Application of Response Surface Methodology for Optimization of Siderophore Production

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ABSTRACT

Background: In the present study a statistical model (Response Surface Methodology) was proposed for optimization of siderophore production by using Enterobacter hormaecheii.

Methods: The rhizospheric soil was used for isolation and isolates were screened for siderophore production by chrome-azurol S (CAS) assay. One potent isolate producing maximum siderophore was selected and characterized by 16S rRNA gene sequencing. The culture conditions were optimized for maximum siderophore production by using Central Composite Design. The response surface curves were used to predict the optimum levels of the factors affecting the yield of siderophore.

Result: By using rhizospheric soil,eight isolates were obtained and one potent organism was identified as Enterobacter hormaecheii subsp. oharae (Accession No. MT 775835) by BLAST. The maximum siderophore production (98%) was obtained in succinate medium and the optimum values of variables were found as pH 7, time 60 hrs, temp. 28°C and succinic acid conc. 0.40%. RSM was used to analyze the data by developing 3D surface plots and the residuals plots. ANOVA was used to determine regression coefficients.

Key words: ANOVA, Central composite design, Chrome-azurol S, Enterobacter hormaecheii subsp. oharae, Regression.

INTRODUCTION

Iron is the fourth most abundant element in the earth’s crust, vital for growth of living organisms as it acts as cofactor for enzymes involved in various metabolic processes (Saha et al., 2016). Iron is an important bioactive metal indispensable for the growth and metabolism of bacteria. It is usually abundant in the environment, particularly in clay soils. The major roles of iron in plants and animals include the biosynthesis of chlorophyll, redox reactions in ATP, ribonucleotide synthesis, formation of heme, cell cycle regulation and detoxification (Silva-Stenico et al., 2005). Despite being most abundant element in earth’s crust, the availability of iron is limited due to very low solubility of the dominant ferric iron (Fe³⁺) in soil and become unavailable to plants as a micronutrient (Troeh et al., 2005). Some bacteria have the capability to produce low molecular weight (500-1000Da) metal chelating compound including iron, called as siderophore (Gupta and Gopal, 2008).

Siderophores chelate iron from mineral phases by formation of soluble Fe³⁺ complexes that can be taken up by energy dependent membrane transport mechanism and make it available to plants or bacterial cells (Ali and Vidhale, 2013). The term Siderophore was coined by Lankford (1973) which in Greek means “iron carrier” (Ahmed and Holmström, 2014). Siderophores are low molecular weight, non-ribosomal peptides, secreted under low iron stress conditions and capture iron from the environment (Budzikiewicz, 1993). These are compounds with small peptidic molecules having side chains and functional groups which have high-affinity ligand to bind ferric ions and transport them through the cell membrane (Niehus et al., 2017). In nature, more than 500 types of siderophores are studied; of which 270 have been structurally characterized (Boukhalfa et al., 2003). Various species of bacteria belonging to genus Escherichia, Pseudomonas, Azotobacter, Bacillus, Rhizobium, Salmonella, Klebsiella, Vibrio, Aeromonas, Aerobacter, Enterobacter, Yersinia and Mycobacterium are known to produce siderophores. Besides bacteria, several common species of fungi e.g., Penicillium, Mucor, Rhizopus, Saccharomyces, actinomycetes e.g., Nocardia, Streptomyces and algae e.g., Anabaena are also known to produce siderophores (Kannahi and Senbagam, 2014). Though, the primary application of siderophore is to provide soluble iron to microbes for its growth. They also play various roles in fields such as agriculture, bioremediation, biosensor and medicine.
produces siderophores. In the next phase, RSM through statistical technique of CCD had been selected for optimization of siderophore production. In this study, the effect of pH, incubation temperature, time and succinic acid conc. for maximizing siderophore production had been evaluated.

