

## Characterization of Protein involve in Nitrogen Fixation and Estimation of CO Factor

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### ABSTRACT

Iron is essential to the majority of microorganisms; it is an important cofactor in many cellular processes and enzymes. However in an aerobic environment and at biological pH, iron is primarily found as insoluble oxyhydroxides and is unavailable to microorganisms. Many bacteria have the ability to produce siderophores, low molecular weight compounds that have a high affinity for  $Fe^{3+}$ . Siderophores are part of a multi-component system that actively transports the iron-siderophore ,catechol complex into the cytoplasm.The different microorganism sp. are characterized by their ability to fix nitrogen for the host plant and the plant provides the microorganism with nutrients. Under iron-limiting conditions, *Bacillus sp.*, *Pseudomonas sp.* , *Yeast sp.*, *Rhizobium* and *Azotobactorsp* .are known to produce siderophores. *Rhizobium sp.*produces Catechol type,dihydroxamate or trihydroxamatesiderophore. ; *Bacillus sp.* produces Iron-Perchlorate, dihydroxamate or a trihydroxamate type siderophore; *Yeast sp.* produces Catechol type siderophore ; *Pseudomonas sp.* produces Iron type siderophore ; *Azotobactorsp* produces Iron type siderophore.This siderophores have been purified and chemically characterized. Results indicate that this strain is producing differerent co factors, which has not been described in a member of the different microorganism family.

**Keywords** : Siderophores ; High Pressure Liquid Chromatography (HPLC); SDS-PAGE Analysis ;Column Chromatography; Spectral Scan Analysis;Thin Layer Chromatography (TLC);Arnow's Assay;Iron-Perchlorate Assay ; Chrome Azurol S (CAS) Assay; Glycerol Stocks.

### INTRODUCTION

Nitrogenase proteins of different microorganisms are characterized by two-dimensional polyacrylamide gel electrophoresis. These strains are tested further by in vitro acetylene reduction assays. Nitrogenase component is required for the iron- catechol cofactor, which is a part of the active site of nitrogenase . These nitrogenase are

required for  $N_2$  fixation in vivo but not for  $N_2$  fixation in vitro.Iron is a vital element required by virtually all living organisms including bacteria, fungi with the exception of only a few, including *Streptococcus sanguis*, some *Lactobacillus* species, and *Borrelia burgdorferi*.. It is important in many cellular processes including the electron

transport chain and in deoxyribonucleotide synthesis and acts as a cofactor for many enzymes, such as ribotidreductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase .It is the fourth most abundant element in the earth's crust following oxygen, silicon, and aluminum. However, at biological pH and under aerobic conditions, iron is oxidized to insoluble oxyhydroxides polymers, which are unavailable to a microorganism. In fact, the maximum free ferric iron concentration in an environment is about  $10^{-18}$  M, while most bacteria must maintain an internal iron concentration of around  $10^{-6}$  M. This iron limitation poses a serious threat to microorganisms, as they are unable to survive without adequate supplies of iron. To overcome this iron deficiency, bacteria have adapted to aerobic environments by evolving several sophisticated mechanisms for acquiring iron in almost any environment, including inside a host, in the soil, or other habitats. Siderophore-mediated iron transport systems, which will be the focus of this work, are one such mechanism used by many bacteria to obtain iron. Other mechanisms include acquisition of iron from heme or heme compounds and systems that acquire iron from transferrin or lactoferrin, which will be discussed in more detail below. These systems are regulated by iron concentration and are only expressed under iron-deficient conditions and suppressed under high iron concentrations. Many Gram-negative bacteria are known to produce one or more siderophores and the components for their transport. Such systems have been well studied in *Escherichia coli* .Siderophores are part of a multi-component system for transporting ferric iron into a cell. Other components include a specific outer membrane receptor protein, the TonB-ExbB-ExbD protein complex in the inner membrane, a periplasmic binding protein, and an inner membrane ATP-dependent ABC-type transporter, all of which are described in detail below. Gram-positive bacteria also secrete siderophores under

iron stress, but mechanisms for their transport are not as well understood as the siderophore-mediated transport systems of Gram-negative bacteria. Many nitogen fixating microorganism are also known to produce siderophores and this work will focus on the siderophore mediated iron-transport system of those microorganism.

### Siderophores

The term 'siderophore' is Greek for "iron carrier" and is so named because these molecules produced by microorganisms have an extremely high affinity for ferric iron; thus, siderophores bind ferric iron and transport it into the bacteria. cell. They are low molecular weight (350-1500 Daltons) organic molecules, which can compete for ferric iron in ferric hydroxide complexes. There are over 500 described siderophores that are classified based on their chelating group specific for ferric iron. There are two main siderophore classes, the catechol-type and the hydroxamate-type. Catechol-type siderophores bind ferric iron with adjacent hydroxyls of catechol rings, and are almost always derived from 2,3-dihydroxybenzoic acid (DHBA) . The best-studied example of a catechol-type siderophore is enterobactin , which is produced by *E. coli*. Hydroxamate-type siderophores contain a carboxyl group attached to an adjacent nitrogen, which chelates ferric iron. An example of this type is ferrichrome , a fungal siderophore produced by *Ustilagosphaerogena* . Hydroxamates are generally more complex structurally and are also considered more hydrophilic in nature. In addition to these classes, a miscellaneous class of siderophores has also been established. Siderophores belonging to this class may contain both catechol and hydroxamate groups, which is the case for heterobactin, which is produced by *Rhodococcuserythropolis* , or other groups responsible for iron chelation. The binding capabilities vary depending on the siderophore; enterobactin has a stability constant ( $K_f$ ) of  $10^{52}$  for ferric iron, while ferrichrome exhibits a  $K_f$  of  $10^{29}$  . Several assays have been developed to

detect the presence of siderophore as well as to determine the chemical type of siderophore produced by a microorganism. Siderophores are secondary metabolites and are assembled by nonribosomal cytoplasmic peptide synthases. Currently little is known about siderophore excretion. One 43 kDa inner membrane protein thought to be involved in enterobactin secretion, EntS, has been described and shows some homology to known export pumps, such as TetA that exports tetracycline. The gene, *entS*, which codes for this protein is found in the region of genes required for enterobactin synthesis and mutant strains that produce defective EntS secrete much less intact enterobactin than the wild-type. Work is ongoing to better characterize the systems responsible for siderophore export.

#### **MATERIALS AND METHODS**

Take test tubes and label them as Blank and 1 to 6. Make dilutions of Protein (BSA) standards with concentrations of 200, 160, 120, 80, 40,  $\mu\text{g}/200\ \mu\text{l}$  by transferring respective amount of BSA from the standard protein solution (1 mg/ml) and adjusting it to a total volume of 200  $\mu\text{l}$  by adding distilled water as mentioned. Add 3 ml of Alkaline Copper reagent to each test tube including the Blank and Unknown tubes. Mix well. Keep at room temperature for 10 minutes. Add 0.5 ml of 1N Folin's reagent to each test tube. Vortex the tubes and keep in Boiling Water Bath for 10 minutes. Switch on the Spectrophotometer, select the wavelength at 660 nm and let it warm before taking the absorbance (OD). First take the OD of Blank and make it zero. Remove Blank tube and take the OD of all the tubes and record it. Wash the cuvette after taking OD of each sample. Plot a Standard Curve of absorbance at 660 nm on "Y" axis versus concentration of protein  $\mu\text{g}/200\ \mu\text{l}$  on "X" axis. Record the value "x" of Unknown from graph corresponding to the optical density reading of the test sample.

