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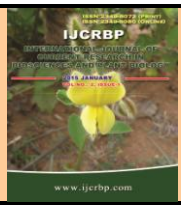

Original Research Article

Characterization of Protein Involved in Nitrogen Fixation and Estimation of Co-Factor

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A b s t r a c t

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, cathechol complex into the cytoplasm. Under iron-limiting conditions, *Bacillus species*, *Pseudomonas species*, yeast species, *Rhizobium* and *Azotobactor species* are known to produce siderophores. *Rhizobium sp.* produced catechol type, dihydroxamate or trihydroxamate siderophore; *Bacillus species* produced iron-perchlorate, dihydroxamate or a trihydroxamate type siderophore; yeast species produced catechol type siderophore; *Pseudomonas species* produced iron type siderophore; *Azotobactor species* produced Iron type siderophore. These siderophores have been purified and chemically characterized. Results indicate that these strains were produced different co-factors, which has not been described in member of different microorganism family.

K e y w o r d s

Azotobactor species

Co-factors

Nitrogen fixation

Siderophores

Yeast

Introduction

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 bacterial 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* (Brickman and McIntosh, 1992; Chakraborty et al., 2003; Furrer et al., 2002; Raymond et al., 2003; Sprencelet al., 2000).

Nitrogenase protein of different microorganisms is 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- cathechol cofactor, which is a part of the active site of nitrogenase. Nitrogenase is required for N2 fixation *in vivo* but not for N2 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* (Guiseppi and Fridovich, 1982; Archibald, 1983). 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 ribotidereductase, 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. Many nitrogen fixing microorganism are also known to produce siderophores and the present study is aimed to focus on the siderophore mediated iron-transport system of selective microorganisms.

Materials and methods

Protein estimation

Protein in different microbial samples was estimated by adopting Lowry's method.

Sonication process

The samples from each large production conical flask were collected and kept in to the small beaker and kept for 1 h in rotary 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 (Potassium Phosphate- 40mM, Sodium Phosphate -30Mm, Sodium Chloride-400mM, Tris-HCl- 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 h at 28°C.

Congo red dgar: The culture was 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 identification if a contaminant is present. This media has the following composition: (1% Mannitol, 0.05% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 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 were prepared from this medium. *Rhizobium* stain was incubated for 48 h at 28°C.

N free Mannitol agar media: The culture is maintained on N free Mannitol agar media (Mannitol =10 g, CaCO3 =5 g, K_2HPO_4 =0.5 g, $MgSO_4.7H_2O$ =0.2 g, NaCl= 0.2 g, ferric chloride (Trace), $MnSO_4.4H_2O$ (Trace), distilled water =1000 ml). This media was used for the large production of *Azotobacter species*. The pH of the media was brought to 7 before autoclaving and incubated for 48 h at 28ºC.

Pikovskaya's broth: The culture was maintained on N free Mannitol agar media [$Ca_3(PO_4)$ 2- 5.0 g, $(NH_4)_2SO_4$- 0.5 g , KCl -0.2 g, $MgSO_4$. $7H_2O$- 0.1 g, $MnSO_4$- Trace, $FeSO_4$- Trace, yeast extract- 0.5 g, distilled water-1000 ml). This media was used for the large production of phosphate solubilizing bacteria, *Bacillus sp.* and *Pseudomonas sp.* The pH of the media is brought to 7 before autoclaving and incubated for 48 hours at 28ºC.

Yeast extract broth: YEPD or yeast extract peptone dextrose, also often abbreviated as YPD, was a complete medium for yeast growth. The composition of the media was yeast extract, peptone, double-distilled water and glucose or dextrose and used as solid medium by including

agar. The YEPD typically contained 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 consisted of .3% (mass/volume) yeast extract, 1% peptone, 1% glucose/dextrose and 2% agar with the rest being distilled water. The broth version of YEPD typically contained 1% yeast extract, 2% peptone, 2% glucose/dextrose with 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 h or until OD_{600nm} = 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 was used as an iron-restricted media. Media was prepared by dissolving 5.0 g K_2HPO_4 and 5.0 g L-asparagine in 954 ml H_2O and pH was adjusted to 6.8. After autoclaving, 9.94 ml of each of the following solutions was added to the 954 ml sterile media: 50% glucose, 0.005% $ZnCl_2$, 0.001% $MnSO_4$, and 0.4% $MgS0_4$. To minimize the amount of contaminating iron, all media and components were prepared with deionized-distilled water and all glassware used for media storage and for growth of the culture were treated with concentrated HNO_3 and rinsed with double distilled H_2O.