**MATERIALS AND METHODS**

**Collection of soil samples and enrichment**

In the present study, rhizospheric soil of sugarcane, mango and capsicum were collected from Baramati Pune, Maharashtra, India (18.1792°N, 74.6078°E). The intact plant with root was dug out carefully. Then 250 gm of clumps of soil tightly bound to the roots were collected from all the sites and carefully stored in sterile polyethylene bag and used for the isolation.

Standard serial dilution method was used for isolation. Soil samples were air dried to remove the excess moisture. One gm of each soil sample was then suspended in 9 ml sterile distilled water followed by transfer of one ml solution from each tube sequentially in next tube, a dilution range of 10⁻¹ to 10⁻⁶ was prepared. 0.1 mL of sample from 10⁻⁴ dilution was inoculated in sterile minimal and Ashby's broth which were deprived of carbon source and kept for enrichment for 72 hrs on rotary shaker. The loopfull sample from these broths was streaked on sterile minimal medium and sterile Ashby's agar plates and incubated for 24 hrs at 37°C. The isolated colonies were characterized on the basis of biochemical tests described in Bergey's manual of determinative bacteriology. On the basis of morphological characters various biochemical tests were conducted like amylase, gelatinase, catalase, citrate utilization, indole, Voges Proskaure, methyl red, nitrate reduction, H₂S production and sugar fermentation test (glucose, mannitol, lactose, sucrose, maltose and xylose). The isolate was further identified upto species level by 16S ribosomal RNA gene sequencing (Panda et al., 2017). The selected isolate was further characterized on the basis of biochemical tests described in Bergey's manual of determinative bacteriology. On the basis of morphological characters various biochemical tests were conducted like amylase, gelatinase, catalase, citrate utilization, indole, Voges Proskaure, methyl red, nitrate reduction, H₂S production and sugar fermentation test (glucose, mannitol, lactose, sucrose, maltose and xylose). The isolate was further identified upto species level by 16S ribosomal RNA gene sequencing (Panda et al., 2017). Motility was determined by using Hanging drop technique (Bisen, 2014). The selected isolate was further characterized on the basis of biochemical tests described in Bergey's manual of determinative bacteriology. On the basis of morphological characters various biochemical tests were conducted like amylase, gelatinase, catalase, citrate utilization, indole, Voges Proskaure, methyl red, nitrate reduction, H₂S production and sugar fermentation test (glucose, mannitol, lactose, sucrose, maltose and xylose). The isolate was further identified upto species level by 16S ribosomal RNA gene sequencing (Panda et al., 2017).
Application of Response Surface Methodology for Optimization of Siderophore Production

CCD was used to find the optimum concentration of the selected variable. The effect of selected variables on the responses was analyzed to maximize the siderophore production (Murugappan et al., 2012). CCD was used to study the interaction between the significant components and also to determine their optimum levels. It involved steps such as procedures to find the optimum region, the responses in the optimum region of variables, estimation of the optimal conditions and verification of the data (Tanyildizi et al., 2005).

A set of 31 runs for four variables (A to D) at high and low levels were constructed by CCD using MINITAB software (Table 1). The concentrations of the four factors i.e., pH (6-8), temperature (22-34°C), incubation time (24-96 hrs.) and succinic acid conc. (0.28-0.52%) were optimized. The frequency of high and low values of each variable was maintained according to the rules of CCD. These parameters were optimized for siderophore production using succinate media. Siderophore produced by the isolate in different experimental setup was quantified after incubation.

Statistical analysis of the yield and interpretation of the data was carried out for the RSM experimental design using Minitab statistical software. The analysis of variance (ANOVA) was used to value the effects and determine regression coefficients of model. The response surface plots were adopted to represent the effect of the independent variables on siderophore production. These response curves were then used to predict the optimum level of the factors (Kwak et al., 2006). Minitab software was used throughout the study for designing the experiments of RSM using CCD, regression and graphical analysis.