#### **Sonication process**

The samples from each large production conical

flask were collected and kept in to the small beaker and kept for 1 hour in rotaty shaker with ice cubes and centrifuged tubes were taken and centrifuged for 10 min at 10000 rpm speed. Then the supernatant of the samples were discarded and added lysis buffer and continued the process for 3 times. Then after that the supernatants were collected and go for further experiments. The lysis buffer (PotassiumPhosphate- 40mM, Sodium Phosphate -30Mm, Sodium Chloride-400mM, TrisHCl- 25mM, pH- 7) is used for sonication of the samples.

#### **Microorganism Strains and Growth Conditions**

The different microorganism stains are obtained from the different culture media. The culture was grown for 24-48 hours at 28°C.

#### **Congo Red Agar**

The culture is maintained on a modified Mannitol Yeast Agar supplemented with Congo Red dye. This is used because Rhizobia do not generally take up this dye as readily as other organisms, which can help identify if a contaminant is present. This media has the following composition: (1% mannitol, 0.05%  $\text{K}_2\text{HPO}_4$ , 0.02%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01% NaCl, 0.1% Yeast Extract (Difco), and 3% Bacto-agar (Difco). A volume of 0.25 ml of 1% Congo Red solution was added per 100 ml of media prepared. The pH of the media was brought to 6.8 with 6 M NaOH before autoclaving. Both agar plates and slants is prepared from this medum. *Rhizobium* stain is incubated for 48 hours at 28°C.

#### **N Free Manitol Agar Media**

The culture is maintained on N free Manitol Agar Media which is containing (Mannitol =10 gm,  $\text{CaCO}_3$  =5 gm,  $\text{K}_2\text{HPO}_4$  =0.5gm,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  =0.2 gm, NaCl= 0.2 gm, Ferric chloride (Trace),  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  (Trace), Distilled Water =1000 ml). This media is used for the large production of *Azotobactorsp* microorganism. The pH of the media is brought to 7 before autoclaving and incubated for 48 hours at 28 °C .

### **Pikovskaya's Broth**

The culture is maintained on N free Manitol Agar Media which is containing( Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>- 5.0 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>- 0.5 g ,KCl -0.2 g, MgSO<sub>4</sub>. 7H<sub>2</sub>O- 0.1 g, MnSO<sub>4</sub>- Trace, FeSO<sub>4</sub>- Trace, Yeast Extract- 0.5 g, Distilled Water-1000 ml). This media is used for the large production of Phosphate Solubilizing Bacteria (**Bacillus sp**, **Pseudomonas sp** ) microorganism. The pH of the media is brought to 7 before autoclaving and incubated for 48 hours at 28 °C .

### **Yeast Extract broth**

YEPD or yeast extract peptone dextrose, also often abbreviated as YPD, is a complete medium for yeast growth. It contains yeast extract, peptone, double-distilled water, and glucose or dextrose. It can be used as solid medium by including agar. The yeast extract will typically contain all the amino acids necessary for growth. By being a complete medium, YEPD cannot be used as a selection medium to test for auxotrophs. Instead, YEPD is used as a growth medium to grow yeast cultures. The agar version of YEPD typically consists of .3% (mass/volume) yeast extract, 1% peptone, 1% glucose/dextrose, 2% agar, with the rest being distilled water. The broth version of YEPD typically contains 1% yeast extract, 2% peptone, 2% glucose/dextrose, and the rest is distilled water.

### **Glycerol Stocks**

Glycerol stock cultures of this strain were also prepared and stored at -80°C. Cultures were grown in 50 ml Luria-Bertani (LB) broth for 5-6 hours or until OD<sub>600nm</sub> = 0.5-0.6 and 0.8 ml aliquots were added to 0.2 ml sterile 75% glycerol in 2 ml vials.

### **Fiss-Glucose Minimal Media**

Siderophores are only produced under iron-limiting conditions, Fiss-glucose minimal media is was used as an iron-restricted media. Media was prepared by dissolving 5.0 g K<sub>2</sub>HPO<sub>4</sub> and 5.0 g L-asparagine in 954 ml H<sub>2</sub>O and pH was adjusted to

6.8. After autoclaving, 9.94mL of each of the following solutions was added to the 954 ml sterile media: 50% glucose, 0.005% ZnCl<sub>2</sub>, 0.001% MnSO<sub>4</sub>, and 0.4% MgSO<sub>4</sub>. To minimize the amount of contaminating iron, all media and components are prepared with deionized-distilled water and all glassware used for media storage and for growth of the culture were treated with concentrated HNO<sub>3</sub> and rinsed with ddH<sub>2</sub>O.

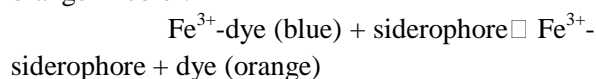
### **Preparation of Inoculum (Seed Culture)**

The microorganism stains are grown in different broth on a rotary shaker for 18-20hours prior to inoculating iron-restricted media.

### **Detection of Siderophore Production**

#### **Chrome Azurol S (CAS) Assay**

The CAS Assay is the universal chemical assay for siderophore detection and is based on a siderophore's high affinity for ferric iron. CAS plates are blue in color because chrome azurol S dye is complexed with ferric iron. When siderophore is present, the following reaction occurs, which releases the free dye, which is orange in color.



The different microorganisms were grown in Fiss-glucose minimal media containing no added iron, minimal media supplemented with 0.5 μM FeSO<sub>4</sub> (low iron) and minimal media supplemented with 20 μM FeSO<sub>4</sub> (high iron control). Cultures are grown for 24 hours on a rotary shaker and the supernatant from each was collected by centrifugation at 13,500 rpm. Using a 2 cork borer, wells were bored into a CAS plate and 60 μl aliquots of each culture supernatant was pipetted into a separate well. Sterile media are also added to a well as a control. The plates are then incubated at room temperature. Depending on the culture, color formation may take 30 minutes to 5 hours. Formation of an orange halo around the well indicates that the culture is producing a siderophore.

### **Iron-Perchlorate Assay for Detection of Hydroxamic Acids**

If siderophore is detected with the CAS assay, then further assays are employed to determine what type of siderophore is being produced. The iron-perchlorate assay is a colorimetric assay used for detection and estimation of hydroxamate-type siderophores. Because this assay is done under acidic conditions, it does not detect the presence of a catechol-type siderophore, which react at alkaline pH. Culture supernatants are collected as described previously and 0.5 ml supernatant is added to 2.5 ml 5 mMFe(ClO<sub>4</sub>)<sub>3</sub> in 0.1M HClO<sub>4</sub> solution and allowed to incubate at room temperature for approximately five minutes. If a hydroxamate-type siderophore is being produced, an orange-red color will form, which varies in intensity based on how much siderophore is produced. Absorbance is measured at 480 nm, with uninoculated media mixed with reagent used as a blank.