Preparation of inoculum (seed culture): The microorganism stains are grown in different broth on a rotary shaker for 18-20 h prior to inoculating iron-restricted media.

Detection of siderophore production

Chrome Azurol S (CAS) assay: The CAS assay was the universal chemical assay for siderophore detection and is based on a siderophore's high affinity for ferric iron. CAS plates were 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.

Fe^{3+}-dye (blue) + siderophore Fe^{3+}-siderophore + dye (orange)

The different microorganisms were grown in Fiss-glucose minimal media containing no added iron, minimal media supplemented with 0.5 µM $FeSO_4$ (low iron), and minimal media supplemented with 20 µM $FeSO_4$ (high iron control). Cultures were grown for 24 h on a rotary shaker and the supernatant from each was collected by centrifugation at 13,500 rpm. Using a 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 were then incubated at room temperature. Depending on the culture, color formation may take 30 min to 5 h. 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 was detected with the CAS assay, then further assays were employed to determine what type of siderophore was 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 were collected as described previously and 0.5 ml supernatant is added to 2.5 ml 5 mMFe $(ClO_4)_3$ in 0.1 M $HClO_4$ 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 was measured at 480 nm, with uninoculated media mixed with reagent used as a blank.

Estimation of siderophore concentration: The iron-perchlorate assay was colorimetric and amount of siderophore produced by a culture could be visually estimated based on the intensity of the orange-red color formation. To better estimate the concentration of siderophore in a sample, $OD_{480\ nm}$ 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 was producing a siderophore that contained catechol groups, Arnow's method was 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 was 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 distilled H_2O), and 1 ml 1 M NaOH. These were allowed to incubate for 5 min for the reaction to fully occur. Absorbance was 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) remains colorless with the addition of reagents.

Siderophore detection using thin layer chromatography (TLC): Culture supernatant or concentrated samples of siderophore were spotted on Selecto Scientific 10 × 20 silica gel plates and spots were allowed to dry. The plates were then run in an n-butanol: acetic acid: dH_2O (12:3:5) solvent system until the solvent front reaches the top of the plate. Plates were then dried and sprayed with 0.1 M $FeCl_3$ 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 were separated on the basis of hydrophobicity using these plates.

Effect of temperature on siderophore production

The different microbial stains grown at 25-30°C might not be the optimal temperature for siderophore production, so, optimizing the temperature is essential step. 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, 308°C, 40°C and 50°C). After 24 h, 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 ml seed inoculum. The cultures were grown for 24 h at 28°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 30 min. 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 were prepared according to the Atkin's method (Atkin et al., 1970), except that because a concentrated sample is used, only 20-50 µl aliquot was used and the corresponding amount of double distilled H_2O 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 was used for gel filtration to separate compounds based on molecular weight. It was prepared by suspending 50 g LH-20 in methanol and de-aerating with shaking for around 20 min. The material was then packed into a 50 × 1.5 cm column (packed to the top of the column) and was 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 were collected and were tested for their siderophore content using TLC plates. Fractions positive for siderophores was combined in a 100 ml boiling flask and evaporated to dryness using a rotary evaporator. The dried sample was then re-dissolved in ~3 ml double distilled H_2O. If the solution is cloudy, it can be syringe-filtered (0.45 µm pore size) into a 15 ml polypropylene tube. The sample was estimated using the iron-perchlorate assay and stored at –20°C.