RESULTS AND DISCUSSION
Collection of soil samples and enrichment of the isolates

The different rhizospheric soil samples were successfully collected from the different regions of Baramati. The soil samples collected were found to be the rich source of microbial diversity. Total eight isolates were obtained and they were further screened on the basis of siderophore production. Rhizospheric soil samples are the rich source of microorganisms having the ability to produce variety of biocompounds (Pahari and Misra, 2017).

Estimation of siderophore production by qualitative and quantitative assay

Yellow coloration around the test colony was observed which indicated positive result for qualitative test.

Quantitative estimation of siderophore production was done in terms of percent siderophore units (% SU). Maximum siderophore production was 98%.

Pattan et al. (2017) also used qualitative and quantitative methods for the estimation of siderophore production.

Qualitative estimation by CAS assay and quantitative by CAS shuttle assay are popularly used to estimate siderophore production. Similar results were reported by

![Fig 2a: Qualitative estimation of siderophore production by CAS assay b: Quantitative estimation of siderophore production by CAS shuttle assay.](image)

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**Table 1**: CCD experimental setup for siderophore production.

| Run Order | pH | Temperature (°C) | Time (hrs.) | Succinic Acid conc. (%) | Run Order | pH | Temperature (°C) | Time (hrs.) | Succinic Acid (gms) |
|-----------|----|-----------------|-------------|-------------------------|-----------|----|-----------------|-------------|---------------------|
| 1         | 6  | 34              | 96          | 0.28                    | 17        | 8  | 34              | 96          | 0.52                |
| 2         | 7  | 28              | 60          | 0.64                    | 18        | 6  | 34              | 96          | 0.52                |
| 3         | 7  | 28              | 60          | 0.16                    | 19        | 6  | 22              | 24          | 0.52                |
| 4         | 6  | 22              | 24          | 0.28                    | 20        | 7  | 28              | 12          | 0.40                |
| 5         | 5  | 28              | 60          | 0.40                    | 21        | 6  | 22              | 96          | 0.52                |
| 6         | 8  | 22              | 24          | 0.28                    | 22        | 7  | 28              | 60          | 0.40                |
| 7         | 7  | 28              | 60          | 0.40                    | 23        | 7  | 40              | 60          | 0.40                |
| 8         | 7  | 28              | 132         | 0.40                    | 24        | 7  | 28              | 60          | 0.40                |
| 9         | 6  | 34              | 24          | 0.52                    | 25        | 8  | 34              | 24          | 0.52                |
| 10        | 8  | 34              | 96          | 0.28                    | 26        | 7  | 16              | 60          | 0.40                |
| 11        | 7  | 28              | 60          | 0.40                    | 27        | 8  | 22              | 96          | 0.52                |
| 12        | 8  | 22              | 24          | 0.52                    | 28        | 7  | 28              | 60          | 0.40                |
| 13        | 8  | 22              | 96          | 0.28                    | 29        | 9  | 28              | 60          | 0.40                |
| 14        | 7  | 28              | 60          | 0.40                    | 30        | 7  | 28              | 60          | 0.40                |
| 15        | 6  | 34              | 24          | 0.28                    | 31        | 6  | 22              | 96          | 0.28                |
| 16        | 8  | 34              | 24          | 0.28                    |            |    |                 |             |                     |
Biochemical characterization of the selected isolate.

Enterobacter hormaechei subsp. oharae DSM 16687(T) by using 16S r RNA gene analysis technique. The 16S rRNA FASTA analysis showed 99.12% similarity with the strain Enterobacter hormaechei (Table 2).

The Neighbor-Joining method was used to inferred evolutionary history (Saitou and Nei, 1987). The optimal tree with the sum of branch length 0.46613182 is shown. Associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances used to infer the phylogenetic trees, with branch lengths in the same units. The Tajima-Nei method was used to compute the evolutionary distances (Kimura, 1980) and is in the units of the number of base substitutions per site. A gamma distribution (shape parameter = 1) was used to modeled the rate variation among sites. The positions showing upto 95% coverage of site were excluded. MEGA6 was used to conduct evolutionary analysis (Tamura et al., 2013).

