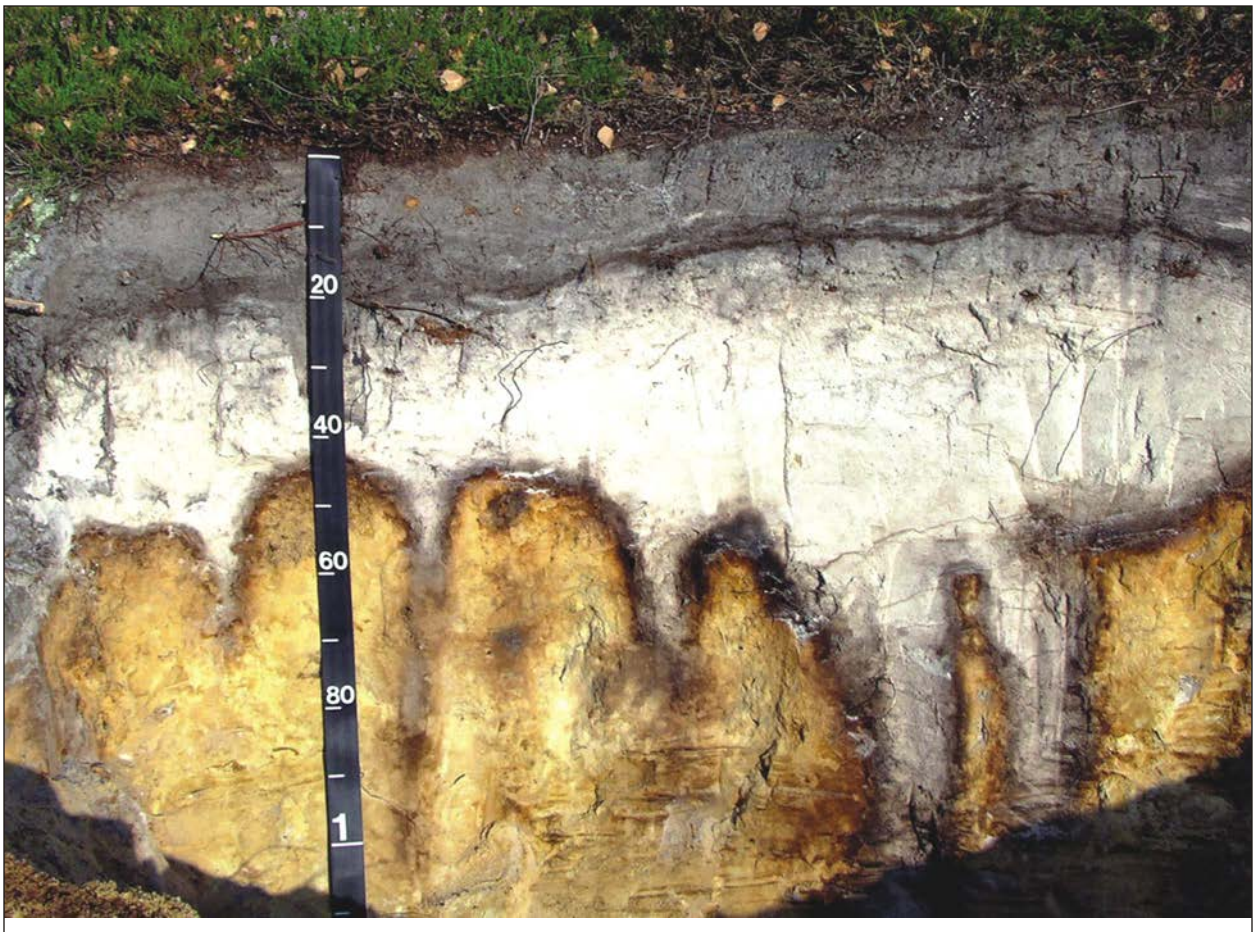

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Production of Hydroxamate-Type of Siderophores by *Rhizobium* strains from *Sesbania sesban* (L.) Merr.