### **Estimation of Siderophore Concentration**

The iron-perchlorate assay is colorimetric and amount of siderophore produced by a culture can be visually estimated based on the intensity of the orange-red color formation. To better estimate the concentration of siderophore in a sample, OD<sub>480 nm</sub> was measured and compared to a standard curve prepared using a known concentration of ferrichrome .

### **Arnow's Assay for Catechol-type Siderophore**

To determine whether a culture is producing a siderophore that contains catechol groups, Arnow's method is used (Arnow 1937). This is also a colorimetric assay and can be used to estimate catechol concentration using a known catechol as a standard. The assay is performed by mixing the following in order: 1 ml culture supernatant, 1 ml 0.5 M HCl, 1 ml nitrite-molybdate reagent (prepared by dissolving 10 g sodium nitrite and 10 g sodium molybdate in 100 ml ddH<sub>2</sub>O), and 1 ml 1 M NaOH. These are allowed to incubate for 5 minutes for the reaction

to fully occur. Absorbance is measured at 500 nm with uninoculated media instead of supernatant used in the blank. Catechol groups can be detected because they form a yellow color in nitrous acid, which turns pink-red when excess sodium hydroxide is present. A control (either a culture grown in high iron or uninoculated media) will remain colorless with the addition of reagents.

### **Siderophore Detection Using Thin Layer Chromatography (TLC)**

Siderophore can also be detected by using thin-layer chromatography. Culture supernatant or concentrated samples of siderophore (see later sections) are spotted on Selecto Scientific 10 x 20 silica gel plates and spots are allowed to dry. The plates are then run in an n-butanol:acetic acid:dH<sub>2</sub>O (12:3:5) solvent system until the solvent front reaches the top of the plate. Plates are then dried and sprayed with 0.1 M FeCl<sub>3</sub> in 0.1 N HCl. The formation of a wine-colored spot indicates a hydroxamate-type siderophore, while a dark gray spot indicates production of a catechol-type siderophore. Siderophores are separated on the basis of hydrophobicity using these plates.

### **Effect of Temperature on Siderophore Production**

While the different stains grow optimally at 25-30°C, this may not be the optimal temperature for siderophore production. Five 250 ml flasks were prepared with 50 ml modified Fiss-glucose minimal media and inoculated with *the microorganism stains* seed culture. The flasks were grown at different temperatures (10°C, 20°C, 30°C, 40°C, and 50°C). After 24 hours, growth was measured and siderophore production was estimated for each culture.

### **Purification of Siderophore**

#### **Batch Cultures**

In order to obtain enough purified siderophore for chemical characterization, large volumes of culture were grown in the optimized medium. Typically, 5-6 L of medium was prepared and

each liter was inoculated with 10 a ml seed inoculum. The cultures were grown for 24 hours at 28°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 30 minutes. The supernatant was then acidified to pH 2.0 with 6 M HCl in order to make the siderophore less soluble in water.

#### **Spectral Scan Analysis**

A spectral scan (300-700 nm) was done on the purified siderophore to determine whether this hydroxamate-type siderophore was a dihydroxamate or trihydroxamate . Samples are prepared according to the Atkin's method, except that because a concentrated sample is used, only 20-50 µl aliquot is used and the corresponding amount of ddH<sub>2</sub>O is added to bring the sample volume to 0.5 ml. At an acidic pH, ferric dihydroxamates show an absorbance maximum in the range of 500-520 nm, while trihydroxamates show an absorbance maximum in the 420-440 nm range.

#### **Column Chromatography**

Sephadex LH-20 column material separates compounds based on their hydrophobicity and is also used for gel filtration to separate compounds based on molecular weight. It is prepared by suspending 50 g LH-20 in methanol and deaerating with shaking for around 20 minutes. The material is then packed into a 50 x 1.5 cm column (packed to the top of the column) and is equilibrated with four bed volumes of methanol. The concentrated sample can then be loaded onto the column and eluted with methanol. Approximately 65 150-drop fractions are collected and are tested for their siderophore content using TLC plates. Fractions positive for siderophore are combined in a 100 ml boiling flask and evaporated to dryness using a rotary evaporator.

The dried sample is then re-dissolved in ~3 ml ddH<sub>2</sub>O. If the solution is cloudy, it can be syringe-filtered (0.45 µm pore size) into a 15 ml polypropylene tube. The sample can be estimated

using the iron-perchlorate assay and should be stored at -20°C.

#### **Preparation of Samples for SDS-PAGE**

A 2 ml seed cultures of the different microorganism are used to inoculate 50 ml of modified Fiss-glucose media with no added iron and 50 ml of Fiss-glucose media with 20 µM FeSo<sub>4</sub> added. Cultures were grown for 24 hours on a rotary shaker at 28°C. Cells were harvested by centrifugation at 7,000 rpm for 10 minutes and supernatant was discarded. Some of the whole cell pellet was saved and stored in an eppendorf tube at -80°C. The remaining pellet was resuspended in 10 ml 10 mM Tris buffer (pH 8.0) and sonicated in an ice bath using a large probe (5-0.7 second bursts of one minute with one minute pauses). Sonicated samples were centrifuged at 7,000 rpm for 10 minutes and the supernatant was poured into ultracentrifuge tubes. These were centrifuged at 30,000 rpm (Beckman 50.2Ti rotor) for 90 minutes and the resulting membrane pellets were stored at -20°C.

#### **SDS-PAGE Analysis of Samples**

A 10% separating gel was prepared (Table 1) and ingredients were deaerated for 10 minutes before addition of 10% ammonium persulfate solution that polymerizes the gel. The separating gel was pipetted into a gel-casting unit and was allowed to polymerize for 30 minutes. A stacking gel was prepared (Table 1) and was also deaerated 10 minutes before adding 10% ammonium persulfate. This gel was pipetted on top of the solidified separating gel and a comb was placed. This gel was allowed to polymerize for 30 minutes and then the gel was placed into an electrophoresis unit. The upper and lower chambers were filled with tank buffer.

Whole cell pellets and membrane pellets are prepared by adding an equal volume of 2x gel loading buffer (~20 µl) in an eppendorf tube. Additionally, a protein molecular weight standard was prepared by adding 2 µl BioRad SDS-PAGE broad-range molecular size marker with 8 µl of 2x

gel loading buffer. All samples were then placed in a boiling water bath for five minutes. Samples were loaded onto the gel (5 µl of whole cell pellets and standard and 15 µl of membrane pellets), and the gel was run at 30 milliamps per gel for approximately one hour or until the dye front reached the bottom of the gel. The gel was placed in Coomassie Blue Stain for 30 minutes, destained, and stored in 5% acetic acid.

**High Pressure Liquid Chromatography (HPLC)**

The concentrated siderophore was further purified using a BioRad Biologic Duoflow HPLC system with a Waters 7.8 mm x 300 mm Novapak HR C<sub>18</sub> hydrophobic column as the stationary phase and deaerated, filtered ddH<sub>2</sub>O and filtered 90% methanol as mobile phases. These were filtered using a Millipore filtration system with 0.45 µm membranes. The UV detector was set at 280 nm. The column was equilibrated with 3 bed volumes ddH<sub>2</sub>O prior to use. Sample volume injected onto the column varied between 0.5 ml-1 ml. Several preliminary runs were made to determine the gradient at

which the siderophore eluted from the column. Various programs were created to best separate the pure siderophore from impurities in the sample. A chromatogram was generated after each run and fractions showing peaks on chromatograms were tested for their siderophore content using TLC. After the entire sample had been run through HPLC, any fraction containing siderophore was pooled and concentrated using a rotary evaporator.