Table 2: Biochemical characterization of the selected isolate.

| Sr. No. | Biochemical test | Result |
|---------|------------------|--------|
| 1       | Indole           | +      |
| 2       | Methyl red       | -      |
| 3       | Voges-Proskauer  | +      |
| 4       | Citrate          | +      |
| 5       | Starch hydrolysis | +    |
| 6       | Gelatinase       | -      |
| 7       | Catalase         | +      |
| 8       | Oxidase          | -      |
| 9       | Glucose          | AG     |
| 10      | Lactose          | -      |
| 11      | Sucrose          | -      |
| 12      | Maltose          | +      |
| 13      | Mannitol         | +      |
| 14      | Xylose           | -      |
| 15      | Nitrate reduction | +     |
| 16      | H₂S production   | -      |

(+) Positive, (-) Negative, (A+G) Acid and gas production.

Characterization of the efficient siderophore producing isolate

Further the bacterial strain having the ability to produce siderophore was successfully characterized upto species level by using morphological, biochemical and molecular methods. The colony characters of the selected isolate were determined and were found to be Gram negative, rod shaped, non-motile and non-endospore forming. The isolate showed positive results for Voges-Proskauer, citrate, starch hydrolysis, catalase and nitrate reduction tests. It showed negative methyl red, gelatinase, oxidase and H₂S production and was able to ferment glucose with acid and gas production. On the basis of results obtained of biochemical tests by using Bergey's manual of determinative bacteriology, the organism was identified upto genus level as Enterobacter sp. The isolate was identified as Enterobacter hormaechei.

Patel et al., (2017) reported that the use of the CAS assay as a comprehensive, exceptionally responsive and most convenient. Their study showed that 37% of bacteria were positive and mainly belonged to Enterobacter, Pseudomonas and Bacillus, showed presence of orange halo around the colonies. For quantitative estimation, the isolates were grown in iron-deficient succinate medium and CAS assay was employed and OD value was measured at 630 nm. Kumar et al. assayed siderophore production qualitatively by using CAS assay. Their results showed positive for the strains Bacillus thuringiensis VIT KVS and Enterobacter soli VIT VK6.

Shah et al., (2016) also used similar approach for the quantitative estimation of siderophore production. They also used CAS shuttle assay for quantitative estimation of siderophore using succinate medium and found that P. aeruginosa RZS9 produced 63.38% SU. Marathe et al., (2015) used same method for the quantitative estimation of siderophore produced by Pseudomonas which was determined by CAS-shuttle assay. The isolated strain of Pseudomonas sp. showed 71% siderophore production. Mokracka et al., (2004) also reported efficient siderophore production using Enterobacter sp.

Fig 3 a: 16s r RNA sequence of selected isolate b: Phylogenetic tree showing evolutionary relationships of taxa.
Optimization of siderophore production and statistical analysis of the CCD (by RSM and ANOVA)

The CCD under the RSM was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. The average siderophore production given in Table 2 was subjected to multiple linear regression analysis. The effect of pH, time, temperature and succinic acid on siderophore production was described in the form of following equation as

\[
\text{Yield} = 0.276 + 0.0824 \text{pH} – 0.00657 \text{ (Temperature)} + 0.002437 \text{ Time (hrs.)} – 0.035 \text{ Succinic Acid}
\]

For siderophore production, 49.55% of variability in the response could be explained by the model (Table 3). The closer the R\(^2\) value to unity, the better the empirical model fits the actual data. The model explains 50.45% of the total variations were due to other factors which were excluded in the model. However, the R\(^2\) (pred) of 29.30% which is less than R\(^2\) indicates that the model is overfit. In this study, the adjusted R\(^2\) (41.79%) was close to the R\(^2\) (49.55%) value. The higher the adjusted R\(^2\) implies better the model. The relationships between the response and the predictors, pH (P value 0.003) and Time (P value 0.002) are significant. The relationship between the response, siderophore production and the predictor, temperature (P value 0.128) and succinic Acid (P value 0.867) is not statistically significant because their p-value is higher than the α-level. A commonly used α-level is 0.05.

