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Optimization of Siderophore Production in Lead Resistant Bacterium as a Promising Bioremediation Strategy

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A B S T R A C T

Presently, there is an increased interest in exploiting the ability of siderophore producing micro-organisms for bioremediation of heavy metals. So far, association of siderophore production and chelation of heavy metals like Cu, Cr and Pb, in addition to iron, is well established. However, to the best of our knowledge, no study has reported increased efficiency of heavy metal bioremediation on optimization of its siderophore production. The current study was thus carried out with an objective to screen a lead resistant microbial strain and study the increase in its bioremediation potential on optimization of siderophore production. Among the eleven isolates screened in this study, Pseudomonas spp. was the most potential siderophore producing lead tolerant isolate. Optimization studies indicated maximum siderophore production in iron restricted minimal media (pH 7) on incubation at 37°C and shaking conditions (120 rpm). Overall, 6.6% increase was observed in lead bioremediation potential of Pseudomonas spp. under optimum conditions for siderophore production (which increased by 16.19%). Immobilization of Pseudomonas spp. in calcium-alginate beads further improved its bioremediation potential by 6% attaining 85.6% lead removal. Thus, our study confirmed that siderophore production is proportionally linked to chelation and consequent bioremediation of lead in Pseudomonas spp. isolated in this study.

Keywords: Bioremediation, Heavy metals, Immobilization, Lead, *Pseudomonas*, Siderophores

1. Introduction

Heavy metal contamination is an ever-increasing environmental scenario as a consequence of human activities. Industries including electrochemical, tanneries, shipping and construction release many heavy metals into the environment (Tchounwou et al., 2012). In the long list of environmental contaminants, lead is regarded as one of the extremely dangerous metal in ionic form due to its property of biomagnification (Collin et al., 2022). In spite of the associated environmental hazards, characteristics like poor conductivity to electricity, resistance to corrosion, high malleability and ductility has encouraged the persistent use of lead in industries (Wani et al., 2015). Among the most commonly used commodities, lead is used in batteries, cable sheaths, cosmetics, newspaper printing and paints (IARC, 2006). Automobile exhausts release lead salts into the environment that pollute soils, water as well as surface waters over a long distance (Masindi&Muedi, 2018). In the natural food web, lead interferes with biochemical mechanisms of the lowest members like phytoplankton by disrupting its oxygen accumulation capacity in marine environments (Botte et al., 2022). In humans, lead bioaccumulation triggers premature mortality, cardiovascular diseases and neurological disorders, among others (Jaishankar et al., 2014; Assi et al., 2016). Exposure to lead due to its increased application in industries and disposal in environment is thereby a serious concern, and researchers constantly seek strategies to remediate lead polluted sites.

Bioremediation techniques are favored over physical and chemical remediation techniques due to their sustainability and economical applications. During bioremediation, either live cells of microorganisms, biomolecules or secondary metabolites (enzymes) are employed for detoxification and decontamination of heavy metals (Pande et al., 2022; Sharma, 2021). Ideally, micro-organisms tolerant to high concentration of heavy metals is selected for bioremediation purpose. These organisms uptake the metal ions from the soil and metabolize them into less or non-toxic forms. Alternatively, they help in bioremediation by mechanisms like adsorption, complexation or precipitation (Pande et al., 2022; Henao and Herrera, 2021).

In the past two decades, the detoxification mechanism of microbial siderophores has gained attention in the field of bioremediation (O'Brien et al., 2014; Hesse et al., 2018). Siderophores are microbial proteins that mainly functions in the sequestration of ferric ions in iron limiting soil, though it can also chelate other metal ions like Pb, Zn, Cd, Mg, Cr with low affinity (Hesse et al., 2018). It is known that siderophore production can not only be induced by ferric ions but also by other metal ions (O'Brien et al., 2014; Ahmed and Holmström, 2014). Siderophores chelate metal ions forming complexes that are not identified by cellular receptors leading to a collapsed transport system (Kramer et al., 2019). An advantage of employing a heavy metal tolerant siderophore producing strain in bioremediation is the higher accumulation of metal ions which can be subsequently detoxified by microbial enzymes or other physiological processes (Roskova et al., 2022).

