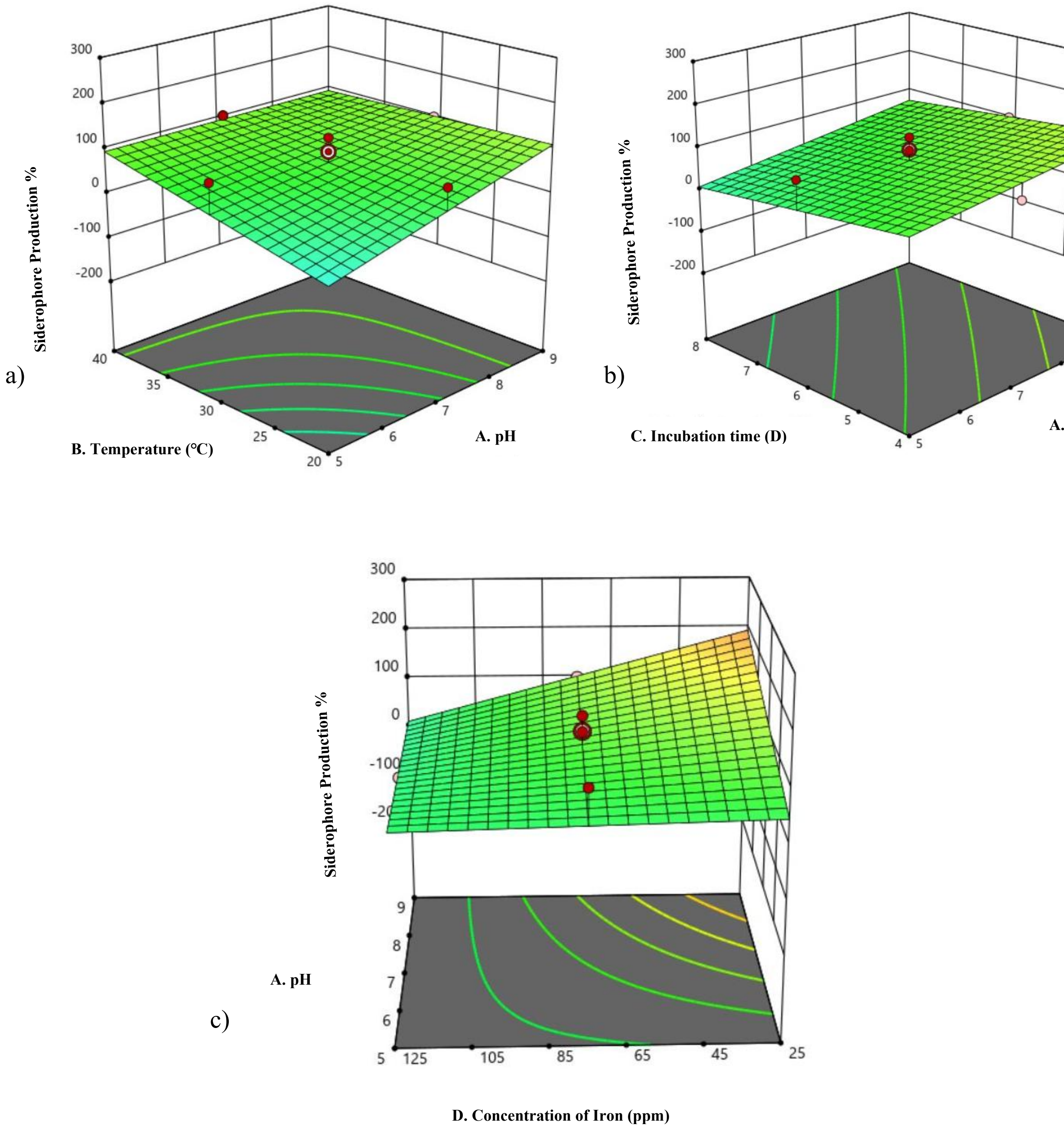
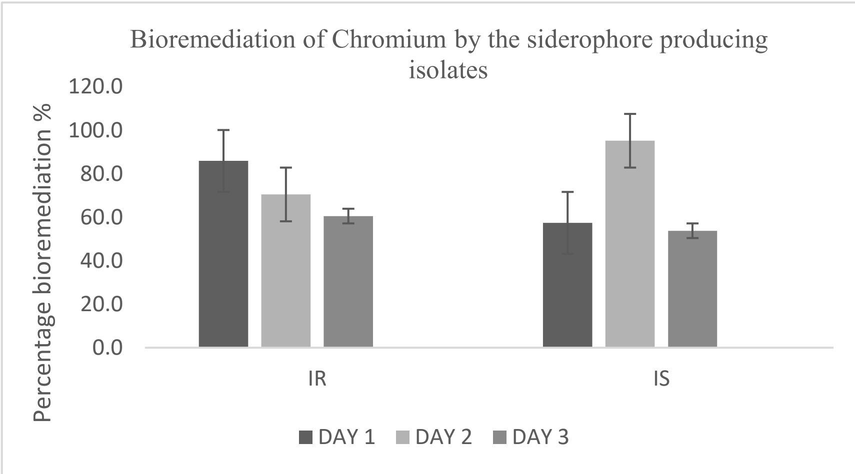
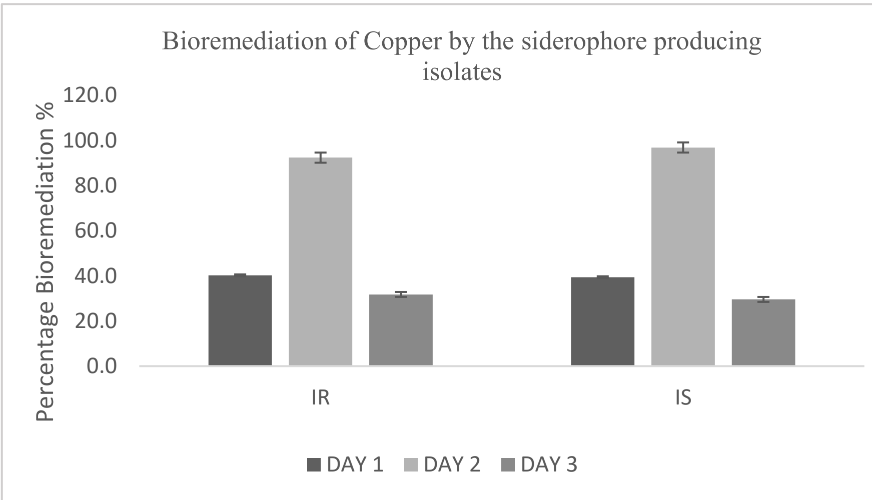


