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# Detection and Characterization of Siderophores Producing Bacteria and Fungi with Potential to Chelate and Qualification & Quantification Assay

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Abstract—***Siderophores are low molecular weight, Fe ion specific chelating agents which have been elaborated by microorganisms growing under low Fe stress. The present work aimed to detect and study the biodiversity of the Parnera hill forest and identify suitable different microorganisms which produce alternative, environment-friendly iron chelating agent i.e. Siderophores. To fulfill these objectives, the soil samples are collected from Parnera hill forests from different geographical locations during variable times and seasons. Isolated microorganisms were detected and identified for Siderophore production by CAS agar assay, level of Siderophore production is determined using various culture media. Also detailed study was carried out to identify types of siderophore produced using Arnow's and Tetrazolium test, Spectrophotometric assay, $FeCl_3$ test, Vogel test. The qualitative analysis of culture filtrates revealed the presence of Hydroxamate nature of siderophores for isolates S1-3, S6-12, S10-1, S9-10, S13-7, S13-8 and Catecholate nature of siderophores for isolates S1-1, S6-12 and S9-10 whereas, S1-3, S10-1, S13-8 and S14-19 isolates produced Carboxylate nature of siderophores The quantification of Catecholate nature of siderophores was also carried out: S6-12 showed maximum 80.76% siderophores production and S9-10 showed 70.15% siderophores production, whereas S1-1 showed least siderophores production 23.07% in 144 hours incubation. Therefore, isolates S6-12 and S9-10 are the most promising microbes and may be helpful for mass production of siderophores from microbes.***

Keywords— *Siderophores, chelating agents, CAS agar, Hydroxamate, Catecholate, Carboxylate*

## I. INTRODUCTION

Siderophores [Greek: "iron carrier"] are extracellular, small [low molecular weight <1000 Daltons] compounds which selectively bind iron[Fe3+]. Life is contingent on a number of essential elements of which iron enjoys a status of notable importance. Iron is the 4$^{th}$ most abundant element on the earth's crust following oxygen, silicone and aluminium. Though iron is one of the most abundant element in the earth's crust, it is not readily bioavailable. Iron exists in the ferric [Fe3+] state, in most aerobic environments such as soil, which tends to form insoluble rust-like solids. In order to be used up, nutrients must not only be available but also they must be soluble. Microorganisms, both aerobic and facultative anaerobic and monocotyledonous plants under low-iron stress conditions generally produce the siderophores [27]. Iron is an absolute requirement for many biochemical processes of microorganisms. Plants and microbial species have developed a chelation approach to stimulate metal availability during stress situations [9], [10]. The fungal species in soil and plant endophytes synthesize hydroxamate and carboxylate type siderophores of several classes- coprogens, fusarinines and ferrichromes [11], [12]. The siderophores produced by fungi play a significant role in transporting iron to plants, bacterial and actinomycetes members [13], [14]. Siderophores sequester ferric iron from environment under iron stress conditions. No system analogues to siderophores have been found for any other metal ion, thus making iron unique in requiring such specific ligands. Siderophores which are widespread among fungi and bacteria are of three types based on the chemical nature of their coordination sites [28]. Hydroxamates type of siderophores that are produced by both bacteria and fungi, consists of N-hydroxylated amide bonds as coordination sites. Catecholates, that are produced only by bacteria, coordinate iron with catecholatehydroxy group. Carboxylates [earlier known as complexones] that are produced by certain bacteria such as *Rhizobium* and *Staphylococcus* strains and fungi belonging to Mucorales, coordinate iron with carboxyl and hydroxyl groups.

Now-a-days, the most common detection method for siderophore production is the universal assay of Schwyn and Neilands (1997). This assay is based on competition for iron between the ferric complex of a indicator dye, Chrome azurol S (CAS), and a chelator or siderophore produced by microorganisms. The iron is removed from CAS by the siderophore, which apparently has a higher affinity for iron(III). The positive reaction results in colour change of CAS reagent.

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The goal of present study is to detect hydroxamate, catecholate and carboxylate nature of bacterial and fungal siderophores. Moreover, quantify the amount of siderophores produced by the organisms that were isolated from Parnera hill Forest, Valsad, Gujarat.