**Extraction of Iron**

When it was necessary to extract iron from a siderophore, the following procedure was used .Siderophore solution and 5% (w/v) 8-quinolinol (8-hydroxyquinolone) in chloroform were combined in a 1:3 ratio in a 60 ml separating funnel. The solution was shaken vigorously, removing air periodically, and then layers were allowed to separate completely. The chloroform layer was on the bottom and was removed. This extraction process was repeated 3-4 times by adding fresh 5% w/v 8-quinolinol in chloroform, removing the bottom layer each time.

**RESULTS**

**Table : 1** Ingredients and amounts used in preparation of 10% SDS-PAGE gel.

Reagents	10% Separating Gel	Stacking Gel
30% Acrylamide Solution	6.66 ml	1.33 ml
Running Gel Buffer	5.0 ml	-----
Stacking Gel Buffer	-----	2.50 ml
10% SDS	0.2 ml	0.1 ml
ddH <sub>2</sub> O	8.0 ml	6.1 ml
TEMED	10 µl	5 µl
10% ammonium persulfate	100 µl	50 µl

**Table :2** Protein estimation of Lowry Method (standardized with BSA Solution)

S. N.	Vol. of BSA (ml)	Conc of BSA (mg/ml)	Vol. of Distilled water (ml)	Vol. of Alkaline Copper reagent(ml)	min Incubation for 10	Vol. of Fc Reagent (ml)	min Incubation for 30	OD At 660nm
1	0	0	1	5		0.6		0.0
2	0.2	40	0.8	5	0.6	0.103		

Characterization of Protein involve in Nitrogen Fixation and Estimation of CO Factor

3	0.4	80	0.6	5		0.6		0.215
4	0.6	120	0.4	5		0.6		0.250
5	0.8	160	0.2	5		0.6		0.418
6	1.0	200	0.0	5		0.6		0.430

**Table : 3** Protein estimation of Lowry Method ( Different microorganism)

S.N.	Vol. of Different samples (ml)	Vol. of Distilled water (ml)	Vol. of Alkaline Copper reagent (ml)	min Incubation for 10	Vol. of Fe Reagent (ml)	min Incubation for 30	O.D at 660nm <i>Bacillus sp</i>	O.D at 660nm <i>Yeast sp.</i>	O.D at 660nm <i>Pseudomonas sp.</i>	O.D at 660nm <i>Rhizobium sp.</i>	O.D at 660nm <i>Azotobactor sp.</i>	
1	0	0	1		0.6		0.0	0.0	0.0	0.0	0.0	0.0
2	0.2	40	0.8		0.6		0.125	0.242	0.215	0.287	0.255	
3	0.4	80	0.6		0.6		0.278	0.390	0.356	0.324	0.410	
4	0.6	120	0.4		0.6		0.420	0.465	0.372	0.520	0.551	
5	0.8	160	0.2		0.6		0.574	0.625	0.487	0.594	0.656	
6	1.0	200	0.0		0.6		0.615	0.782	0.612	0.840	0.804	
7	1.0	unknown	0.0	0.6	0.12	0.24	0.33	0.48	0.13			

**Table 4 :** Iron-Perchlorate Assay for Detection of Hydroxamic Acids (Standarized estimation)

Serial no	Conc of Hydroxamic acids (ug)	Vol of sample (ml)	Vol of dw (ml)	Fe(ClO <sub>4</sub> ) <sub>3</sub> + Fe(ClO <sub>4</sub> ) <sub>3</sub> (ml)	O.D at 420nm
1.	0	0	1	1	0.0
2.	40	0.2	0.8	1	0.14
3.	80	0.4	0.6	1	0.21
4.	120	0.6	0.4	1	0.41
5.	160	0.8	0.2	1	0.37
6.	200	1.0	0	1	0.46

**Table 4.1:** Protein estimation of Different microorganism.

Serial no	Concentration of sample (ug)	Vol of sample (ml)	Vol of dw (ml)	Fe(ClO <sub>4</sub> ) <sub>3</sub> + Fe(ClO <sub>4</sub> ) <sub>3</sub> (ml)	O.D at 420nm <i>Bacillus sp.</i>	O.D at 420nm <i>Yeast sp.</i>	O.D at 420nm <i>Pseudomonas sp.</i>	O.D at 420nm <i>Rhizobium sp.</i>	O.D at 420nm <i>Azotobactor sp.</i>
1.	0	0	1	1	0.0	0.0	0.0	0.0	0.0
2.	40	0.2	0.8	1	0.19	0.02	0.06	0.01	0.03
3.	80	0.4	0.6	1	0.28	0.06	0.10	0.30	0.06
4.	120	0.6	0.4	1	0.18	0.07	0.13	0.04	0.08
5.	160	0.8	0.2	1	0.43	0.15	0.16	0.07	0.06
6.	200	1.0	0	1	0.55	0.27	0.23	0.08	1.04
7.	Unknown	1.0	0	1	0.38	0.28	0.25	0.21	1.10

**Table : 5** Estimation of Siderophore Concentration of different microorganism

Serial No.	Conc of the sample (ug/ml)	O.D at 600 <i>Bacillus sp.</i>	O.D at 600 <i>Yeast sp.</i>	O.D at 600 <i>Pseudomonas sp.</i>	O.D at 600 <i>Rhizobium sp.</i>	O.D at 600 <i>Azotobactor sp.</i>
1.	0	0.0	0.0	0.0	0.0	0.0
2.	40	1.20	1.14	1.17	0.26	0.22
3.	80	1.68	1.64	1.91	1.45	1.61
4.	120	2.28	1.77	2.33	2.11	2.23
5.	160	1.98	0.81	1.88	2.34	1.87
6.	200	1.54	1.16	1.78	2.56	1.91

**Table : 6** Arnow's Assay for Catechol-type Siderophore (standarized with catechol)

Serial no.	culture catechol(ml)	Concentration (ug/ml)	Distilled water (ml)	0.5M HCl(ml)	Nitrite-molybdate(ml)	1M NaOH(ml)	O.D at 500 nm
1.	0	0	1	1	1	1	0.0
2.	0.2	40	0.8	1	1	1	0.061
3.	0.4	80	0.6	1	1	1	0.107
4.	0.6	120	0.4	1	1	1	0.293
5.	0.8	160	0.2	1	1	1	0.361
6.	1	200	0	1	1	1	0.44