An earlier study demonstrated promising results in decontamination of arsenic by siderophore producing *Pseudomonas azotoformans* (Nair et al., 2007). Valuable insights into the use of siderophore-producing *P. aeruginosa* in bioremediation was also provided by Braud et al. (2010) by demonstrating its

enhanced growth in metal-contaminated media compared to isogenic mutants that do not produce pyoverdine. In a recent study, Huo et al. (2021) described the heavy metal bioremediation potential of siderophore producing rhizobacteria. These studies evidently provide compelling evidence to the role of siderophores in bioremediation of heavy metals. However, whether increasing siderophore production can enhance bioremediation potential of a strain proportionally, is an interesting insight not attempted to be explored in existing literature. In this context, the current study was carried out with an aim to obtain a potential siderophore producing lead tolerant isolate from natural environment. We further studied the effect of optimization of siderophore production and immobilization of live cells on the bioremediation potential of the isolate.

2. Materials and Methods

2.1. Collection of samples

In the present study, 2 waste water samples were collected from areas near rubber and tannery industries situated in Vasai and Mazgaon Dockyard, Mumbai, India respectively. These samples were collected in sterile glass bottles and transported to the laboratory, where they were processed immediately.

2.2. Enrichment and isolation of lead resistant microorganism

The lead resistant microorganisms were enriched in Nutrient Broth (NB) and Sabouraud's Broth (SAB) containing different concentrations (10, 50, 100 and 1000 ppm) of lead acetate (Chatterjee et al., 2012). The growth medium was incubated for 1 week at room temperature. The resistant organisms were isolated from flasks with highest concentration of lead acetate that showed significant turbidity. Isolation was done on agar plates containing appropriate concentration of lead acetate. The colony characteristics of all the isolates were studied.

2.3. Screening of siderophore producers

The lead resistant isolates obtained in our study were further screened for production of siderophores using modified Chrome Azural S (CAS) medium (Schwyn and Neilands, 1987). Actively growing (24h old) cultures were spot inoculated on CAS agar plates and incubated at Room Temperature (RT; ~28°C) for 24h. Isolates showing orange-yellowish halo zones indicated siderophore production.

2.4. Determination of lead reducing capabilities of siderophore producers

The lead reducing capacity of isolates was determined in Synthetic Effluent (SE) media [composition in g/L: glucose (5), NaCl (0.1), KCl (0.1), $MgSO_4.7H_2O$ (0.02), $(NH_4)_2SO_4$ (0.02) and $CaCl_2.2H_2O$ (1.8), $FeCl_3$ (0.002), pH 7.2]. For this purpose, 5ml suspension of isolates capable of lead reduction and siderophore production were inoculated in SM media containing 1000 ppm of lead acetate and incubated at RT for 1 week. The Optical Density (OD) of isolates was adjusted to 0.1 at 600 nm using a colorimeter. A control flask was also set up with un-inoculated media and incubated under same conditions.

2.5. Estimation of residual lead using Dithizone method

The residual lead in the supernatant of inoculated SM media, obtained after centrifuging at 10,000 rpm for 10 mins, was estimated by the Dithizone method. Lead nitrate was used as standard solution for the assay. To carry out the assay, the supernatant (30 ml) was mixed with 5 ml of water and 10 ml of ammonium citrate- cyanide reducing solution, in a separating funnel. Dithizone solution (5 ml) was then added and shaken for 10 sec. The pink colored lead dithizonate complex thus formed was estimated colorimetrically at 430 nm. The residual lead was estimated using a standard graph of concentration of lead (in ppm) v/s absorbance (Powell and Kinser, 1958).

2.6. Identification of potential isolate

The isolate showing maximum lead reduction as well as siderophore production was selected for further studies. It was identified using biochemical, cultural and morphological tests described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

2.7. Characterization of siderophore

Different tests were carried out to characterize the siderophore produced by the potential isolate. These tests are indicated below.