Siderophore are reported to have many favourable applications for mankind and Mother Nature. They are useful in treatment of antibiotic resistant bacteria- or used to treat acute iron intoxications. They are also helpful in the treatment of malaria. Removal of transuranic elements such as aluminium and vanadium from human body is also possible with help of siderophores. Siderophores are documented bio-control agents against bacterial and fungal phytopathogens. Siderophores are also used in pulp treatment, for bioremediation of mercury, to solubilize a varied array of heavy metal such as Cd, Zn, Ni, Cu, Pb and actinides like Pu, Th, and U produced in industries, nuclear power stations and mining. Siderophores are also reported to diminish oxidative stress in microorganisms. Siderophores are also used for the classification of microorganisms based on the type of siderophore they produce, which is known as siderophore typing or sidero-typing [17] - [26].

## II. METHODOLOGY

### *A. Sampling*

Soil samples were collected from Parnera Hill Forest of Valsad, Gujarat. Two sets of samples were collected during the course of three months. The first set of samples was collected in October (ending of rainy season) whereas second set of samples was collected in February (mid of winter season), each time total 14 samples were collected from same location right from bottom to top.

### *B. Physicochemical Analysis of the Soil Samples*

*1) pH and Temperature:* pH of each soil samples were determined by using the pH strip. For this purpose, 0.5g of soil sample was added to 10ml of distilled water in sterile test tube. Temperature of each soil sample was determined by using Thermometer. Moreover, colour of each soil sample was also noted.

### *C. Isolation of Microorganism*

Enrichment of microorganisms was done in nutrient broth (1g of soil sample in 10ml sterile nutrient broth) and incubated at room temperature for 48-72hours. After enrichment isolation of the microorganisms were carried out on Minimal medium(MM) agar. MM agar contained per litre: 10g glucose, 1.47g glutamic acid, 3.0g potassium hydrogenophosphate ($K_2HPO_4$), 1.0g potassium dihygrogenophosphate ($KH_2PO_4$), 0.5g ammonium chloride ($NH_4Cl$), 0.1g ammonium nitrate ($NH_4NO_3$), 0.1g sodium sulphate ($Na_2SO_4$), 10mg magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 1mg manganese sulphate tetrahydrate ($MnSO_4.4H_2O$), 0.5mg calcium chloride ($CaCl_2$) and 20g agar.

All enriched samples were streaked on minimal media agar plate using four-flame method and incubated for 3-4 days at room temperature. After incubation in SET 1 a total of 12 isolates were found while in SET 2 a total of 20 isolates were found (including bacteria and fungi). The colony characteristics of each isolates were noted down followed by Fungal mounting and Gram's staining.

### *D. Detection of Siderophore Production*

Ability of different isolates to produce iron binding compounds siderophore was detected by universal CAS assay in solid medium as per Schwyn and Neilands 1987. It is a universal test for the detection and determination of siderophores as 0.02µm of siderophore can be determined. One litre of CAS blue agar was prepared using 60.5 mg CAS dissolved in 50 ml distilled water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3$ .6 $H_2O$, 10 mM HCI). Under stirring, this solution was slowly added to 72.9 mg H.D.T.M.A. (Hexa decyl tri methyl-ammonium bromide) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved for 20 min and also autoclaved a mixture of 750 ml water, 15 g agar, 30.24 g Pipes and 12 g of a solution of 50% (w/w) NaOH to raise the pH to the pKa of Pipes (6.8). The dye solution was finally poured along the glass wall and agitated with enough care to avoid foaming. Petri dishes (9.5 cm in diameter) were prepared with 30 ml of appropriate medium for culturing different isolates. The CAS reaction rate was determined by measuring the intensity of colour-change in the CAS-blue agar. The control plates of CAS-agar uninoculated were incubated under the same conditions of CAS and no colour change in the CAS-blue agar was observed, even after long incubation periods (10-15 days).

### *E. Chemical Characterization of Siderophores*

1) *Detection of Hydroxamate Siderophores:* The cell-free supernatants were used for the assay of hydroxamate nature by the following two tests-

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a) *$FeCl_3$ test ( Neilands, 1981):* 1 ml of freshly prepared aqueous 2% $FeCl_3$ solution was added to 1 ml of culture supernatant. The formation of orange colour indicates the presence of ferric hydroxamate, showing maxima at 420-450 nm.

b) *Tetrazolium test (Snow,1954):* To a pinch of tetrazolium salt, 1-2 drops of 2N NaOH was added and 1ml of the test sample. Instant appearance of deep red colour indicated presence of a hydroxamate siderophores.These test is based on the capacity of hydroxamic acids to reduce tetrazolium salt, by hydrolysis of hydroxamate group in presence of strong alkali.