**Table : 7** Arnow's Assay for Catechol-type Siderophore ( Different microorganism)

Serial no.	culture supernatant(ml)	Conc. (ug/ml)	Distilled water	0.5M HCl (ml)	Nitrite - molybdate(ml)	1M NaOH (ml)	<i>Bacillus sp.</i> (O.D at 500 nm)	<i>Yeast sp.</i> (O.D at 500 nm)	<i>Pseudomoas sp.</i> (O.D at 500 nm)	<i>Rhizobiu m sp.</i> (O.D at 500 nm)	<i>Azotoba ctor sp.</i> (O.D at 500 nm)
1.	0	0	1	1	1	1	0.0	0.0	0.0	0.0	0.0
2.	0.2	40	0.8	1	1	1	0.14	0.01	0.03	0.16	0.04
3.	0.4	80	0.6	1	1	1	0.27	0.04	0.04	0.39	0.07
4.	0.6	120	0.4	1	1	1	0.37	0.07	0.08	0.87	0.06
5.	0.8	160	0.2	1	1	1	0.43	0.12	0.10	0.14	0.12
6.	1	200	0	1	1	1	0.59	0.21	0.22	0.08	0.13
7.	1	unknown	0	1	1	1	0.25	0.13	0.23	0.48	0.07

**Table: 8** Thin Layer Chromatography (TLC) of different microorganism

Serial no.	Sample	Distance traveled (cm)	Total distance (cm)	Rf
1.	<i>Bacillus sp.</i>	4.1	10	0.41
2.	<i>Yeast sp.</i>	4.4	10	0.44
3.	<i>Pseudomonas sp.</i>	3.7	10	0.37
4.	<i>Rhizobium sp.</i>	2.4	10	0.24
5.	<i>Azotobactor sp.</i>	3.8	10	0.38

**Table 9** : Effect of Temperature on Siderophore Production of different microorganism

Serial No.	Temperature Intervals	O.D at 600 <i>Bacillus sp.</i>	O.D at 600 <i>Yeast sp.</i>	O.D at 600 <i>Pseudomonas sp.</i>	O.D at 600 <i>Rhizobium sp.</i>	O.D at 600 <i>Azotobacter sp.</i>
1.	10	1.13	1.08	1.02	0.14	0.24
2.	20	1.96	1.45	1.98	1.15	1.66
3.	30	2.16	1.87	2.05	2.77	2.73
4.	40	1.43	0.22	1.55	1.1	1.44
5.	50	0.32	0.04	0.46	0.86	1.03

**Table : 10** Spectral Scan Analysis at different absorbance of different microorganism

Serial no.	Sample	O.D at 300 nm	O.D at 500 nm	O.D at 700 nm
1.	<i>Bacillus sp.</i>	0.03	0.105	0.23
2.	<i>Yeast sp.</i>	0.08	0.169	0.281
3.	<i>Pseudomonas sp.</i>	0.17	0.302	0.432
4.	<i>Rhizobium sp.</i>	0.26	0.59	0.956
5.	<i>Azotobacter sp.</i>	0.06	0.175	0.323

**Table: 11** SDS-PAGE Analysis and estimate the molecular weight of diifferent sample of different microorganism

Serial no.	Sample no	Migration Distance(cm)	Molecular Weight (kDa)
1.	Standard (BSA)	2.1	66.2
2.	<i>Bacillus sp.</i>	4.7	70.9
3.	<i>Yeast sp.</i>	5.6	71.8
4.	<i>Pseudomonas sp.</i>	4.2	70.4
5.	<i>Rhizobium sp.</i>	2.9	69.1
6.	<i>Azotobacter sp.</i>	2.3	68.5

**Table : 12** HPLC analysis of Iron siderophore in *Azotobacter sp.*

Ret. Time(min)

Results

Peak No.	Peak ID	Ret Time	Height	Area	Conc
1		2.815	80.714	742.200	70.6185
2		9.440	59.500	308.800	29.3815
<b>Total</b>			140.214	1051.000	100.000

**Table : 13** HPLC analysis of Catechol siderophore in *Bacillus sp.*

Peak No.	Peak ID	Results			
		Ret Time	Height	Area	Conc
1		1.265	149.000	792.500	0.6161
2		2.123	1060.859	23815.740	18.5146
3		2.465	1111.251	12206.581	9.4895
4		2.565	1027.220	8244.678	6.4095
5		2.782	570.152	8943.251	6.9526
6		3.973	177.036	1388.193	1.0792
7		4.190	6187.593	66193.109	51.4593
8		5.415	28.647	271.300	0.2109
9		5.882	330.143	5969.500	4.6408
10		7.515	35.308	378.300	0.2941
11		8.432	35.565	428.800	0.3334
<b>Total</b>			10712.773	128631.952	100.000

**Table : 14** HPLC analysis of Iron siderophore in *Yeast sp.*

Peak No.	Peak ID	Results			
		Ret Time	Height	Area	Conc
1		0.507	4556.787	55865.703	49.0847
2		1.065	1063.568	10123.097	8.8944
3		1.915	656.915	15603.900	13.7099
4		2.490	1502.367	8382.700	7.3652
5		2.765	126.231	399.500	0.3510
6		2.982	1188.813	18196.900	15.9882
7		5.248	412.968	5243.000	4.6066
<b>Total</b>			9507.648	113814.801	100.000

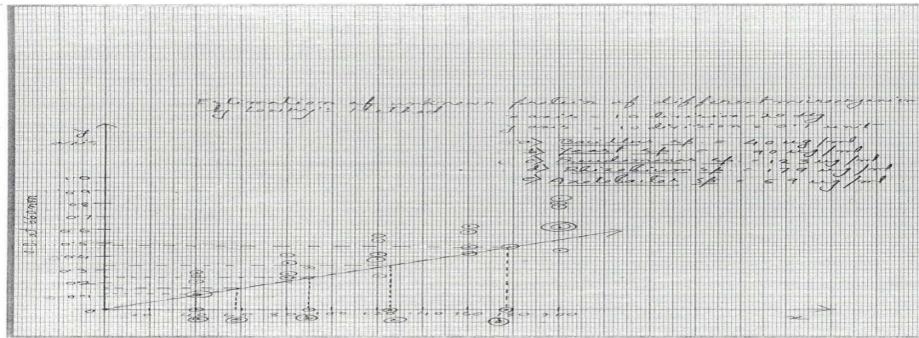
**Table : 15** HPLC analysis of Iron siderophore in *Pseudomonas sp.*

Peak No.	Peak ID	Results			
		Ret Time	Height	Area	Conc
1		0.090	42.733	167.300	0.0173
2		0.257	7132.000	34651.398	3.5731
3		1.473	159.483	1556.245	0.1605
4		1.965	4091.650	75550.250	7.7904
5		2.207	3352.850	30909.537	3.1873
6		2.507	22766.410	192504.172	19.8502
7		2.632	18866.893	143750.938	14.8230
8		2.865	69263.328	399587.469	41.2037
9		3.215	3504.480	52163.066	5.3788
10		3.698	1090.881	16196.483	1.6701
11		4.048	1495.034	13306.080	1.3721
12		5.532	40.062	331.200	0.0342
13		5.782	342.908	3620.000	0.3733
14		7.065	67.000	877.400	0.0905
15		8.015	79.783	1862.843	0.1921
16		8.582	101.087	2751.056	0.2837
<b>Total</b>			132396.582	969765.438	100.000