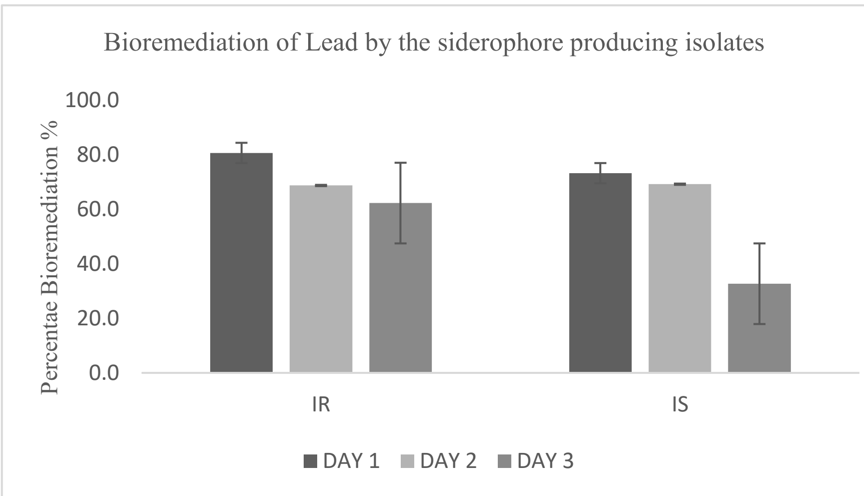
Figure 3a-c: Optimization of Culture conditions for the production of siderophores by strain IR



a)



b)



c)

Figure 4a-c: Bioremediation of a) Chromium, b) Cadmium and c) Lead by the isolates IR and IS