2) *Detection of Catecholate Siderophores:* The cell-free supernatants were used for the assay of catecholate nature by the following two tests-

a) *$FeCl_3$ test (Neilands, 1981):* 1 ml of freshly prepared aqueous 2% FeCl3 solution was added to 1 ml of culture supernatant. The formation of wine coloured complex indicates the presence of ferric chloride. The formation of wine coloured complex was monitored at 495 nm.

b) *Arnow's test (Arnow,1937):* To 1ml of culture filtrate, was added 1ml of 5N HCl, 1ml of nitrite molybdate (10 gm sodium nitrite + 10 gm sodium molybdate in 100 ml distilled water) yellow colour results at this point. 1ml of 1 M NaOH (a red colour results) and enough distilled water was added to make the volume to 5ml. Absorbance was noted at 630nm.

Precaution: The sample should not be too strongly buffered, as the function of the HCl is to generate nitrous acid from sodium nitrite. The nitrous acid is required for nitrosation of the catechols ring. The purpose of excess alkali is to convert the initially formed yellow complex to one absorbing at longer wavelengths. If the sample contains too much of an ester, such as ethyl acetate, the latter will consume the alkali and the yellow colour would persist.

3) *Detection of Carboxylate Siderophores:* The cell-free supernatants were used for the assay of carboxylate nature by the following two tests-

a) *Chemical test (Vogel):* To 3 drops of 2N NaOH add 1 drop of phenolphthalein. Water was added until light pink colour develops. On addition of culture supernatant, disappearance of colour indicates presences of carboxylate siderophores.

b) *Spectrophotometric assay(Shenker et.al 1992):* To 1ml of culture supernatant, was added 1ml of 250 M $CuSO_4$ and 2ml of acetate buffer at $_pH$ 4.The copper complex formed was observed for absorption at 269nm.

### *F. Quantification of Siderophores*

Minimal medium was prepared and used for siderophore production. 24hr old culture of microorganisms were used to inoculate minimal medium and incubated for 24-48hrs at $30^0$ C with constant shaking at 120 r.p.m. Following the inoculation, fermented broth was centrifuged (10,000 r.p.m for 15 mins) and cell free supernatant was subjected to detection and estimation of siderophores. Quantitative estimation was done by CAS-Shuttle assay. In which 0.5ml of culture supernatant was mixed with 0.5ml of CAS reagent and absorbance was measured at 630 nm against the reference consisting of 0.5ml of uninoculated broth and 0.5ml of CAS reagent. Siderophore content in the fermented broth were calculated using the following formula:

$$\% \text{ Siderophore units} = \frac{A_r - A_s}{A_r} \times 100$$

Where $A_r$=Absorbance of reference at 630nm (CAS reagent)

$A_s$=Absorbance of sample at 630nm.

## III.RESULT AND DISCUSSION

### *A. Sample Collection*

The soil samples were collected from Parnera Hill forest, Valsad, Gujarat. The samples were collected twice at three months interval. The first set of samples were collected in October (ending of rainy season) while second set of samples were collected in February (mid of winter season) each time 14 samples were collected from same location right from bottom to top.

### *B. Physicochemical Analysis of the Soil Samples*

For the present work, we have collected total 14 soil samples each time from same location at three months interval right from bottom to top. The physicochemical characteristic of soil samples is shown in table I & II.

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TABLE I
PHYSICOCHEMICAL CHARACTERISTICS OF SOIL SAMPLES – SET I

| Set – I (October 2020) | | | |
|---|---|---|---|
| **Sample Number** | **pH** | **Temperature** | **Colour** |
| 1 | 6 | $36^0$C | Dark brown |
| 2 | 6 | $36^0$C | Reddish brown |
| 3 | 7 | $36^0$C | Reddish brown |
| 4 | 7 | $36^0$C | Reddish brown |
| 5 | 6 | $36^0$C | Brown |
| 6 | 7 | $35^0$C | Brown |
| 7 | 7 | $35^0$C | Dark reddish brown |
| 8 | 6 | $36^0$C | Dark brown |
| 9 | 6 | $36^0$C | Brown |
| 10 | 7 | $36^0$C | Black |
| 11 | 6 | $35^0$C | Brown |
| 12 | 8 | $35^0$C | Light brown |
| 13 | 6 | $35^0$C | Light brown |
| 14 | 7 | $36^0$C | Light brown |