2.7.1. Ferric chloride test

The cell free supernatant (1 ml) was treated with equal volume of ferric chloride and incubated at 37°C for 20 mins. The formation of yellow color indicated presence of hydroxamate type siderophore.

2.7.2. Arnow's method

The cell free supernatant was treated with nitrate molybdate in acidic conditions using 0.5 N HCl and then with NaOH to neutralize the remaining acidity. The formation of red colour indicated presence of catechol type siderophore.

2.7.3. Vogel's method

Phenolphthalein reagent (1-2 drops) was added to 3 ml of 0.1 N NaOH to obtain a light pink colored solution. The disappearance of pink color on addition of cell free supernatant indicated presence of carboxylate type siderophore.

2.8. Optimization of siderophore production

To optimize siderophore production, the effect of pH (4, 5, 6, 7, 8, 9), temperature (4°C, 25°C, 37°C, 55°C), incubation conditions (static and shaker) and metal chelators (citrate and EDTA) were studied. In addition, the effect of addition of 1 % carbon source (glucose) and 1 % nitrogen source (asparagine) was also determined. Optimization was carried out using One Factor at a Time (OFAT) method in iron restricted minimal media. The observations were noted after incubation at 37°C for 24 h under static conditions. Siderophores in the media were estimated using CAS shuttle assay, for which equal volumes (0.5 ml) of cell free supernatant of 24 h old culture was mixed with CAS reagent and checked for development of yellow- orange colour. The color intensity was measured colorimetrically at 630 nm. A control was also maintained using un- inoculated media under same conditions (Murugappan et al., 2012; Tank et al., 2012). Siderophore produced was calculated by using the following formula,

$$\%\ Siderophore\ units\ (SU) = \frac{Ar - As}{Ar}\ x\ 100$$

Where, 'Ar' is the absorbance of reference at 630nm and 'As' is the absorbance of sample at 630nm.

2.9. Immobilization of potential isolate

The immobilization of potential isolate was done by calcium alginate method. The culture suspension (2 ml) was added to 50 ml of 3 % sodium alginate. This mixture was added to chilled 3 % calcium chloride with the help of 1 ml pipette, drop by drop, to form beads. The beads were kept at 10°C for 24 h for hardening, and then washed with saline before further use (Trellis and Rivero, 2020).

2.10. Comparative studies of bioremediation potential of test isolate under different conditions

Firstly, the increase in lead reduction potential of the isolate under optimized conditions as compared to standard conditions was calculated. For this purpose, one flask was incubated at standard conditions used initially in our study and another flask was incubated at optimized conditions for siderophore production. The experiment was carried out simultaneously to maintain same external laboratory and incubation conditions.

Comparative studies were also carried out to study lead remediation potential of live and immobilized cells of potential isolate under same laboratory conditions. The SE media containing 1000 ppm lead nitrate was mixed with 5 g of beads with immobilized cells. Similarly, another flask was inoculated with 5 ml of culture to study the efficiency of free cells. The flasks were incubated at shaking conditions at RT for 7 days. A control set up was also maintained with un- inoculated media under same conditions. After incubation, the samples were centrifuged at 10,000 rpm for 10 mins and the supernatant was used for estimation of residual lead by dithizone method (Powell and Kinser, 1958).

3. Results and Discussion

3.1. Enrichment and isolation of lead resistant organisms

Two water samples were collected in the present study for isolation of siderophore producing lead tolerant microorganisms. Sample 1 (collected from area near tannery industry, Dockyard road) had a whitish opaque appearance, with a slightly pungent odour. Sample 2 (collected from area near rubber industry, Vasai road) had a brown translucent appearance with no particular smell. The turbidity observed after enrichment of lead reducing microorganisms in these samples is represented in Table 1. The flask with highest concentration of lead showing turbidity (500ppm) was selected for isolation of tolerant organisms. A total of 11 lead resistant microorganisms, including 6 bacteria and 5 fungi were obtained in our study. These isolates were further tested for their tolerance to higher concentration of lead. All 11 isolates were found to be tolerant to 1000 ppm lead concentration, beyond which they were inhibited. These isolates were re-isolated to ensure purity of culture and maintained on NA (bacterial isolates) or SAB (fungal isolates) agar slants containing 50 ppm lead acetate. The colony characteristics of bacterial and fungal isolates obtained from samples are represented in Tables 2 and 3 respectively.