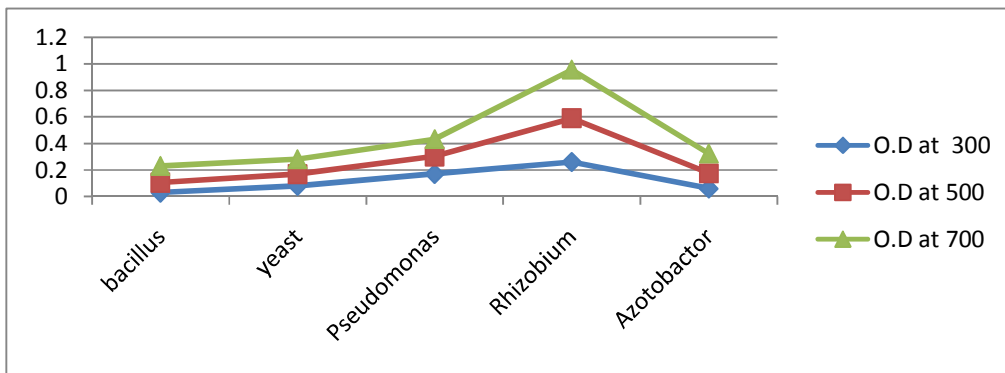
**Table : 16** HPLC analysis of Iron siderophore in *Rhizobium sp.*

Peak No.	Peak ID	Results			
		Ret Time	Height	Area	Conc
1		1.832	1370.416	31616.482	53.8896
2		2.482	3236.403	22120.217	37.7034
3		3.265	387.000	2230.800	3.8023
4		5.365	202.909	2701.500	4.6046
<b>Total</b>			5196.727	58668.999	100.000

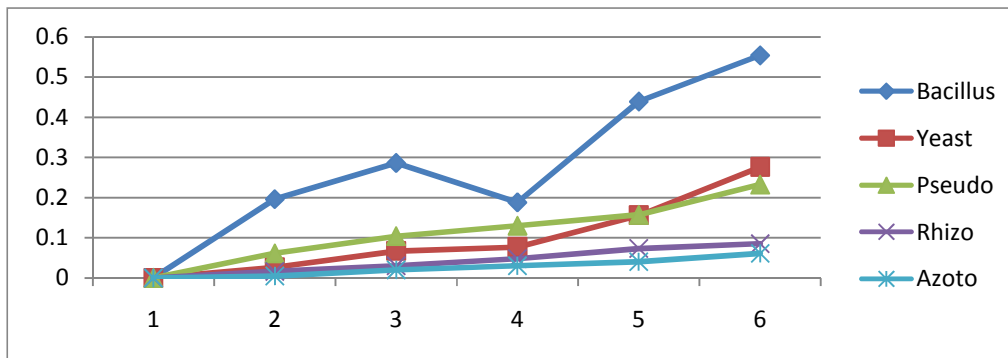
**Graph Analysis**



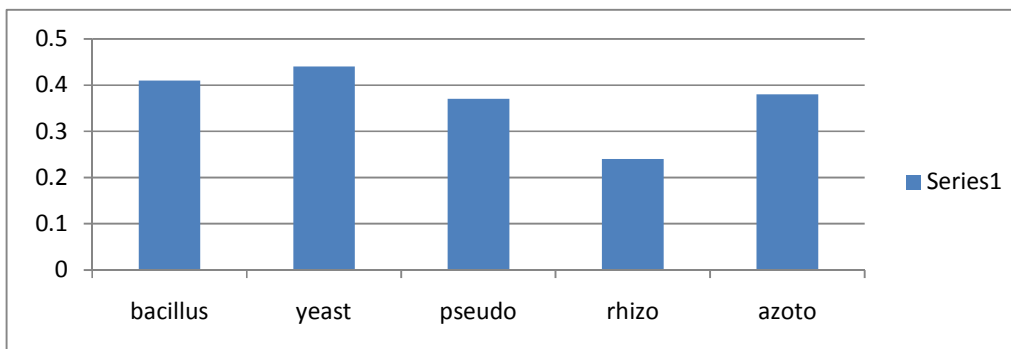
**Graph :1** Protein estimation of Lowry Method of different microorganism



**Graph : 2** Spectral Scan Analysis at different absorbance of different sample of microorganism

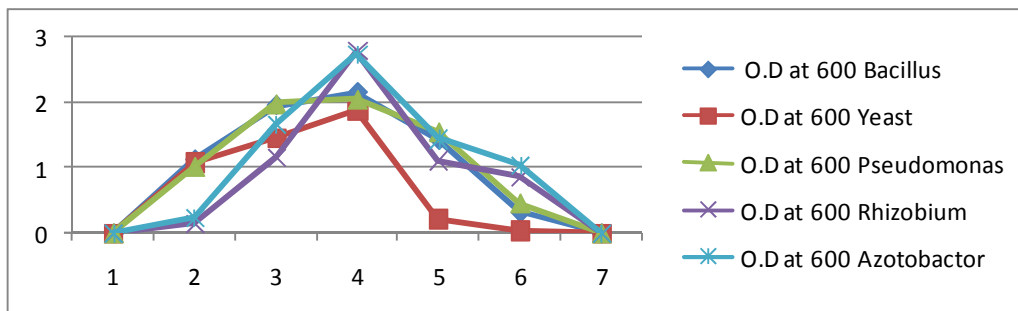


**Graph : 3** Iron-Perchlorate Assay for Detection of Hydroxamic Acids of different microorganism

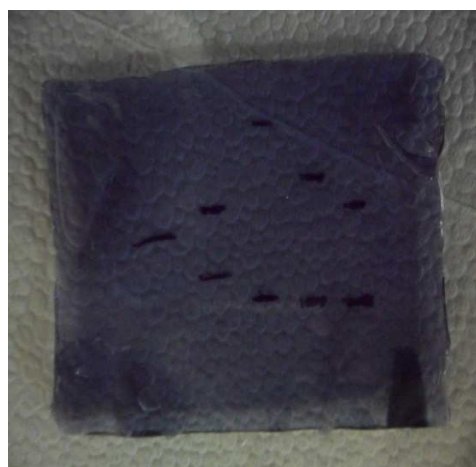


**Graph : 4** Thin Layer Chromatography (TLC) analysis of diiferent microorganism

Characterization of Protein involve in Nitrogen Fixation and Estimation of CO Factor

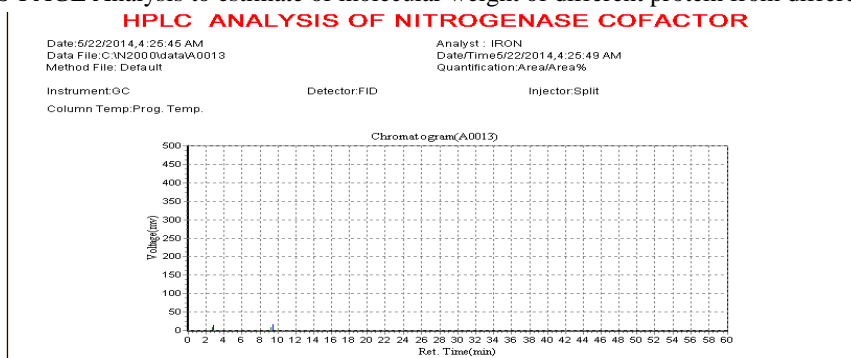


Graph : 5 Effect of Temperature on Siderophore Production of different microorganism