TABLE II
PHYSICOCHEMICAL CHARACTERISTICS OF SOIL SAMPLES – SET II

| Set - II (February 2021) | | | |
|---|---|---|---|
| **Sample Number** | **pH** | **Temperature** | **Colour** |
| 1 | 6 | $24^0$C | Reddish brown |
| 2 | 6 | $24^0$C | Brown |
| 3 | 6 | $24^0$C | Brown |
| 4 | 6 | $23^0$C | Black |
| 5 | 6 | $24^0$C | Brown |
| 6 | 6 | $26^0$C | Brown |
| 7 | 6 | $24^0$C | Light brown |
| 8 | 6 | $24^0$C | Brown |
| 9 | 6 | $24^0$C | Brown |
| 10 | 6 | $24^0$C | Black |
| 11 | 6 | $24^0$C | Brown |
| 12 | 6 | $24^0$C | Brown |
| 13 | 6 | $24^0$C | Brown |
| 14 | 6 | $24^0$C | Brown |

It has been observed that there was a wide change in the temperature of the soil samples as the first set of samples was collected at the end of the rainy season and other set at the mid of the winter season. The temperature of the soil sample at the ending of rainy season was around $35^0$ C-$36^0$ C whereas at the mid of the winter the temperature was lowered down to $24^0$ C- $26^0$ C and these may affect the biodiversity of soil.

*C. Isolation of Siderophores producing Microorganisms*

Total 12 isolates were obtained from SET1 and 20 isolates were obtained from SET 2. Out of those 8 isolates showed coloured zone on CAS agar plate, considered as siderophore positive. They were named as S1-1, S1-3, S6-12, S10-1 (SET 1); S9-10, S13-7, S13-8, S14-19 (SET 2). These eight isolates were selected for the further studies.

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Fig. 1 CAS agar

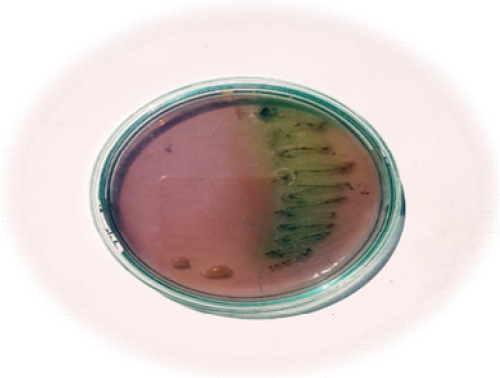

Fig. 2 CAS agar Positive Plate

*D. Siderophores Qualification and Quantification*

Results of various tests for chemical nature of siderophores (hydroxamate, catecholate and carboxylate) produced by 15 days old cultures at $30^0$C are shown in following tables.

Detection of chemical nature of siderophores (Table 3) indicates that out of all four siderophore producers, three isolates synthesized hydroxamate siderophores as evidenced by positive $FeCl_3$ and Tetrazolium test. S1-3, S6-12 and S10-1 have shown positive $FeCl_3$ and Tetrazolium test at 72, 96,120 and 144 hours respectively. Whereas S1-1 shows, negative FeCl3 and Tetrazolium test indicating absence of hydroxamate siderophores.

TABLE III

HYDROXAMATE NATURE OF SIDEROPHORES – SET I

| **Set – I (October 2020)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$FeCl_3$ Test** | | | | **Tetrazolium Test** | | | |
| **Samples** | **72 hrs** | **96 hrs** | **120 hrs** | **144 hrs** | **72 hrs** | **96 hrs** | **120 hrs** | **144 hrs** |
| S1-1 | - | - | - | - | - | - | - | - |
| S1-3 | + | + | + | + | + | + | + | + |
| S6-12 | + | + | + | + | + | + | + | + |
| S10-1 | + | + | + | + | + | + | + | + |

***** + = Positive; - = Negative

Our results clearly demonstrate (Table 4) that out of four siderophores producers, two isolates synthesized catecholate siderophores as evidenced by positive $FeCl_3$ and Arnow's test. S1-1 and S6-12 have shown positive $FeCl_3$ and Arnow's test, whereas S1-3 and S10-1 showed negative result which indicates the absences of catecholate siderophores. The formation of