Table 1 - Enrichment of lead reducing microorganisms

Concentration of lead (in ppm)	Sample 1		Sample 2	
	Nutrient broth	Sabourauds' broth	Nutrient broth	Sabourauds' broth
10	+++	+++	+++	+++
50	+++	+++	+++	+++
100	+++	++	++	++
500	+++	+	++	++
1000	-	-	-	-

Key: +++ highly turbid; ++moderately turbid; +lessturbid; - no turbidity

The effluents of both rubber and tannery industries contain many heavy metals including lead. In rubber industries, the hose is vulcanized in a lead casing to improve the tensile strength of final products (Datta and Ingham, 2001). In tannery industries too, lead is the most commonly used chemical in various processes, after chromium and zinc (Shaibur, 2023). Hence, the water sources near these industries are polluted with lead and other heavy metals leading to natural enrichment of tolerant strains. In addition, the iron limiting conditions (due to presence of iron complexes with other reactive contaminants) as well as presence of heavy metals in polluted industrial regions induces the secretion of siderophores by microorganisms (Hesse et al., 2018). Consequently, collection of samples from these areas increases the probability of isolation of potential strain with dual property of heavy metal tolerance and siderophore production.

Table 2 - Colony characteristics of bacterial isolates observed on Nutrient agar

Colony characteristics	Isolates obtained from Sample 1			Isolates obtained from Sample 2		
	A	B	C	D	E	F
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex	Flat	Convex
Size	1 mm	2 mm	2 mm	2 mm	3 mm	2 mm
Shape	Circular	Circular	Circular	Circular	Irregular	Circular
Color	Off white	Greenish	White	White	White	Off white
Opacity	Translucent	Translucent	Translucent	Opaque	Opaque	Opaque
Consistency	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Gram nature	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Morphology	Short rods	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli

Table 3 - Colony characteristics of fungal isolates observed on Sabourauds agar

Colony characteristics	Isolates obtained from Sample 1		Isolates obtained from Sample 2		
	1	2	3	4	5
Margin	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Convex	Convex	Flat	Convex	Convex
Size	1 mm	1 mm	4 mm	2 mm	3 mm
Shape	Circular	Circular	Circular	Circular	Circular
Color	White	White	White	White	Off white
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Mucoid	Mucoid	Mucoid	Viscous	Mucoid
Morphology	Filamentous	Filamentous	Filamentous	Filamentous	Filamentous

3.2. Screening of siderophore producers among lead tolerant isolates and determination of their lead reducing capabilities

Siderophore production was detected in 3 bacterial and 2 fungal isolates on CAS agar plates. The halo zone of siderophore production around the spot inoculated cultures on CAS agar plate is represented in Figure 1. The zone diameter of siderophores and percent reduction in lead by test isolates is indicated in Table 4. Based on the observations, isolate B was selected as a potential bacterium with maximum tolerance to lead and siderophore producing ability.

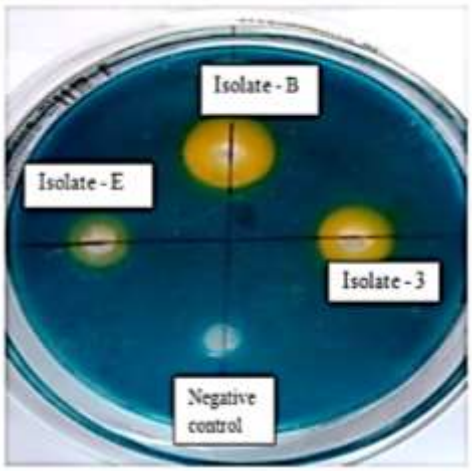

Fig. 1 - Orange- yellowish halo zones around siderophore producers on CAS agar plates