Preparation of samples for SDS-PAGE: Two ml seed cultures of the microorganisms were used to inoculate 50 ml of modified Fiss-glucose media with no added iron and 50 ml of Fiss-glucose media with 20 µM FeSo4 added. Cultures were grown for

24 h on a rotary shaker at 28°C. Cells were harvested by centrifugation at 7,000 rpm for 10 min 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 min and the supernatant was poured into ultracentrifuge tubes. These were centrifuged at 30,000 rpm (Beckman 50.2Ti rotor) for 90 min and the resulting membrane pellets were stored at –20°C.

SDS-PAGE analysis of samples: A 10% separating gel was prepared and ingredients were de-aerated for 10 min 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 min. A stacking gel was prepared and was also de-aerated 10 min 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 min 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 were prepared by adding an equal volume of 2× 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 2× 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 Coomasie Blue Stain for 30 min, 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 × 300 mm Novapak HR C_{18} hydrophobic column as the stationary phase and de-aerated, filtered double distilled H_2O 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 double distilled H_2O 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: 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 and 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 species, Yeast species, Pseudomonas species, Azotobactor species* and *Bacillus species* produced siderophores. 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.

From *Rhizobium species,* yeast, *Pseudomonas species, Azotobactor species* and *Bacillus species* produced different unknown proteins which were accordingly present with different concentration (Table 1). The results showed that unknown protein

was present in *Rhizobium* species with high concentration; low concentration protein was present in yeast (Table 2). Iron-perchlorate assay for detection of hydroxamic acids of different microorganisms detected that protein was present in *Bacillus species* with high concentration and low concentration protein was present in *Azotobactor species* (Fig. 1). The other microorganisms produced different concentration of siderophore protein.

Table 1. Protein estimation in different microorganisms. (Lowry's method).

S. No	O.D at 660 nm *Bacillus species*	O.D at 660 nm *Yeast species*	O.D at 660 nm *Pseudomonas species*	O.D. at 660 nm *Rhizobium species*	O.D at 660 nm *Azotobactor species*
1.	0.0	0.0	0.0	0.0	0.0
2.	0.125	0.242	0.215	0.287	0.255
3.	0.278	0.390	0.356	0.324	0.410
4.	0.420	0.465	0.372	0.520	0.551
5.	0.574	0.625	0.487	0.594	0.656
6.	0.615	0.782	0.612	0.840	0.804
7.	0.12	0.24	0.33	0.48	0.13

Table 2. Arnow's assay for catechol-type siderophores produced by different microorganisms.

S. No.	*Bacillus sp.* (O.D at 500 nm)	*Yeast sp.* (O.D at 500 nm)	*Pseudomoas sp.* (O.D at 500 nm)	*Rhizobium sp.* (O.D at 500 nm)	*Azotobactor sp.* (O.D at 500 nm)
1.	0.0	0.0	0.0	0.0	0.0
2.	0.14	0.01	0.03	0.16	0.04
3.	0.27	0.04	0.04	0.39	0.07
4.	0.37	0.07	0.08	0.87	0.06
5.	0.43	0.12	0.10	0.14	0.12
6.	0.59	0.21	0.22	0.08	0.13
7.	0.25	0.13	0.23	0.48	0.07

The strains of microorganisms used in the study were 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 hydroxamic acids, *Bacillus species* and *Pseudomonas species* showed appreciable results whereas, it was observed low in *Azotobactor species*.

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. The results are given in Figs. 2, 3, 4 and 5. 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% $(NH_4)_2SO_4$ 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. The present study has shown that hydroxamate production was best in *Bacillus species*, and that siderophore production decreases in *Azotobacter species*. This was consistent with the optimum iron concentration for siderophore production of other microorganism.