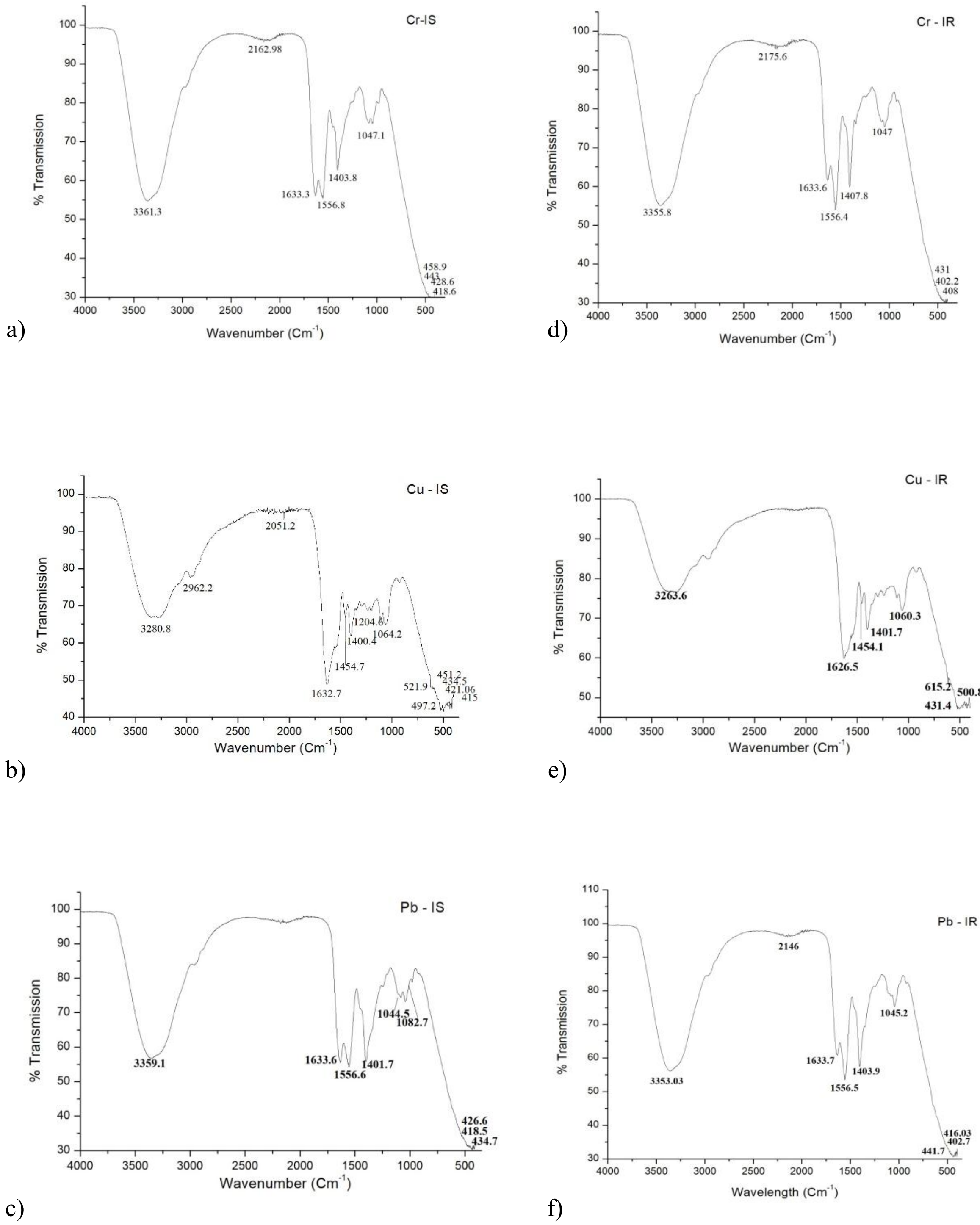


Fig 5: FTIR of heavy metals after bioremediation of a) Chromium b) Copper c) Lead by strain IS and d) Chromium e) Copper f) Lead by strain IR

Tables:

S. No.	Strain name	Name of the isolate	Siderophore Production Index (SPI)
1	SR	*Stutzerimonas stutzeri*	1.15
2	IR	*Bacillus safensis*	1.52
3	IS	*Proteus mirabilis*	1.16
4	CL	*Cytobacillus kochii*	No halo zone was observed
5	IL	*Shouchella clausii*	No halo zone was observed
6	SL	*Bacillus velezensis*	1.3
7	SS	*Yet to be identified*	No halo zone was observed

Table 1: Screening for siderophore production by the isolates

Metal	**Wavenumber observed in FTIR (Cm^{-1})**		
	IR	**IS**	**Standard reference from NIST/ChemicalBook/ Spectrabase**
Chromium	3365.5, 1632.9, 1536.52, 1453.94, 1236.8, 1063.8, 429.4, 415.42	3361.3, 2162.9, 1633.3, 1556.8, 1403.8, 1047.1, 458.9, 443.04, 428.1, 418.6	**Potassium dichromate** 2964, 1305, 966, 950, 939, 934, 908, 902, 891, 784, 667 and 556 **Chromium Oxide** 3480, 1660, 632, 571, 545, 410
Copper	3263.6, 1626.5, 1454.1, 1401.7, 1060.3, 615.2, 500.8, 431.4	3280.8, 2962.2, 2051.2, 1632.7, 1454.7, 1400.4, 1204.6, 1108.9, 1064.2, 521.9, 497.2, 434.5, 421.6, 453, 415.5	**Copper sulphate** 3400, 3290, 1100, 1000, 950, 880,790, 720, 620, 600, 500, 495 **Copper Oxide** 660, 450
Lead	3353.03, 2146, 1633.7, 1556.5, 1403.9, 1045.2, 441, 416, 402.7	3359, 1633.8, 1556.6, 1401.7, 1082.7, 1044.5, 434.7, 426.6, 418.5	**Lead acetate** 3436, 1559, 1409, 1341, 1050, 1019, 936, 664, 618, 473 **Lead Oxide** 480, 300

Table 2: FTIR analysis of culture supernatant after bioremediation by the isolates IR and IS

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