M. Sridevi and K.V. Mallaiah
Department of Microbiology, Acharya Nagarjuna University,
Nagarjuna Nagar-522 510, Guntur, Andhra Pradesh, India

Abstract: Twenty six *Rhizobium* strains isolated from root nodules of *Sesbania sesban* (L.) Merr. were screened for their ability to produce siderophores. Among the twenty six strains, twenty strains produced hydroxamate-type of siderophores in Fiss-glucose mineral medium. The maximum amount of siderophore was produced by *Rhizobium* strain 22. The production of siderophore started at 8 h and reached maximum after 24 h. The addition of limited amount of iron in the media increased growth as well as siderophore production. Fiss-glucose mineral medium supplemented with 1% sucrose and 0.1% $(NH_4)_2SO_4$ increased siderophore production. UV-spectrophotometric scanning of purified siderophore showed absorption maxima in the range of 500-520 nm and indicating that it belongs to dihydroxamate-type of siderophore. Tryptophan and tyrosine were identified as conjugated amino acids of the purified siderophore. The outer membrane protein profiles of the *Rhizobium* strain 22 grown in high iron containing medium revealed an unique protein band of molecular mass 45 kDa.

Key words: Siderophore production, *Sesbania sesban*, *Rhizobium* species, catechol production, hydroxamate production, SDS-PAGE

INTRODUCTION

Plant-Growth-Promoting-Rhizobacteria (PGPR) are involved in plant growth promotion through the production of phytohormones, solubilization of insoluble phosphates and biocontrol of plant pathogens by the production of siderophores (Mandal *et al*., 2007). Rhizobia are now considered as PGPR. Many *Rhizobium* sp. are known for their production of siderophores, but only few of them have been structurally characterized. These include anthranilate, citrate, rhizobactin and other carboxylates, vicibactin as well as unidentified catechols and hydroxamates (Carson *et al*., 2000). Many other strains of rhizobia have not been examined for siderophore production. Basically, siderophores are considered to be of two types, viz., catechol and hydroxamic acids (Neilands, 1981). Further, hydroxamates are classified either into mono, di and trihydroxamates based on maximum absorption in UV-spectrophotometer scanning (Carson *et al*., 2000).

Sesbania sesban (L.) Merr. is an important green manure crop, widely cultivated in South India. Very little is known about siderophore synthesizing capacity of *Rhizobium* strains from this host. Hence, the present work was taken up to study the siderophore synthesizing capacity of 26 *Rhizobium* strains isolated from root nodules of *S. sesban* and also attempted to optimize the cultural and nutritional conditions of the *Rhizobium* strain, which produced maximum amount of siderophores.

MATERIALS AND METHODS

Microorganism and Growth Conditions

Twenty six *Rhizobium* strains were isolated from root nodules of *S. sesban*, collected from different regions of Andhra Pradesh, India. The identity of the strains as *Rhizobium* was confirmed by

Corresponding Author: K.V. Mallaiah, Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, AP 522510, India

Bergey's Manual of Systematic Bacteriology (Jordan, 1984) as well as plant-infection test (Vincent, 1970). The study was conducted in December 2006, in the Department of Microbiology, Acharya Nagarjuna University.

For siderophore production, Fiss-glucose mineral medium (K_2HPO_4, 5.0 g; L-asparagine, 5.0 g; glucose, 5.0 g; $ZnCl_2$, 0.05 g; $MnSO_4$, 0.01 g; $MgSO_4$. 7 H_2O, 4.0 g L^{-1}) was used (Vellore, 2001).

Atkin's Assay

Hydroxamate-type of siderophores was detected and estimated in the culture supernatant by ferric-perchlorate assay (Atkin *et al*., 1970).

Arnow's Assay

Catechol-type of siderophores was detected and estimated in culture supernatant by Arnow's assay (Arnow, 1937).

Optimization of Cultural Conditions

Siderophore Production as a Function of Time

The culture was grown in Fiss-glucose mineral medium with constant shaking (120 rpm) at 30±2°C for 36 h. Samples were withdrawn every 4 h intervals and measured for growth (Optical Density at 600 nm) and siderophore production (OD at 480 nm).

Influence of Media Components

Fiss-glucose mineral medium was modified to determine the siderophore production. Each of four media components was tested for its effect of varying their concentration in the minimal media. Also the minimal medium was supplemented with 1% mannitol or 1% sucrose as an alternate carbon sources. NH_4Cl or $(NH_4)_2SO_4$ (0.1%) was also added to the media as an extra nitrogen sources and the effects were evaluated. After 24 h incubation, growth and siderophore production were measured in each media type.