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wine colour complex on addition of 1-5ml of freshly prepared 2% $FeCl_3$ indicated the presence of hydroxamate siderophores, showing maxima at 495nm.S1-1and S6-12 showed positive $FeCl_3$ with absorbance 0.59and 0.92 at 495nm respectively. Catecholate siderophores on reaction, in succession with nitrous acid, molybdate and alkali yield orange chromogen that absorbs maximally at 620nm.The quantification of siderophores was also carried out at different hours (72,96,120 and 144 hours). S1-1 revealed maximum (61.53%) siderophore production activity at 72 and 96 hours of incubation. Significant production of siderophores 61.53% was occurred at the stationary phase of growth by S1-1 isolate and production was decreased with the time increase. With time the $_pH$ also increased which may be not favourable for siderophore production. While opposite was observed in the second isolate S6-12, which showed maximum (80.76%) production activity at 144 hour of incubation.

TABLE IV

CATECHOLATE NATURE OF SIDEROPHORES – SET I

| **Set – I (October 2020)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **$FeCl_3$ Test** | | | | | **Arnow's Test** | | | |
| **Sample** | **72 Hrs** | **96 Hrs** | **120Hrs** | **144Hrs** | **72 Hrs** | **96 Hrs** | **120 Hrs** | **144 Hrs** |
| S1-1 | + | + | + | + | 61.53% | 61.53% | 42.30% | 23.07% |
| S1-3 | - | - | - | - | - | - | - | - |
| S6-12 | + | + | + | + | 32.69% | 51.92% | 51.92% | 80.76% |
| S10-1 | - | - | - | - | - | - | - | - |

*+=Positive; - = Negative; % = Quantification of Siderophore Concentration

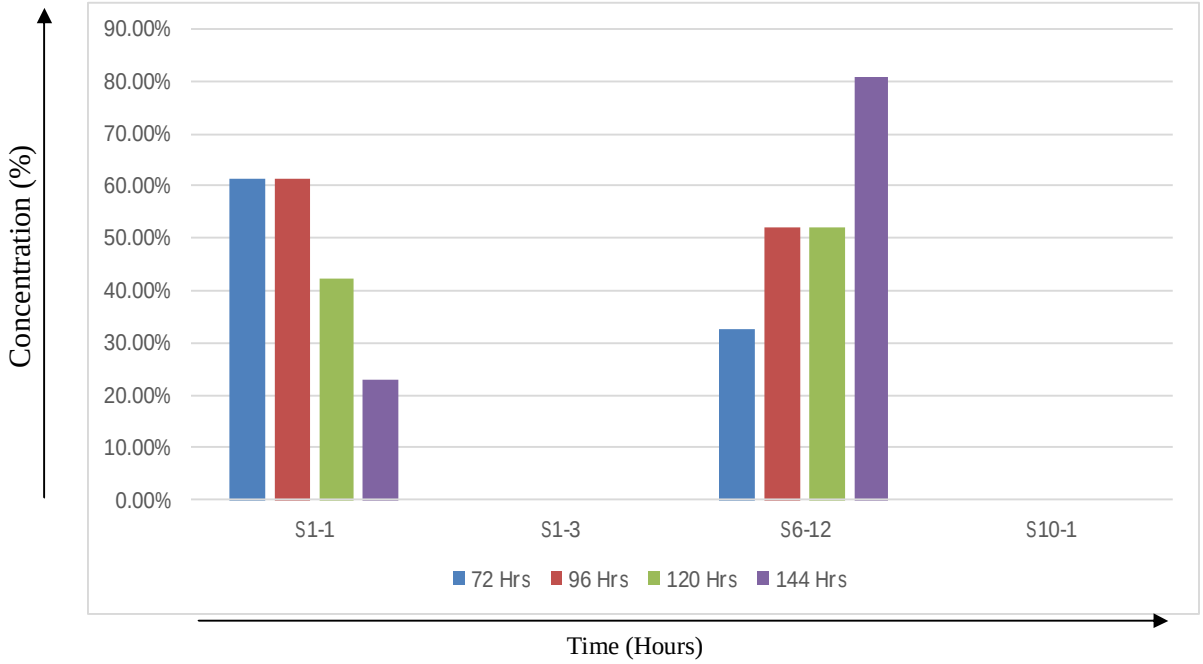


Figure 3: Siderophore concentration produced by different isolates (Set - I)

The present results (Table 5) indicates that isolates S1-3 and S10-1 other than S1-1 and S6-12 formed carboxylate siderophores as they gave Chemical test (Vogel) and Spectrophotometric assay positive at 72,96,120 and 144 hours.