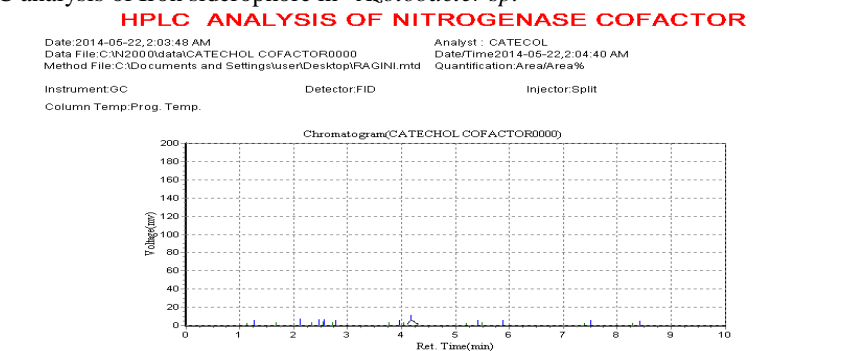


Standard (BSA) -	66.2 kDa
<i>Bacillus sp.</i> -	70.9 kDa
<i>Yeast sp.</i> -	71.8kDa
<i>Pseudomonas sp.</i> -	70.4 kDa

Graph 6 : SDS-PAGE Analysis to estimate of molecular weight of different protein from different microorganism



Graph : 7 HPLC analysis of Iron siderophore in *Azotobacter sp.*

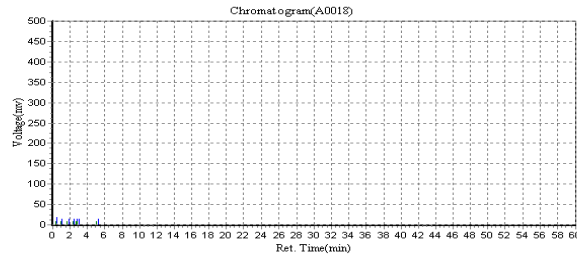


Graph : 8 HPLC analysis of Catechol siderophore in *Bacillus sp.*

Characterization of Protein involve in Nitrogen Fixation and Estimation of CO Factor

**HPLC ANALYSIS OF NITROGENASE COFACTOR**

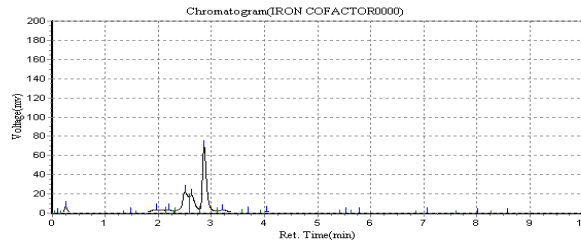
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 Quantification:Area/Area%  
 Detector:FID  
 Injector:Split



**Graph : 9** HPLC analysis of Iron siderophore in *Yeast sp.*

**HPLC ANALYSIS OF NITROGENASE COFACTOR**

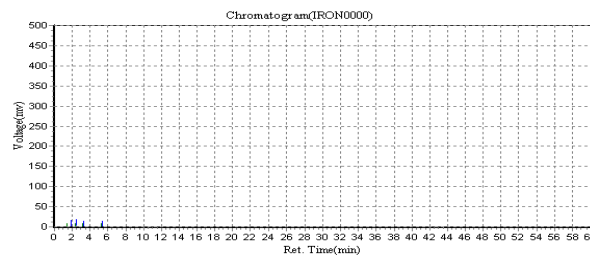
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 Detector:FID  
 Injector:Split



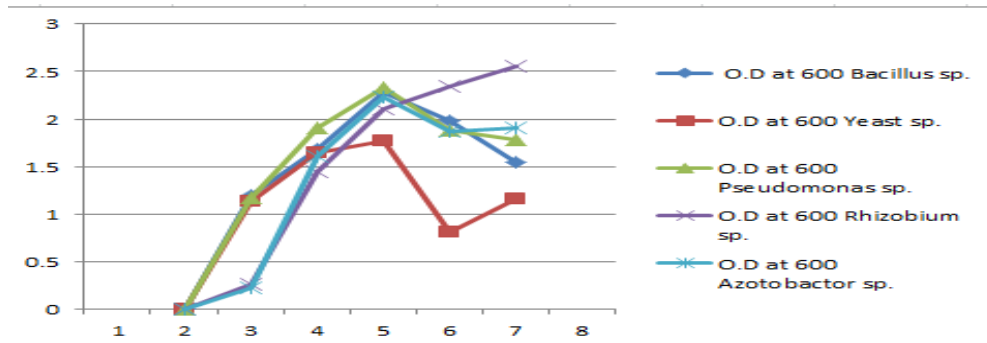
**Graph : 10** HPLC analysis of Iron siderophore in *Pseudomonas sp.*

**HPLC ANALYSIS OF NITROGENASE COFACTOR**

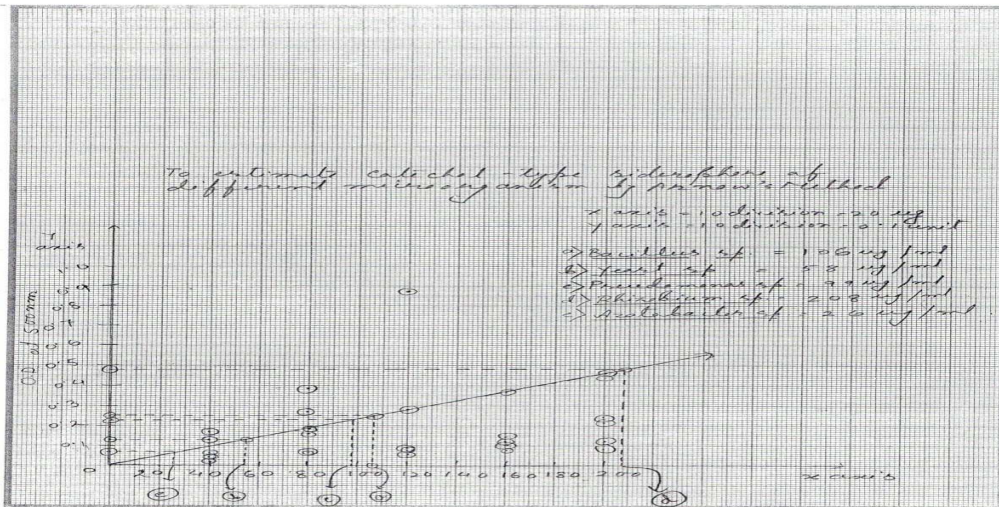
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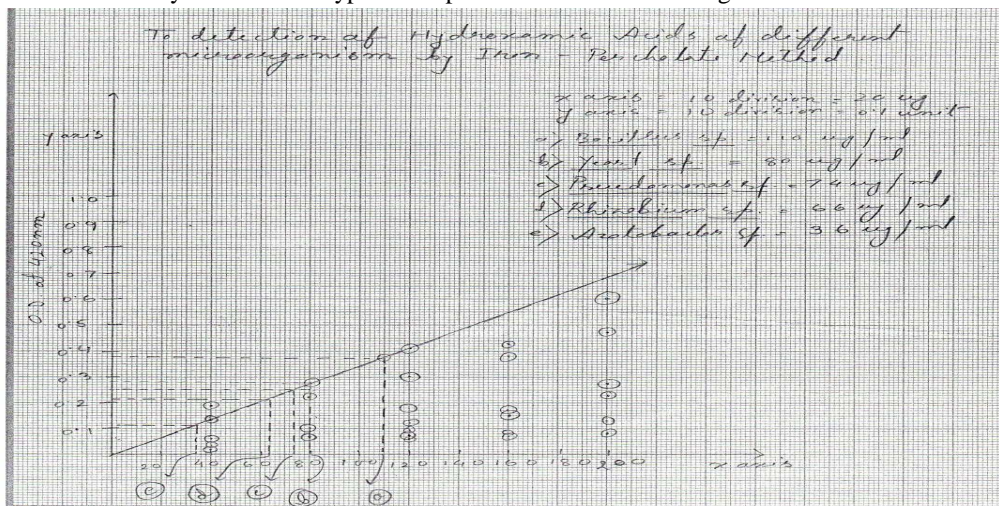
**Graph : 11**HPLC analysis of Iron siderophore in *Rhizobium sp.*