Table 4 - Observed zone diameter on CAS agar plates and percent reduction in lead concentration by siderophore producers

Sr. No.	Isolate	Average Zone size in 'mm'	Lead reduction
Bacterial isolates			
1	B	14.33	74 %
2	C	10.67	70 %
3	E	9.67	64.3 %
Fungal isolates			
4	3	12.33	57.9 %
5	4	10.33	61.97 %
6	Negative control	-	-

Though our study focuses on the role of siderophores in heavy metal detoxification, it is important to note that non-siderophore mediated heavy metal tolerance mechanisms (direct uptake of metal ions, ion exchange and direct reduction) also exist in natural environment (Ojuederie and Babalola, 2017). In the present study, we considered high tolerance to lead as a primary trait during screening of isolates, irrespective of siderophore production, since our basic aim was bioremediation of lead. In the natural environment, multi- species interaction in an eco-system may increase the survival and tolerance of microbial strains in heavy metal contaminated sites (O'Brien et al., 2014). Hence, strains with low tolerance to heavy metals may also thrive at polluted sites due to factors like exopolysacharide secretion, siderophore production or biofilm formation. With this basic understanding, it is possible to comprehend the theory of Hesse et al. (2018) that natural selection in the environment favorssiderophore mediated detoxification at heavy metal polluted sites. At the same time, it can be stated that our observations showing siderophore production among 5/11 (45.45%) lead tolerant isolates complements the above theory by Hesse et al. (2018).

In the present study, isolate B showed largest zone size on CAS agar plate indicating maximum siderophore production among the 5 isolates. Interestingly, it also showed maximum lead reducing ability (74%) on estimation using dithizone method. Similarly, the zone sizes of isolates E and 4 can also be related to their observed lead reduction potential (Table 4). However, such observations were not true for other isolates. For instance, isolate 3 showed larger zone size (12.33 mm) but less lead reducing ability (57.9%) compared to isolate C that showed smaller zone size (10.67 mm) but higher lead reducing ability (70%). These observations indicate that siderophore independent detoxification mechanisms may be functional in isolates C and 3 and there is a high probability that isolate B follows siderophore dependent mechanism for reducing lead. Overall, these observations made our selection of isolate easier to study if improving siderophore production improves its bioremediation potential.

3.3. Identification of potential isolate and characterization of siderophore

In the present study, isolate B was identified as *Pseudomonas* spp. based on its biochemical characteristics. Characterization of siderophore indicated production of mix-ligand siderophore of hydroxamate and catechol-type.

Specific siderophore producing organisms, rather than the type of siderophores, are associated with different environmental applications (De Serrano, 2017). This implies that microorganisms may adapt to given site with the help of siderophores and its structure is less relevant in carrying out activities like bioremediation, plant growth promotion or biocontrol of pathogens among others (Sandy & Butler, 2009). Pyoverdin and pyochelin are commonly produced mix-ligand siderophores by *Pseudomonas* spp. which are associated with bioremediation of arsenic and strain virulence in previous studies (Nair et al., 2007; Duran et al., 2022). Pyoverdine can form complexes with other metal ions like manganese, nickel and lead with a much greater affinity compared to iron (III) (Khan et al., 2018). A mix-ligand siderophore produced by *Pseudomonas azotoformans* has been reported to form hydrogen bonds

with arsenic, and was able to remove 92.8 % heavy metal from contaminated soil sample. Interestingly, the ability of siderophore was better than chemical chelating agents like citric acid and EDTA that removed 70 % and 77.3 % arsenic respectively (Nair et al., 2007). Phyto-extraction of chromium and lead in maize plants is also reported in a study that inoculated siderophore producing *Pseudomonasaeruginosa* and *Pseudomonasfluorescens*in field soil (Braud et al., 2009).