The RSM model signifies better fit to the experimental data when the f value was large and the p-value is less than 0.05. Based on the above discussion, the high f and low p values with 6.83 and 0.001 respectively indicates that the regression model found in this study was significant (Table 4). The test for lack of fit was also calculated by Minitab software. Lack of fit explains the variation in the data around the fitted model. Table 4 shows the results of the lack of fit and it was found that the f and p values for the lack of fit were 9.25 and 0.006, respectively. Besides, the absence of any lack of fit (p>0.05) also strengthened the reliability of the models. Thus, it exhibits that the model was fitted well to the experimental data.

In the normal probability plot the data doesn’t fit a straight line and this verified that the residuals in the data were not normally distributed. In the normal probability plot of the effects, that are farther from 0 are statistically significant (Chantarangsi et al., 2016). The color and shape of the points differ between statistically significant and statistically

**Table 3:** CCD setup with yield for siderophore.

| Run Order | Yield(% SU) | Predicted Yield(% SU) | Run Order | Yield(% SU) | Predicted Yield(% SU) |
|-----------|-------------|------------------------|-----------|-------------|------------------------|
| 1         | 62.49       | 68.45                  | 17        | 91.07       | 88.87                  |
| 2         | 96.89       | 88.32                  | 18        | 62.79       | 68.31                  |
| 3         | 95.51       | 90.02                  | 19        | 52.74       | 58.74                  |
| 4         | 51.60       | 63.57                  | 20        | 67.57       | 49.84                  |
| 5         | 55.20       | 52.21                  | 21        | 84.36       | 81.93                  |
| 6         | 77.23       | 75.97                  | 22        | 84.93       | 91.96                  |
| 7         | 87.69       | 91.96                  | 23        | 80.20       | 68.97                  |
| 8         | 81.26       | 84.93                  | 24        | 90.37       | 91.96                  |
| 9         | 52.74       | 55.18                  | 25        | 65.25       | 79.26                  |
| 10        | 87.49       | 85.75                  | 26        | 87.55       | 84.73                  |
| 11        | 92.61       | 91.96                  | 27        | 90.11       | 94.09                  |
| 12        | 70.61       | 74.42                  | 28        | 94.77       | 91.96                  |
| 13        | 90.61       | 97.94                  | 29        | 96.24       | 85.18                  |
| 14        | 97.06       | 91.96                  | 30        | 96.29       | 91.96                  |
| 15        | 52.74       | 53.02                  | 31        | 98.80       | 89.05                  |
| 16        | 61.65       | 73.84                  |           |             |                        |

**Table 4:** Optimization of siderophore production by estimated regression coefficients of second-order polynomial model.

| Term         | Coef   | SE Coef | T-Value | P-Value | VIF |
|--------------|--------|---------|---------|---------|-----|
| Constant     | 0.267  | 0.232   | 1.15    | 0.260   |     |
| pH           | 0.0824 | 0.0251  | 3.29    | 0.003   | 1.00|
| Temperature  | -0.00657 | 0.00418 | -1.57   | 0.128   | 1.00|
| Time (hrs.)  | 0.002437 | 0.000697 | 3.50    | 0.002   | 1.00|
| Succinic acid| -0.035 | 0.209   | -0.17   | 0.867   | 1.00|