Influence of Iron

The effect of different concentrations (0.5-100 μM) of iron ($FeCl_3$. $6H_2O$) was added to Fiss-glucose mineral medium to determine growth and siderophore production.

Extraction and Purification of Siderophores

The culture was grown in large volumes for 24 h at 28°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7000 rpm for 30 min. The supernatant was then acidified to pH 2.0 with 6M HCl in order to make the siderophores less soluble in water.

The acidified supernatant was passed through XAD-2 column (30×5 cm) and eluted with methanol. Fractions were collected and tested on Thin Layer Chromatography (TLC) plates using solvent system n-butanol: acetic acid: distilled H_2O (12: 3: 5). The plates were developed with 0.1 M $FeCl_3$ in 0.1 N HCl.

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 (maximum absorption range-500-520) or trihydroxamate (maximum absorption range, 420-440 nm) as suggested by Jalal and Vander Helm (1991).

Amino Acid Analysis

The pure siderophore was hydrolyzed with acid (6N HCl) and alkali (6N NaOH). Amino acid standards were prepared using 20 different amino acids. Identification of the amino acids in the sample was done with TLC using solvent system (methanol:ammonium acetate, 60:40) and sprayed with ninhydrin (0.25% w/v) in acetone.

Analysis of Outer Membrane Receptor Proteins (OMRPs)

The culture was grown in presence and in absence of iron and whole cell proteins and membrane proteins were prepared (Filip *et al*., 1973). SDS-PAGE was carried out by the method described by Laemmli (1970).

RESULTS AND DISCUSSION

During the screening of 26 *Rhizobium* strains from *S. sesban* for siderophore production, it was observed that nine strains produced catechol-type of siderophores and 20 strains produced hydroxamate-type of siderophores. Because, maximum number of strains produced hydroxamate-type of siderophores, further studies were carried out for hydroxamate-type of siderophores. However quantitative studies showed that, only the *Rhizobium* strain 22 produced maximum amount of hydroxamates. The strain started siderophore production at 8-9 h post inoculation, with maximum production at 24 h (Fig. 1).

Fiss-glucose mineral media was used in the preliminary characterization of siderophores produced by *Rhizobium* isolates. However, this media needed to be optimized to achieve maximum siderophore production for purification. Each component added to the media stock was varied separately to determine its effect on siderophore production. No change in any of these components was kept the same in minimal media. A variety of media combinations were tried to optimize siderophore production. This include Fiss-glucose medium supplemented with 1% mannitol, Fiss-glucose supplemented with 1% sucrose, Fiss-glucose supplemented with 0.1% $(NH_4)_2SO_4$, 0.1% NH_4Cl, Fiss-glucose supplemented with 1% mannitol and 0.1% $(NH_4)_2SO_4$, Fiss glucose medium supplemented with 1% mannitol and 0.1% NH_4Cl, Fiss-glucose supplemented with 1% sucrose and 0.1% $(NH_4)_2SO_4$ and Fiss-glucose medium supplemented with 1% sucrose and 0.1 NH_4Cl. Addition of 1% sucrose and 0.1% $(NH_4)_2SO_4$ to the original Fiss-glucose medium greatly increased the amount of siderophore produced. Cultures grown in this media produced almost 4-fold more siderophore than that produced in Fiss-glucose minimal media (Fig. 2).

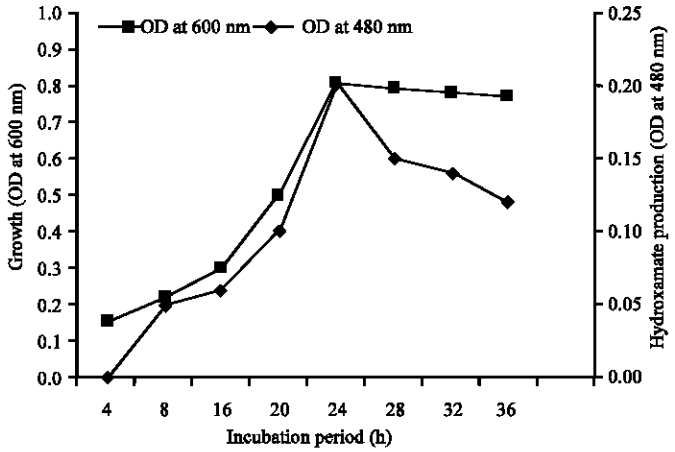

Fig. 1: Effect of Incubation period on growth and siderophore production by *Rhizobium* strain 22