Siderophore production was measured at various temperatures to determine the onset of siderophore production and the peak of production. The strains started producing siderophores at 20ºC and reached peak siderophore production at 30ºC-35ºC (Fig. 2). It was determined that the cultures grew best were *Rhizobium species* and *Azotobacter species* which produced maximum siderophore at 30°C, which is typical for this family. The siderophore production

was less at high temperatures in yeast. 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 was the case for *Bacillus*, as growth was actually a little better at 37°C than 24°C, although the culture grown at 24°C produces almost twice as much siderophore. After all growth conditions had been standardized for microorganisms, 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 microorganisms, SDS PAGE was used. High molecular weight protein was present in *Bacillus species* and yeast and low molecular weight protein was present in *Azotobactor species*. Batch cultures of the different microorganisms were grown to isolate enough siderophore for chemical analyses. Purification of the hydroxamate-type siderophore included passing acidified supernatant through column, followed by passing the concentrated siderophore through a hydrophobic column and finally through HPLC. There were more iron siderophore in *Azotobactor species* and *Rhizobium species* and less in *Pseudomonas species*; more production of catechol was in *Bacillus species*. After each stage of purification, siderophore content was estimated to determine how much siderophore was lost at each step. Less siderophore was lost during purification 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.

Fig. 1: Iron-perchlorate assay for detection of hydroxamic acids of different microorganisms.

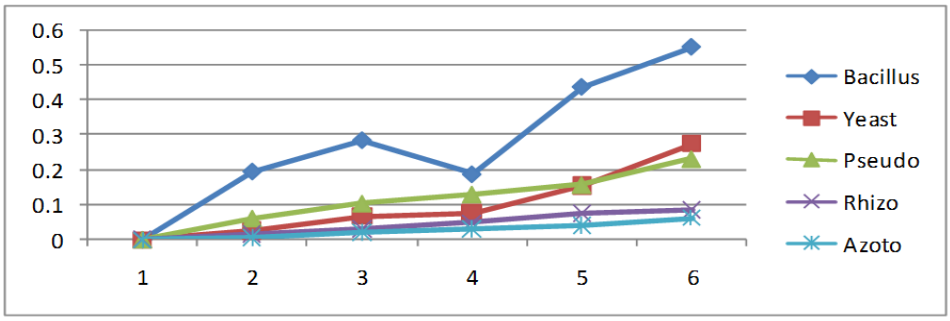
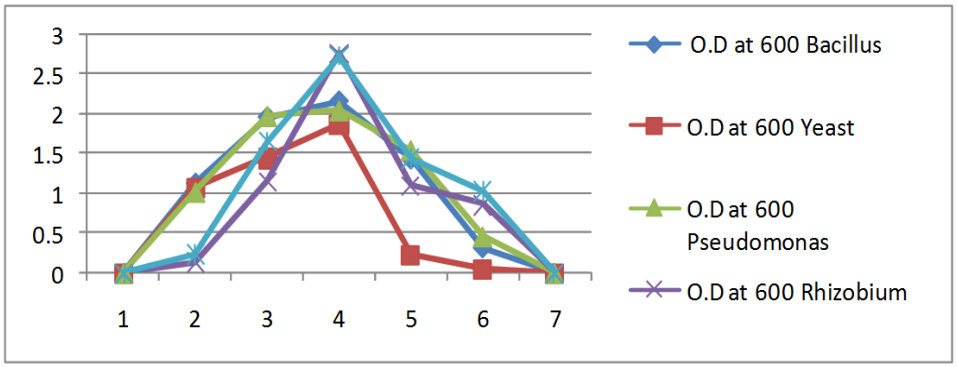


Fig. 2: Effect of temperature on siderophore production of different microorganisms.



Hydroxamate-type siderophores contain a carboxyl group attached to adjacent nitrogen, which chelates ferric iron. An example of this type is ferrichrome, a fungal siderophore produced by *Ustilago sphaerogena* (Emery, 1971). 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 and is produced by *Rhodococcus erythropolis* and 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 (Sprencelet al., 2000; Carran et al., 2001; Vellore, 2001; Furrer et al., 2002; Chakraborty et al., 2003; Raymond et al., 2003).

Fig. 3: Thin Layer Chromatography (TLC) analysis of different microorganisms.