S 0.122858, R-sq 49.55%, R-sq(adj) 41.79%, R-sq(pred) 29.30%

**Table 5:** ANOVA for siderophore production.

| Source          | DF | Adj SS   | Adj MS   | F-Value | P-Value |
|-----------------|----|----------|----------|---------|---------|
| Regression      | 4  | 0.385455 | 0.096364 | 6.38    | 0.001   |
| pH              | 1  | 0.16308 | 0.163086 | 10.80   | 0.003   |
| Temperature     | 1  | 0.037241 | 0.037241 | 2.47    | 0.128   |
| Time (hrs.)     | 1  | 0.184896 | 0.184896 | 12.24   | 0.002   |
| Succinic Acid   | 1  | 0.000432 | 0.000432 | 0.03    | 0.867   |
| Error           | 26 | 0.392449 | 0.015094 |         |         |
| Lack-of-Fit     | 20 | 0.380123 | 0.019006 | 9.25    | 0.006   |
| Pure Error      | 6  | 0.012326 | 0.002054 |         |         |
| Total           | 30 | 0.777904 |          |         |         |
insignificant effects. The normal probability plot of the effects displays negative effects on the left side of the graph and positive effects on the right side of the graph. On this plot, the main effects for the factors A and C are statistically significant at the 0.05 level because their p-values are less than the of 0.05. These points have a different color and shape from the points for the insignificant effects shown in Fig 4a.

A standardized Pareto chart (Fig 4b) consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The bars are exhibited in the order of the size of the effects, with the largest effect on top. The Pareto chart illustrates the order of significance of the variables affecting siderophore production at p ≤ 0.05. In this Pareto chart, the bars that represent the factors C, A, CC and AA cross the reference line that is at 2.120. The variables namely time and pH were found to be statistically significant.

Surface plot is a plot that shows the 3D model of contour plot. A contour plot gives a 2D view of all the points connected to produce contour lines of constant responses.

The response surface plot shows effects of time and succinic acid on siderophore production (Fig 5a). At less incubation time and succinic acid concentration, the yield of siderophore was low. Significant improvement in production could be obtained by increasing time and succinic acid concentration when the incubation time was set at high level i.e. 60 hours and succinic acid concentration at 0.40%, the siderophore production reached a maximum.

![Normal plot of the standardized effects](image1)

![Pareto chart of the standardized effects](image2)

**Fig 4 a:** Normal plot of the standard effect **b:** Pareto charts of the effects for siderophore production.

![Three-dimensional response surface plot showing the effect of the time (hrs.) and succinic acid and temperature (°C) on siderophore production.](image3)

**Fig 5:** Three-dimensional response surface plot showing the effect of the (a) time (hrs.) and succinic acid and (b) temperature (°C) and succinic acid (%) on siderophore productions.

![Three-dimensional response surface plots showing the effect of the temperature (°C) and time (hrs.) and pH and succinic acid (%) on siderophore productions.](image4)

**Fig 6:** Three-dimensional response surface plots showing the effect of the (a) temperature (°C) and time (hrs.) and (b) pH and succinic acid (%) on siderophore productions.
The Fig 5b gives the relationship between temperature and succinic acid on final yield. Maximum production was achieved at temperature 28°C and succinic acid 0.4%.

The 3D response surface plot (Fig 6a) represents a rising ridge surface. It shows that higher siderophore yield was obtained with high time (60 hours) and low temperature (28°C), within the chosen experimental range while Fig 6b gives effect of pH (7) and succinic acid (0.4%) on final yield of siderophore. Similarly the Fig 7a and 7b represents the relationship between pH-time and pH-temperature on siderophore yield respectively. According to Mouafia et al. (2016) the CCD and RSM allowed the creation of a polynomial model for the optimum production of biosurfactant.

CONCLUSION
The study shows that collected rhizospheric soil samples are the thriving source of potent microorganisms able to produce variety of ion chelating compounds. Enrichment increases the number of desired organisms from sample up to detectable levels. For selective enrichment Minimal and Ashby’s broth were used. It was found that enrichment method is very effective for isolation of indigenous rhizobacteria.

