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ISSN: 0975-7538

Research Article

Antifungal activity of catecholate type siderophore produced by *Bacillus* sp.

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ABSTRACT

Siderophore production by *Bacillus* sp. isolated from rhizospheric soil of a medicinally and agriculturally important plant alfalfa (*Medicago sativa* L.) and its antifungal activity on two common plant pathogens (*Fusarium oxysporum* and *Aspergillus niger*) has been described in present work. Siderophores during their production in succinate medium were detected by the Chrome Azurol S (CAS) assay. Arnow's assay was performed to reveal the catecholate type of siderophore. Extraction by ethyl acetate yielded 200 mg/L of siderophore. The purified siderophore was subjected to antifungal assay to check its bio-control potential and was found to inhibit the common plant pathogens *Fusarium oxysporum* and *Aspergillus niger*.

Keywords: *Bacillus* sp.; siderophore; catecholate; bio-control

INTRODUCTION

Living organisms require iron for its metabolic activities and growth. Iron is the fourth most abundant element of the earth crust (Ma, 2005); however, in aerobic soils, iron is mostly precipitated as hydroxides, oxyhydroxides, and oxides so that the amount of iron available for assimilation by living organisms is very low. To survive with a limited supply of iron in bacteria, cellular iron deficiency induces the synthesis of low-molecular weight siderophores, molecules with an extraordinarily high affinity for Fe⁺³ (K_a ranging from 10²³ to 10⁵²) as well as membrane receptors able to bind the Fe-siderophore complex, thereby allowing iron uptake by microorganisms (Neilands, 1981). Siderophores are low molecular weight metal chelating compounds secreted by microorganisms growing under low iron stress (Lankford and Byers, 1973).

Plant growth promoting rhizobacteria or PGPR are free living soil and rhizosphere bacteria that are beneficial to plants (Glick B R, 1995; Kloepper J. *et al.*, 1989) and can affect the growth of plant directly or indirectly. The direct mechanism of growth promotion include a) nitrogen fixation, b) phosphate solubilization, c) sequestering of iron through the mediation of siderophores and d) production of phytohormones such as gibberellins, auxins and cytokinins. The PGPR induces indirect effect by preventing the harmful

effects of one or more phytopathogenic organisms. One of the mechanism by which plant growth promoting bacteria prevent the phytopathogen is by the production and secretion of siderophores that chelates most of the available iron in the rhizosphere and preventing their proliferation because of lack of iron (O'Sullivan *et al.*, 1992). The production of siderophores occurs under conditions of iron-limitation. Such conditions are likely to prevail in the rhizosphere (Loper and Henkels, 1999). The phytopathogens also synthesize siderophores but have lower affinity for iron as compared to the siderophores produced by plant growth promoting rhizobacteria.

Alfalfa (*Medicago sativa* L) is an agriculturally important plant because of its large scale use as green manure and animal feed stock. It also finds its uses as many homeopathic medicines and preparations like alfalfa malt and alfalfa tonic are available for health benefits. Present work is focused on the production, purification and antifungal activity of purified siderophore from *Bacillus* sp. isolated from rhizospheric soil of alfalfa.

MATERIALS AND METHODS

Isolation of microorganism and identification

The siderophore producing organism was isolated on Chrome Azurol S (CAS) agar plate from rhizospheric soil of alfalfa plant of Anand Agriculture University, Anand, Gujarat. The bacterium was identified as *Bacillus* sp. using Biolog (MicrologTM 3 Software) based on their carbon utilization profile and on the basis of morphological and biochemical characteristics per Bergey's Manual of Systematic Bacteriology.

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Contact: +91-8866315393
Received on: 06-11-2013
Revised on: 28-11-2013
Accepted on: 09-12-2013

Siderophore Production

For siderophore production, succinic acid medium (SM) (pH 7.0) consisting of following components (g/L) was used: K₂HPO₄ 6, KH₂PO₄ 3, MgSO₄.7H₂O 0.2, [NH₄]₂SO₄ 1, and succinic acid 4 (Meyer, J M *et al.*, 1978). To remove the traces of iron in the medium, succinic acid medium was treated with equal volume of chloroform containing 1.5% 8-hydroxyquinoline and the traces of hydroxyquinoline was removed by washing thrice with chloroform to obtain deferrated medium. The medium was then autoclaved at 15 psi for 15 min.

Preparation of inoculum

A fixed aliquot was withdrawn from overnight grown culture in deferrated medium and its optical density was measured at 660 nm against media as blank. Appropriate aliquot of inoculum was added to all the experimental flasks to get initial O.D. of 1.

Siderophore assay

Siderophore units were calculated by the method described by Payne (1994) in which 0.5 ml of culture supernatant was mixed with 0.5 ml CAS reagent, and absorbance was measured at 630 nm against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in aliquot was calculated by using following formula:

$$\% \text{ Siderophore units (\% SU)} = \frac{Ar - As}{Ar} \times 100$$

Where, Ar = absorbance of reference (uninoculated SM +CAS reagent) and As = absorbance of sample (supernatant of production medium + CAS reagent) at 630 nm.