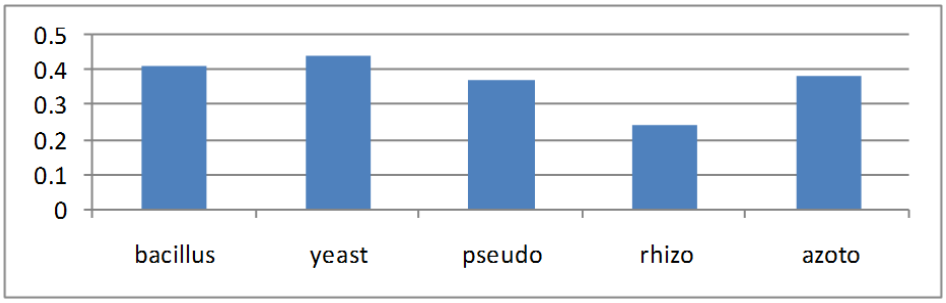


Fig. 4: Estimation of siderophore concentration of different microorganisms.

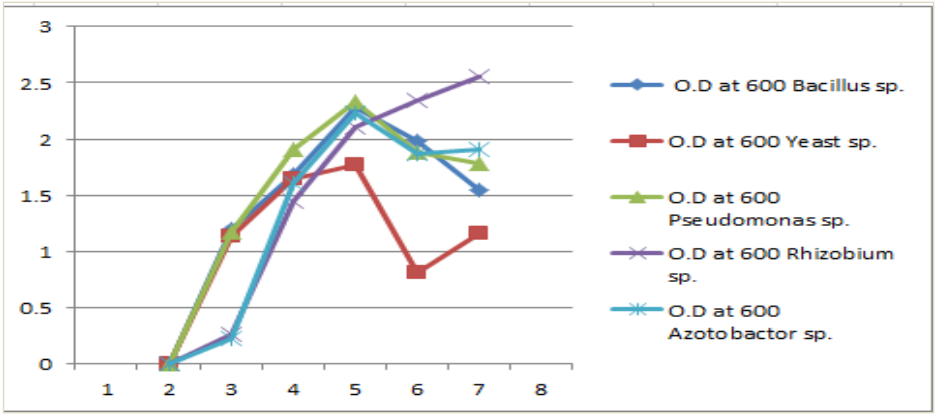
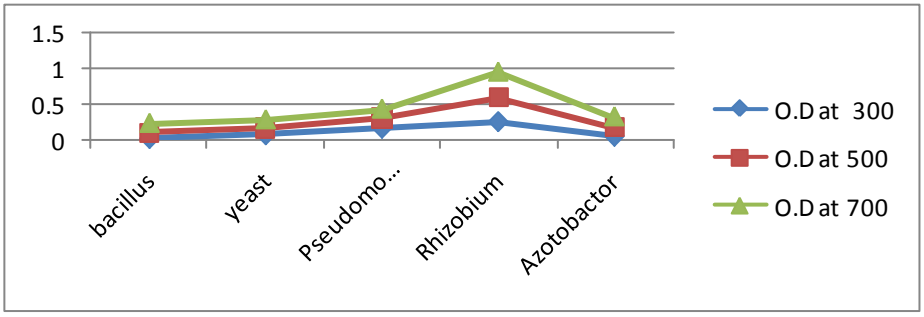


Fig.5: Spectral scan analysis at different absorbance of different samples of microorganisms.



Spectral scans (300-700 nm) indicate whether a dihydroxamate or a trihydroxamate- type siderophore is present. Spectral scans of the purified siderophore isolated from the different microorganisms in the present study showed that both were dihydroxamate-type siderophores, but

their absorbance maximum were different, leading us to initially believe these were structurally different compounds (Fig. 5). In this method dihydroxamate or a trihydroxamate- type siderophore were more produced in *Rhizobium species* at different absorbance and less in *Bacillus species*. Under iron-limiting conditions, *Bacillus species*, *Pseudomonas species*, yeast, *Rhizobium* and *Azotobactor species* produce siderophores. *Rhizobium species* produced catechol type, dihydroxamate or trihydroxamate siderophore; *Bacillus species* produced iron-perchlorate, dihydroxamate or a trihydroxamate type siderophore; Yeast produced catechol type siderophore; *Pseudomonas species* produced iron type siderophore; *Azotobactor species* produced iron type siderophore. These siderophores have been purified and chemically characterized. Results indicate that these strains were producing different co-factors, which has not been described in member of different microorganism family.

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