CAS-HDTMA complex has high affinity towards ferric ion resulting in dark blue color. When the siderophore is added, the siderophore binds with the ferric iron and iron-ligand complex is formed and release of the free dye is accompanied by a color change. Instant decolorization of CAS reagent from blue to orange red was observed with three cultures and it was confirmed by qualitative CAS test. All three isolates were Pseudomonas sp. which produced 71%, 72.33%, 33% units of siderophores in succinate medium, respectively.

Morphological and biochemical tests can be readily used for the identification of organisms up to genus level by using Bergey's manual while 16S rRNA gene sequencing is used for sp. level identification effectively.

The statistical based optimization using CCD and RSM offered an efficient and feasible approach. The optimum conditions for the maximum siderophore production were predicted from the proposed model. Present study showed that maximum yield of siderophore was obtained at pH 7, time 60 hrs, incubation temp. 28°C and succinic acid conc. of 0.40%. The confirmation experiment was performed successfully within the range of levels predicted from the model which gives maximum yield siderophore production by the isolate.

Report suggests that the proposed model accurately predict the maximum siderophore production by providing optimum values of pH, incubation period, temperature and succinic acid concentration. Authors conclude that these types of models are of great importance for developing industrially viable processes.

REFERENCES
Ahmed, E. and Holmström, S.J. (2014). Siderophores in environmental research: roles and applications. Microbial Biotechnology. 7: 196-208.
Akhtar, S. and Ali, B. (2011). Evaluation of rhizobacteria as non-rhizobial inoculants for mung beans. Australian Journal of Crop Science. 5: 1723-1728.
Ali, S.S. and Vidhale, N.N. (2013). Bacterial siderophore and their application: a review. International Journal of Current Microbiology and Applied Sciences. 2: 303-312.
Bisen, P.S. (2014). Microbes in Practice, Edition: First, Chapter: 6 Microbial Staining. IK International, New Delhi. 139-155.
Boukhalfa, H., Lack, J.G., Reilly, S.D., Hersman, L.E. and Neu, M.P. (2003). Siderophore production and facilitated uptake of iron plutonium in P. putida (No. LA-UR-03-0913). Los Alamos National Laboratory. 673: 343-344.
Budzikiewicz, H. (1993). Secondary metabolites from fluorescent Pseudomonads. FEMS, Microbiology Letters. 104: 209-228.
Chantarangsi, W., Liu, W., Bretz, F., Kiatsupaibul, S. and Hayter, A. (2016). Normal Probability Plots with Confidence for the Residuals in Linear Regression. Communications in Statistics - Simulation and Computation. 47: 1-23.
Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39: 783-791.
Gupta, A. and Gopal, M. (2008). Siderophore production by plant growth promoting rhizobacteria. Indian Journal of Agricultural Research. 42(2): 153 -156.
Kannahi, M. and Senbagam, N. (2014). Studies on siderophore production by microbial isolates obtained from rhizosphere soil and its antibacterial activity. Journal of Chemical and Pharmaceutical Research. 6: 1142-1145.
Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 16: 111-120.

Kumar, V., Menon, S., Agarwal, H. and Gopalakrishnan, D. (2017). Characterization and optimization of bacterium isolated from soil samples for the production of siderophores. Resource-Efficient Technologies. 3: 434-439.

Kwak, K.O., Jung, S.J., Chung, S.Y., Kan, C.M., Huh, Y.I. and Bae, S.O. (2006). Optimization of culture conditions for CO₂ fixation by a chemoautotrophic microorganism, strain YN-1 using factorial design. Biochemical Engineering Journal. 31: 1-7.

Lee, W., Van Baalen, M. and Jansen, V.A. (2012). An evolutionary mechanism for diversity in siderophore producing bacteria. Ecology Letters. 15: 119-125.