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TABLE V

CARBOXYLATE NATURE OF SIDEROPHORE – SET I

| **Set - I (October 2020)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Chemical Test (Vogel)** | | | | | **Spectrophotometric Assay** | | | |
| **Samples** | **72hrs** | **96 hrs** | **120hrs** | **144hrs** | **72 hrs** | **96hrs** | **120hrs** | **144hrs** |
| S1-1 | - | - | - | - | - | - | - | - |
| S1-3 | + | + | + | + | + | + | + | + |
| S6-12 | - | - | - | - | - | - | - | - |
| S10-1 | + | + | + | + | + | + | + | + |

***** + = Positive; - = Negative

Detection of chemical nature of siderophores (Table 6) indicates that out of all four siderophore producers, three isolates synthesized hydroxamate siderophores as evidenced by positive FeCl3 and Tetrazolium test. S9-10, S13-7 and S13-8 have shown positive FeCl3 and Tetrazolium test at 72, 96,120 and 144 hours respectively. Whereas S14-19 shows, negative FeCl3 and Tetrazolium test indicating absence of hydroxamate siderophores.

TABLE VI

HYDROXAMATE NATURE OF SIDEROPHORES – SET II

| **Set – II (February 2021)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **$FeCl_3$ Test** | | | | | **Tetrazolium Test** | | | |
| **Samples** | **72 hrs** | **96 hrs** | **120 hrs** | **144 hrs** | **72 hrs** | **96 hrs** | **120 hrs** | **144 hrs** |
| S9-10 | + | + | + | + | + | + | + | + |
| S13-7 | + | + | + | + | + | + | + | + |
| S13-8 | + | + | + | + | + | + | + | + |
| S14-19 | - | - | - | - | - | - | - | - |

***** + = Positive; - = Negative

Our results clearly demonstrate (Table 7) that out of four siderophores producers, one isolates synthesized catecholate siderophores as evidenced by positive $FeCl_3$ and Arnow's test. S9-10 have shown positive $FeCl_3$ and Arnow's test, whereas S13-7, S13-8 and S14-19 showed negative result, which indicates the absences of catecholate siderophores. The quantification of siderophores was also carried out at different hours (72, 96,120 and 144 hours). S9-10 revealed maximum (71.15%) siderophore production activity at 144 hours of incubation.

TABLE VII

CATECHOLATE NATURE OF SIDEROPHORES – SET II

| **SET – II (February 2021)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **$FeCl_3$ Test** | | | | | **Arnow's Test** | | | |
| **Samples** | **72Hrs** | **96Hrs** | **120Hrs** | **144Hrs** | **72Hrs** | **96Hrs** | **120Hrs** | **144Hrs** |
| S9-10 | + | + | + | + | 23.07% | 51.92% | 51.92% | 71.15% |
| S13-7 | - | - | - | - | - | - | - | - |
| S13-8 | - | - | - | - | - | - | - | - |
| S14-19 | - | - | - | - | - | - | - | - |