**Graph 12 :** Estimation of Siderophore Concentration of different microorganism



Graph 13 :Arrow's Assay for Catechol-type Siderophore of different microorganism



Graph 14 :Iron-Perchlorate Assay for Detection of Hydroxamic Acids of different microorganism

## DISCUSSION

Many gram-negative organisms are known to secrete siderophores under iron-limiting conditions, either in the environment or in an animal host in the case of pathogens. Siderophore-mediated iron transport has been studied in many organisms, but most of the research has been done using the iron transport systems of *E. coli*. It is known that many *Rhizobium sp.*, *Yeast sp.*, *Pseudomonas sp.*, *Azotobacter sp.*, *Bacillus sp.* produce siderophore. The agricultural importance of these organisms and the fact that linked to iron-

deficient soil affects many crops, there is a need to study the siderophore-mediated iron transport systems of this family in greater detail. The general mechanism of siderophore transport in these organisms, there are major differences in their transport system .

Through Lowry's Method ,Th different Type of protein can be estimated . From *Rhizobium sp.* ,*Yeast sp.* , *Pseudomonas sp.* , *Azotobacter sp.* ,*Bacillus sp.* produce different unknown protein which were accordingly present with different concentration .

Arrow's Assay is used for detection of Catechol-type Siderophore from different microorganism

sample . depending upon the values of O.D .Arnow's Assay for Catechol-type Siderophore of different microorganism estimated that unknown protein is present in **Rhizobium sp.** with high concentration and low concentration protein is present in **Yeast sp.**

Iron-Perchlorate Assay for Detection of Hydroxamic Acids of different microorganism detected that protein is present in **Bacillus sp** with high concentration and low concentration protein is present in **Azotobactorsp.**The other microorganism produce different concentration of siderophore protein which estimate the Hydroxamic acid presence in different microorganism .

The aim is to identify and estimate the siderophore-mediated iron transport system in these different microorganism and further study the mechanism of iron transport . This strains of the organisms are found to produce a hydroxamate-type siderophore under iron-deficient conditions.

This is determined through the iron-perchlorate assay and TLC . In Iron-Perchlorate Assay for Detection of HydroxamicAcids ,**Bacillus sp.** And **Pseudomonas sp.** gave good results and low in **Azotobactor sp.**

Growth conditions were then optimized in order to achieve maximum siderophore production. Conditions that are optimized were media components, iron concentration in the media , and temperature. It has long been known that members of the some microorganism family prefer mannitol or maltose as a carbon source . Variations on the original Fiss-glucose minimal medium included these sugars, various nitrogen sources, and variations on the four Fiss components added to the media. After trying several media combinations, it was determined that Fiss-glucose minimal media supplemented with 1% maltose and 0.1%  $(\text{NH}_4)_2\text{SO}_4$  had the greatest effect on hydroxamate production by these different microorganism . Cultures grown in this medium

produced almost four times more siderophore than the cultures grown in the original medium. Iron concentration of a medium is also extremely important to how much siderophore a culture will produce, as siderophore production is directly regulated by iron concentration . We have shown that hydroxamate production by this culture is best in **Bacillus sp.**, and that siderophore production decreases in **Azotobactor sp.**. This is consistent with the optimum iron concentration for siderophore production of other microorganism.

Siderophore production was measured at various temperature to determine the onset of siderophore production and the peak of production. This strain begins producing siderophore at 20<sup>0</sup>C and reaches its peak siderophore production at 30<sup>0</sup>C - 35<sup>0</sup>C.It is determined that the culture grew best in **Rhizobium sp. and Azotobactor sp.** and produced maximum siderophore at 30<sup>0</sup>C, which is typical for this family .**Yeast sp.** Production was less at high temperature It has been reported that a bacterial culture can often grow equally well at two temperatures, but siderophore production may be much less at the higher temperature . This is the case for **Bacillus**, as growth is actually a little better at 37<sup>0</sup>C than 24<sup>0</sup>C,although the culture grown at 24<sup>0</sup>C produces almost twice as much siderophore. After all growth conditions had been standardized for Microorganism,siderophore production increased approximately 3.8 times over that produced at the original conditions.

To estimate the molecular weight of different unknown protein of the different microorganism SDS PAGE is used . Through this technique The high molecular weight protien was present in **Bacillus sp., Yeast sp.** and low molecular weight protein was present in **Azotobactor sp.**

Batch cultures of the different microorganisms are grown to isolate enough siderophore for chemical analyses. Purification of the hydroxamate-type siderophore included passing acidified supernatant through a column, followed

by passing the concentrated siderophore through a hydrophobic column and finally through HPLC . Through HPLC analysis , there were more iron siderophore in *Azotobacter sp.* and *Rhizobium sp.* And less in *Pseudomonas sp.* And more production of catechol was in *Bacillus sp.* After each stage of purification, siderophore content is estimated to determine how much siderophore was lost at each step. We determine that less siderophore is lost when it is purified in an iron-complexed form, most likely because it is more stable in this form, which has also been noted for other hydroxamate-type siderophores like.

Following purification through the distinct spots characteristic of a hydroxamate-type siderophore were observed. Often siderophores are degraded during purification but can still bind ferric iron and will give a spot on TLC plates.. The two spots seen on the TLC plates were purified separately and will be referred to as compound 'L' and compound 'H'. Both were subjected to various chemical analyses to determine their chemical structure. The production of siderophores were observed in *Yeast sp.* , *Bacillus sp.* and *Azotobacter sp.*, but less production in *Rhizobium sp.* and were visually similar in appearance.

Spectral scans (300-700 nm) can indicate whether a dihydroxamate or a trihydroxamate-type siderophore is present . Spectral scans of the purified siderophore isolated from the different microorganism that both are dihydroxamate-type siderophores, but their absorbance maximum were different, leading us to initially believe these were structurally different compounds. In this method dihydroxamate or a trihydroxamate- type siderophore were more produced in *Rhizobium sp.* at different absorbance and less in *Bacillus sp.*

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