Marathe, R.J., Phatak, Y.B. and Sonawane, A.M. (2015). Bio prospecting of Pseudomonas aeruginosa for their potential to produce siderophore, process optimization and evaluation of its bioactivity. International Journal of Bioassays. 4: 3667-3675.

Meyer, J.A. and Abdallah, M.A. (1978). The fluorescent pigment of Pseudomonas fluorescens: biosynthesis, purification and physicochechemical properties. Microbiology. 107: 319-328.

Mokracka, J., Koczura, R. and Kaznowski, A. (2004). Yersinia bactin and other siderophores produced by clinical isolates of Enterobacter spp. and Citrobacter spp. FEMS Immunology and Medical Microbiology. 40: 51-55.

Mohali, F.E., Abo Elsoud, M.M. and Moharam, M.E. (2016). Optimization of biosurfactant production by Bacillus brevis using response surface methodology. Biotechnology Reports. 9: 31-37.

Murugappan, R.M., Aravinth, A., Rajaroobia, R., Karthikeyan, M. and Alamelu, M.R. (2012). Optimization of MM9 medium constituents for enhancement of siderophore genesis in marine Pseudomonas putida using response surface methodology. Indian Journal of Microbiology. 52: 433-441.

Neilands, B. (1987). Universal chemical assay for the detection determination of siderophores. Anal. Biochem. 56: 47-56.

Niehus, R., Picot, A., Oliveira, N.M. and Mitri, S. (2017). Foster, The evolution of siderophore production as a competitive trait. Evolution. 71: 1443-1455.

Pahari, A. and Mishra, B.B. (2017). Characterization of siderophore producing Rhizobacteria and its effect on growth performance of different vegetables. International Journal of Current Microbiology and Applied Sciences. 6: 1398-1405.

Panda, S.H., Goli, J.K., Das, S. and Mohanty, N. (2017). Production, optimization and probiotic characterization of potential lactic acid bacteria producing siderophores. AIMS Microbiology. 3: 88-107.

Pattan, J., Kajale, S. and Pattan, S. (2017). Isolation, production and optimization of siderophores (iron chelators) from Pseudomonas fluorescens NCIM 5096 and Pseudomonas aeruginosa from soil rhizosphere and marine water. International Journal of Current Microbiology and Applied Sciences. 3: 919-928.

Payne, S.M. (1994). Detection, isolation and characterization of siderophores. Methods in Enzymology. 235: 329-344.

Raval, A.A. and Desai, P.B. (2015). Screening and characterization of several siderophore producing bacteria as plant growth-promoters and biocontrolling agents. International Journal of Pharmacy and Life Sciences. 6: 4803-4811.

Saha, M., Sarkar, S., Sarkar, B., Sharma, K., Bhattacharjee, S. and Tribedi, P. (2016). Microbial siderophores and their potential applications: A review. Environmental Science and Pollution Research. 23: 3984-3999.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4: 406-425.

Schwyn, B. and Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry. 160: 47-56.

Shaikh, S.S., Wani, S.J. and Sayyed, R.Z. (2016). Statistical-based optimization and scale-up of siderophore production process on laboratory bioreactor. Biotech. 6: 69.

Shen, C. and Zhang, Y. (2017). Staining technology and bright-field microscope use in food microbiology laboratory for the food science student. Springer. Cham. 9-14.

Silva-Stenico, M.E., Pacheco, F.T.H., Rodrigues, J.L.M., Carrihio, E. and Tsai, S. (2005). Growth and siderophore production of Xylella fastidiosa under iron-limited conditions. Microbiological Research. 160: 429-436.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. 30: 2725-2729.

Tanyildizi, M.S., Özer, D. and Elböl, M. (2005). Optimization of α-amylase production by Bacillus sp. using response surface methodology. Process Biochemistry. 40: 2291-2296.

Troeh, F.R. and Thompson, L.M. (2005). Soils and soil fertility. New York, USA: Blackwell. 489.