***** +=Positive; - =Negative; % = Quantification of Siderophore Concentration

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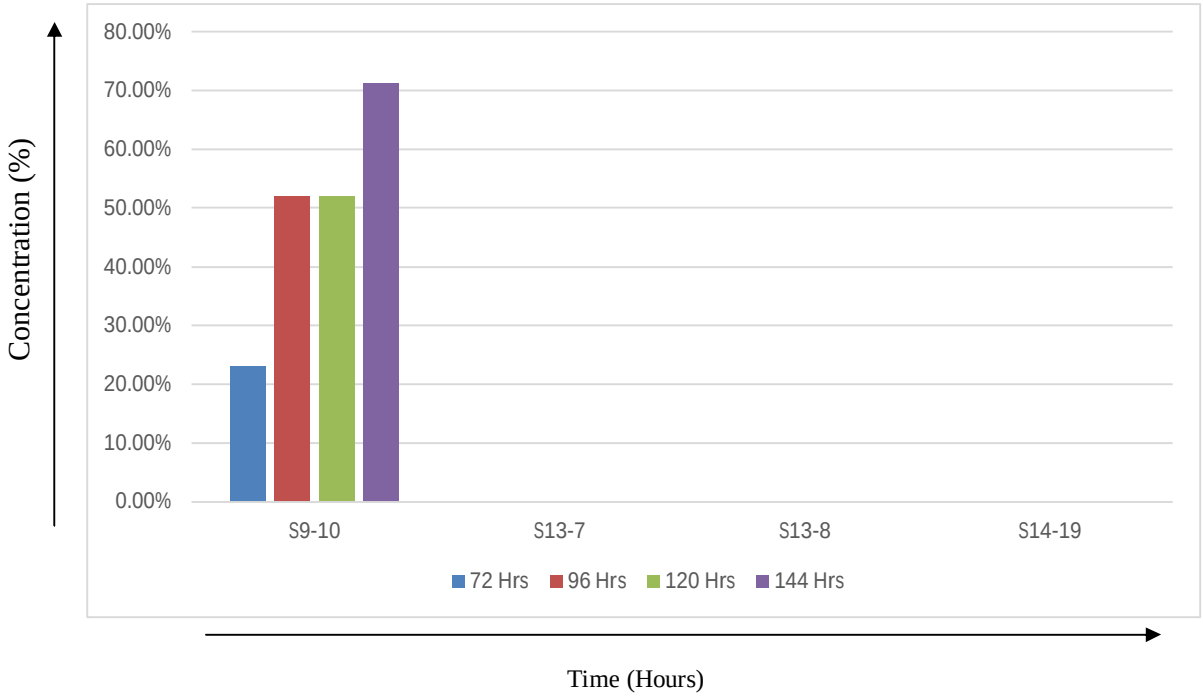


Figure 4: Siderophore concentration produced by different isolates (Set - II)

Our study revealed (Table 8) that S13-8 and S14-19 had positive results for both Chemical test (Vogel) and Spectrophotometric assay, which indicates presence of carboxylate siderophore in the filtrate of culture medium.

TABLE VIII

CARBOXYLATE NATURE OF SIDEROPHORES – SET II

| **Set – II (February 2021)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Chemical Test(Vogel)** | | | | | **Spectrophotometric Assay** | | | |
| Samples | 72Hrs | 96Hrs | 120Hrs | 144Hrs | 72Hrs | 96Hrs | 120Hrs | 144Hrs |
| S9-10 | - | - | - | - | - | - | - | - |
| S13-7 | - | - | - | - | - | - | - | - |
| S13-8 | + | + | + | + | + | + | + | + |
| S14-19 | + | + | + | + | + | + | + | + |

***** + = Positive; - = Negative

From all the results mentioned in above tables it can be stated that 3 isolates i.e. S1-3, S6-12, S10-1 produced hydroxamate siderophores during the end of rainy season and similarly, 3 isolates i.e. S9-10, S13-7, S13-8 shows production of hydroxamate siderophores during the mid of winter season. Thus, we can say that hydroxamate siderophores are found most commonly and its production may be affected by the temperature and environmental changes.

During the end of the rainy season it was found that 2 isolates produced catecholate siderophores. S1-1 showed highest production at 72 hrs (61.53%) and lowest production at 144 hrs (23.07%). S6-12 showed highest production at 144 hrs (80.76%) and lowest production at 72 hrs (32.69%). Whereas, during the mid of winter season only 1 isolate produced catecholate type of siderophores. S9-10 showed highest production at 144 hrs (71-15%) and lowest production at 72 hrs (23.07%). It may be possible that lowering of temperature during winter have affected the siderophores production.

The production of carboxylate type of siderophores at the end of the rainy season was showed by 2 isolates i.e. S1-3 and S10-1. During the mid of winter season, it was observed that 2 isolates S13-8 and S14-19 produced carboxylate siderophores. It can be concluded the production of siderophores majorly depends on the strain and kind of isolates.

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## IV. CONCLUSION

The present study emphasis on the analysis of siderophore production from various microorganisms isolated from soil of Parnera hill forest Valsad, Gujarat. The performance of eight microorganisms was studied in view of their potential to produce different types of siderophores. Hydroxamate nature of siderophores was produced by S1-3, S6-12, S10-1, S9-10, S13-7 and S13-8. The quantification of catecholate nature of siderophores was carried out; the result indicates that S1-1, S6-12 and S9-10 showed positive results. Out of these three S6-12 showed maximum siderophore production (80.76%) after 144 hours of incubation. S1-3, S10-1, S13-8 and S14-19 isolates produced carboxylate nature of siderophores. Therefore, the present study indicates that the ability of these microbes to produce siderophores are good. Modern application of siderophore in agriculture, medical science and environment science are increasing. This study may help for further enhancement of different types of siderophore production for commercial use and more application of it in modern science.