3.4. Optimization of siderophore production

In the present study, siderophore mediated detoxification of lead was highly likely in *Pseudomonas* sp., compared to other isolates. To confirm the same and further study the effect of improved siderophore production on bioremediation potential of the isolate, we optimized the conditions for maximizing its synthesis. Figure 2 represents the optimum conditions for siderophore production by *Pseudomonas* sp. identified in this study. Maximum concentration of siderophore (~ 80% SU) was produced in iron restricted minimal media (pH 7) when it was incubated at 37°C and shaker conditions. The addition of 1 % glucose slightly reduced siderophore production as compared to its concentration in iron restricted minimal medium, whereas asparagine (nitrogen source), citrate and EDTA significantly inhibited its production.

Though siderophore production is mainly induced under iron limited conditions, availability of other nutrients, pH and temperature also affects its synthesis. Earlier studies have reported that increasing the concentration of iron even slightly can reduce siderophore production drastically in *Pseudomonas* spp. (Rachid and Ahmed, 2005; Naik and Dubey 2011). Hence, iron restricted minimal medium was used in this study. The pH and temperature are environmental factors that can affect the solubility of iron and hence siderophore production. Also, pH and temperature controls the metabolic activities of microorganisms and extreme conditions can reduce cell viability and inhibit cellular processes. Also, the formation of metal hydroxides at acidic and alkaline conditions limits siderophore production (Gaonkar and Bhosle, 2013). *Pseudomonasmonteilii* MN759447 isolated from a forest in Uttarakhand optimally produced siderophores (80% units) at pH 7 and 30°C (Srivastava et al., 2022). Similarly, most other studies have reported neutral pH and temperatures between 30°C - 37°C as optimum for siderophore production by *Pseudomonas* spp. (Shaikh et al., 2016; Pattan et al., 2017).

Previously published studies have reported that addition of carbon or nitrogen sources may increase the production of siderophores in Pseudomonas spp. (Verma et al., 2019). Duffy and Defago (1999) reported that presence of glucose strongly supports siderophore production in *P. fluorescens* CHAO. In *Pseudomonas syringae* and *Pseudomonas viridiflava* LMG2352 strains, glucose and asparagine supported maximum siderophore production (Yu et al., 2017). As observed in Figure 2, although glucose significantly supported siderophore production, its concentration was low compared to standard media. Hence, it was not added to optimized media while performing comparative studies described further in this article.