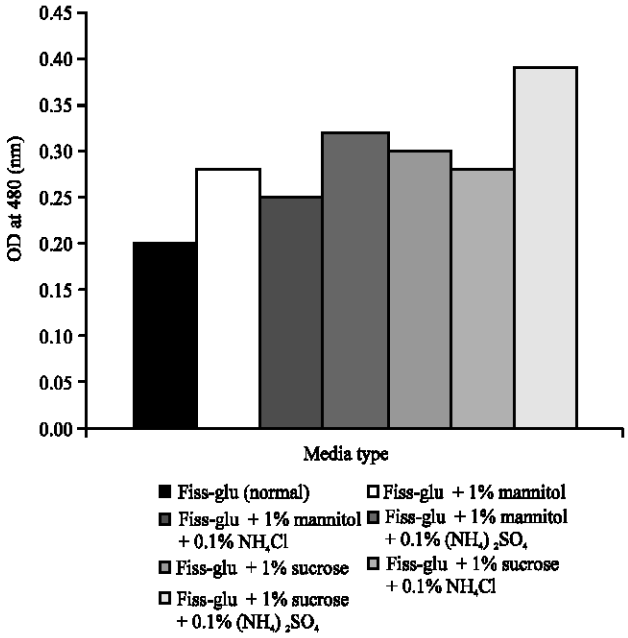

Fig. 2: Hydroxamate production measured with various media

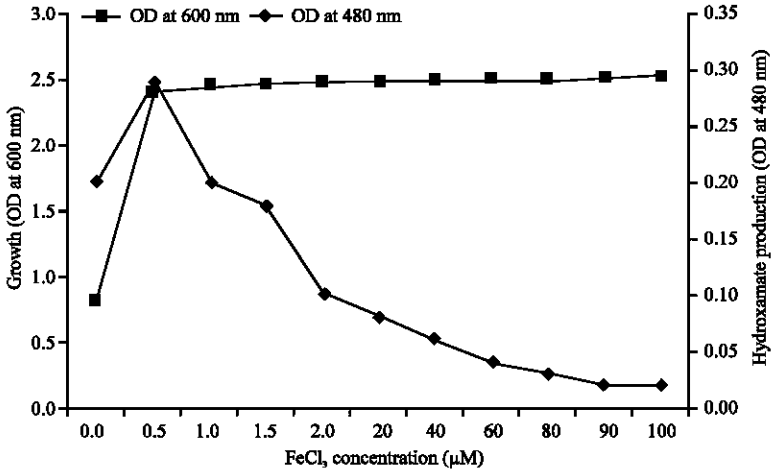

Fig. 3: Effect of iron concentration on growth and hydroxamate production by *Rhizobium* strain 22

The addition of limited amount of iron in the media can increase growth of the culture, which can lead to an increased production of siderophores. However, higher concentrations of iron in the media can completely suppress production of siderophores. In a medium where iron has been completely removed with 2′2-dipyridyl, cell growth can not been sustained and the culture can not produced any siderophores. Siderophore production is highest when a ferric iron concentration of 0.5 μM is added to the modified minimal medium (Fig. 3). At a concentration higher than this, siderophore production decreases. This is consistent with the optimum iron concentration for siderophore production of other rhizobia, in which studies have been found to be less than 1 μM (Carson *et al*., 2000).