## V. ACKNOWLEDGEMENT

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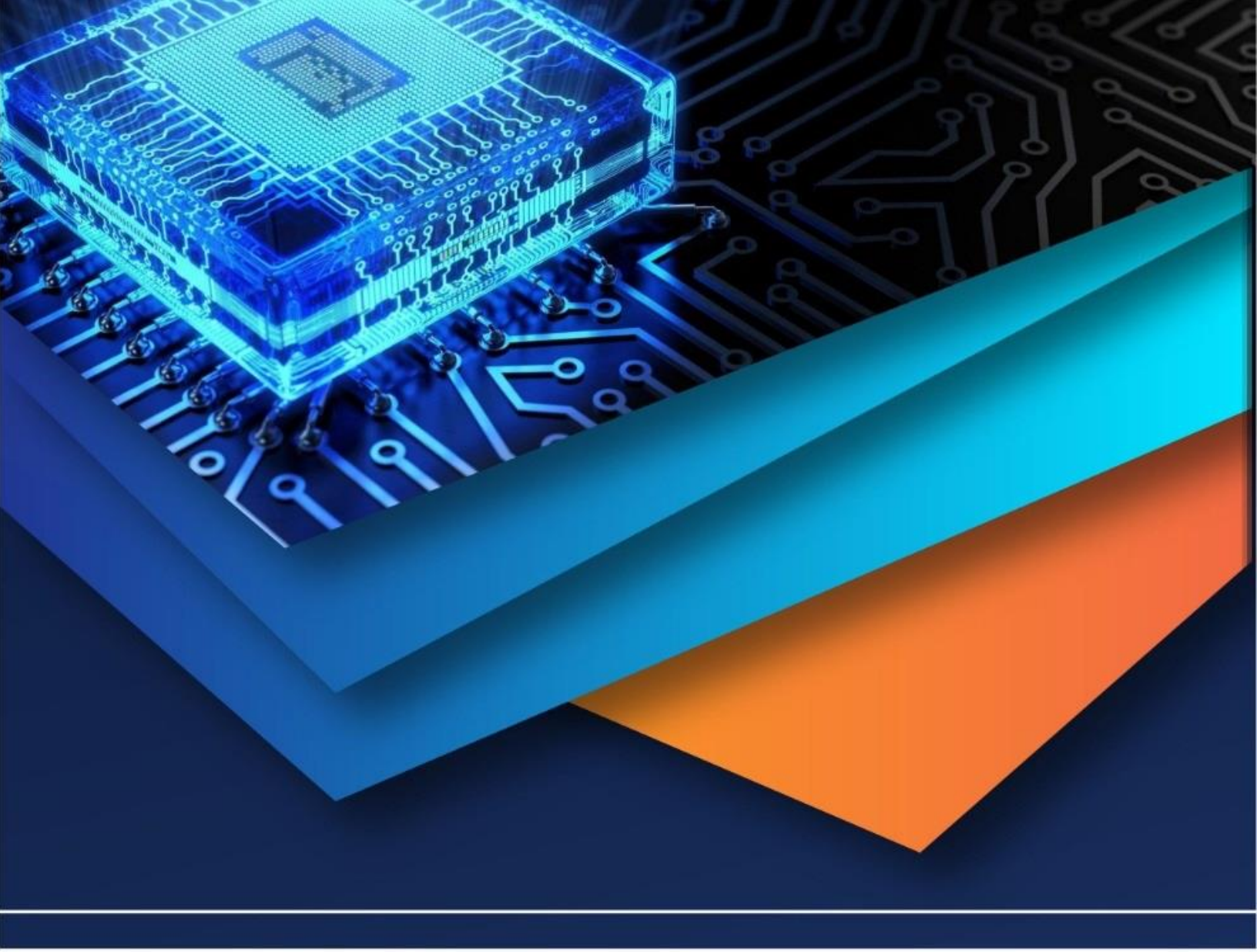

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