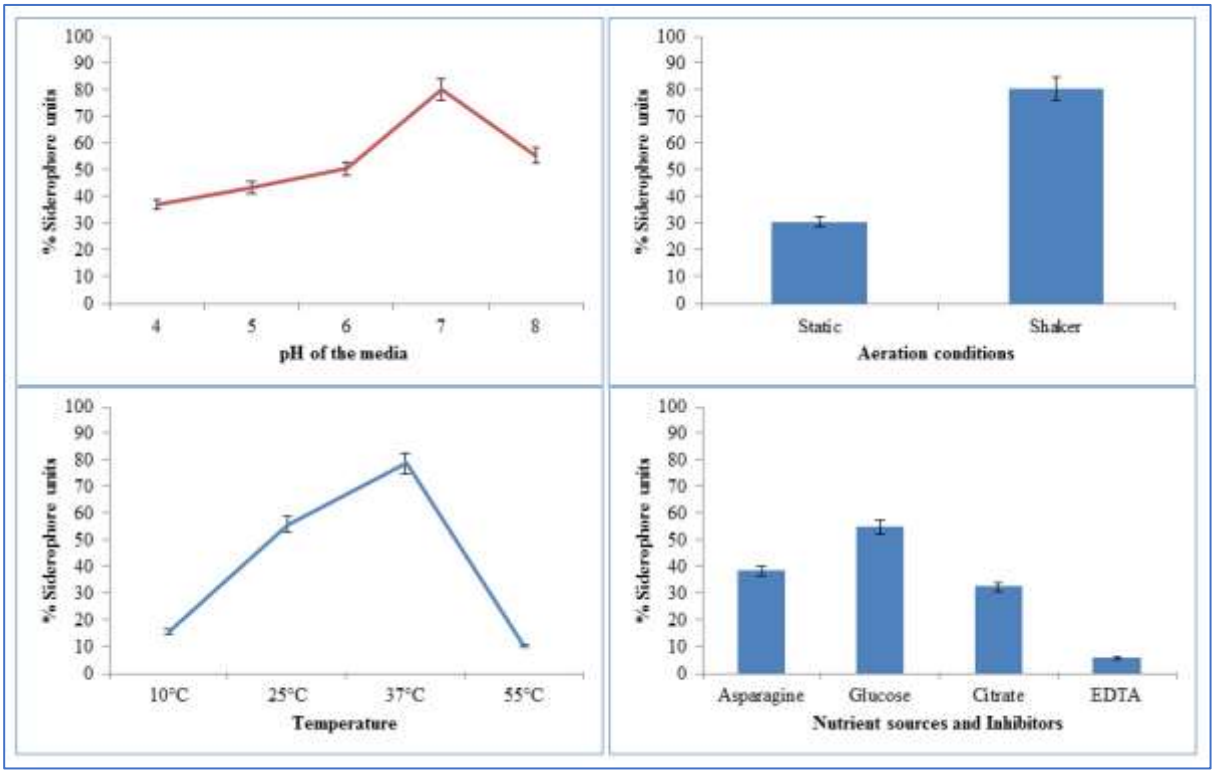

Fig. 2 - Optimized conditions for production of siderophore by Pseudomonas sp.

3.5. Comparative studies of bioremediation potential of test isolate under different conditions

Comparison of siderophore production under standard conditions used initially in the study and optimized conditions showed an increase of 16.19% (from 68.54% SU to 84.73% SU) in this study. The lead reduction capability was also found to increase from initial 74% to 79.6%. Hence, our study shows that increasing the concentration of siderophores can improve bioremediation potential of an isolate, in a scenario where the mechanism is siderophore dependent. Yu et al. (2017) has reported that lead has highest affinity for siderophores compared to other metal ions. In their study, siderophore production by *Bacillus* sp. PZ-1 was enhanced on increasing the concentration of lead in the growth medium. Similarly, Cu and Cd ions stimulated pyoverdine production in *P. aeruginosa* (Sinha and Mukherjee, 2008; Dimpka et al., 2008). However, to the best of our knowledge, none of the published studies have reported siderophore concentration dependent detoxification of heavy metals.

In addition to effect of optimization of siderophores, we also studied the effect of immobilization on lead reduction capacity of *Pseudomonas* sp. isolated in this study. As compared to free cells under optimized conditions, 6% increase was observed in lead reduction capacity of immobilized cells in calcium alginate beads. In general, research studies have reported higher bioremediation capacity of immobilized cells compared to free cells. This is because immobilized cells are protected to some degree from the toxic environment which increases their survival and stability (Mehrotra et al., 2021; Zommere and Nikolajeva, 2017). In addition to lead, immobilized cells of *Halobacteriumcutirubrum*were more effective in removing Cu^{2+}, Cd^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} compared to free cells (Kumar and Raju, 2008).

Overall, the present study indicated that optimizing the production of siderophore in *Pseudomonas* sp. improves its lead reduction capacity. To further improve its potential, the free cells can be immobilized in calcium alginate bead to attain upto 85.6 % lead reduction.

4. Conclusion

The increasing application of lead in commodities of everyday use like batteries and automobiles has led to increased environmental pollution. The bioremediation strategies are in itself an effective and sustainable strategy to overcome the problems associated with heavy metal contamination. At the same time, it is feasible to comprehend that nature uses multiple simultaneously occurring mechanisms to combat changes caused by human interference. The understanding of these mechanisms can greatly benefit us in overcoming environmental challenges. As observed in this study, combining more than one mechanism significantly improved lead reduction by test isolate. Here, Pseudomonas spp. was tolerant to high lead concentration and also produced siderophores. It is possible that both siderophore dependent and independent mechanisms of lead reduction were functional at cellular level of the test isolate. Further combining the potential of this isolate with the advantages offered by an immobilization matrix can make the biotechnological application of this strain highly economical efficient and environment friendly technique. However, more studies are required to confirm siderophore concentration dependent increase in heavy metal reduction by microorganisms before above techniques can be standardized.

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