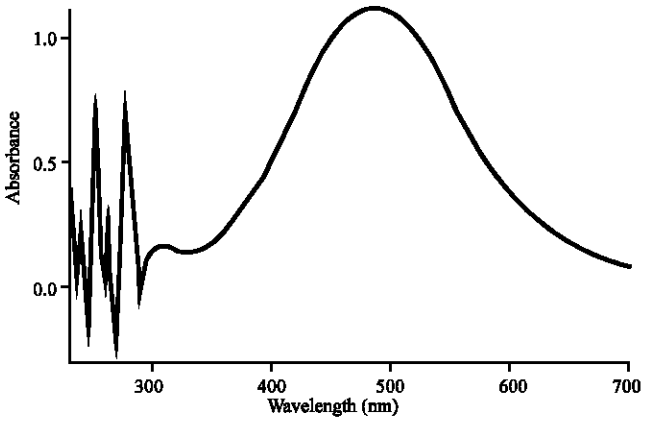

Fig. 4: UV-spectra of the purified compound isolated from *Rhizobium* strain 22

Table 1: Identification of amino acids in purified siderophore

Amino acid	Rf-value
Tryptophan	0.47
Tyrosine	0.32
Siderphore extract	
Spot 1	0.46
Spot 2	0.32

Once the growth conditions had been optimized, it was possible to produced large amount of siderophores by growing the culture in batch culture. Around 4-5 L of cultures were grown under optimized conditions and the acidified supernatant was partially purified through XAD-2 column chromatography. Different fractions were collected and the fraction that gave positive result with ferric-perchlorate assay were collected and subjected to TLC. A wine coloured spot was developed indicating hydroxamate-type of siderophores. The spots were scraped out and the compounds eluted from that were analyzed by UV-spectrophotometric scanning. Spectral scans (300-700 nm) of the purified siderophores showed the absorption maxima at 500 nm indicating dihydroxamate-type of siderophores (Fig. 4). The dihydroxamate-type of siderophores was also produced by *Sinorhizobium meliloti* (Carson *et al*., 2000).

When acid or alkali hydrolyzed siderophores were analyzed by TLC, it was found to contain tryptophan and tyrosine (Table 1). Siderophores are often conjugate of amino acids. Dihydroxamate-type of siderophores were first identified in *Enterobacter aerogenes*, is a conjugate of L-lysine (Gibson and McGrath, 1969).

SDS-PAGE analysis was performed on whole cell pellet and membrane pellet of culture grown in no added iron and high iron in medium to detect a possible Outer Membrane Receptor (OMR) proteins involved in siderophore transport. This protein should be expressed in the no added iron pellets and repressed in high iron conditions. The molecular weights for most described OMRPs in the range of 70-90 kDa, which is the region of focus in the gel (Fig. 5). The SDS-PAGE showed the presence of a band in this molecular weight range that is only present in the no added iron cultures, indicating that it is regulated by the amount of iron in the medium. This protein is likely involved in siderophore transport. This described for other iron regulated OMRPs in rhizobia (Reigh and O'connell, 1993). But, OMRPs of cultures grown in high iron media revealed the presence of single protein band of molecular mass of 45 kDa was observed. From this study, it may conclude that hydroxamate-type of siderophore production is most common among *Rhizobium* strains and strains vary in their production of siderophores. Moreover, the ecological advantage in the synthesis of siderophores may enable *Rhizobium* strains to compete with other species for iron uptake.

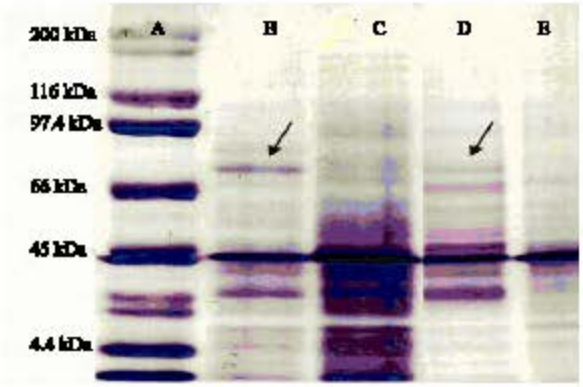

Fig. 5: SDS-PAGE analysis of whole and membrane pellets: (A) Molecular weight standard, (B) *Rhizobium* strain 22 grown in no added iron whole cell pellet, (C) *Rhizobium* strain 22 grown in high iron whole cell pellet, (D) *Rhizobium* strain 22 grown in no iron added membrane pellet and (E) *Rhizobium* strain 22 grown in high iron membrane pellet

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