Qualitative analysis siderophore

Arnou's test (Arnou L E, 1937) and Czsaky's test (Csaky T Z, 1948) were performed to determine the catechol and hydroxamate type of siderophore produced by the organism respectively.

Production of siderophore

The culture was grown in deferrated medium with constant shaking (150 rpm) at 30°C. Samples were withdrawn at every 6 h intervals and measured for growth (O. D. at 660 nm), pH and siderophore production.

Extraction and purification of siderophore

The culture was grown in 1 L volume for 60 h at 30 °C on rotary shaker. The culture supernatant was collected by centrifugation at 8000 rpm for 30 min. The supernatant was then acidified to pH 2.0 with 6M HCl and extracted with 1/4 volume of ethyl acetate thrice. Ethyl acetate fractions were pooled together and concentrated by using rotary vacuum evaporator. Dried fractions were then re-suspended in deionised water and stored at -20°C.

Analysis of siderophore by thin layer chromatography

Purified siderophore was subjected to analysis by thin layer chromatography (5 x 10 cm silica gel 60 F₂₅₄, Merck, Germany) using the solvent system butanol : acetic acid : water (12:3:5) as mobile phase and the R_f values were compared with standard (2, 3 di-hydroxy benzoic acid).

Antifungal activity of purified siderophore

Purified siderophore was checked for their antifungal property against two common fungal plant pathogens *Fusarium oxysporum* and *Aspergillus niger* on potato dextrose agar containing (per liter) potato infusion 200 g, dextrose 20 g and agar 15 g. These fungi were spread on potato dextrose agar and 5 mm wells were prepared. The wells were filled with purified siderophore and incubated at 28°C ± 2°C for 6 – 7 days. After incubation the plates were observed for inhibition of fungal growth.

RESULTS AND DISCUSSION

A number of bacteria like *Pseudomonas*, *Bacillus*, *Enterobacter* (Wani P A *et al.*, 2007), *Rhizobium* (Ahemad M *et al.*, 2010), *Mesorhizobium* (Wani P A *et al.*, 2008), *Bradyrhizobium* (Wani P A *et al.*, 2007), *Brevibacillus* (Vivas A *et al.*, 2006) and *Proteus* (Rani A *et al.*, 2008) are known to have plant growth promoting activity. These bacteria participate in many important biological activities such as biological control of plant pathogens, nutrient cycling and increase in plant growth. Although *Medicago sativa* L (alfalfa, lucerne) is an agriculturally and medicinally important plant, less detailed studies are available on the rhizospheric plant growth promoting organisms of this plant. In present study an attempt was made to isolate siderophore producing microorganism from the rhizosphere of alfalfa as siderophores are involved directly (iron assimilation) and indirectly (biocontrol of plant pathogens) in plant growth promoting activity. The isolated bacterium was identified as *Bacillus* sp. using Biolog Microlog3 4.20.05 based on their carbon utilization profile and on the basis of morphological and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology (Jordan D C *et al.*, 1984).

Further it was studied for siderophore production in iron free modified succinic acid medium (SM). Upon inoculation into SM, siderophore production was checked every 6 h by CAS assay. As shown in Figure 1 maximum siderophore production was obtained at 60 h (84 % Siderophore Units). The siderophore production was accompanied with the growth of the organism and decreased with the decrease in growth after 60 h. An increase in pH with time which indicated that alkaline pH is not favorable for siderophore production. The increase in pH was in the range of 8 – 8.5.

The absorption maxima of cell free supernatant was recorded at 279 nm (Figure 2) which was compared with standard catechol solution. Csaky's test did not

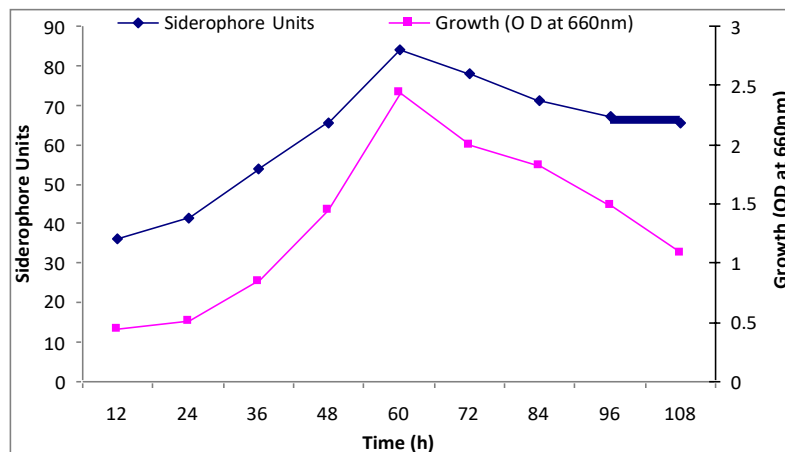


Figure 1: Growth and production of siderophore by *Bacillus sp.* in succinate medium

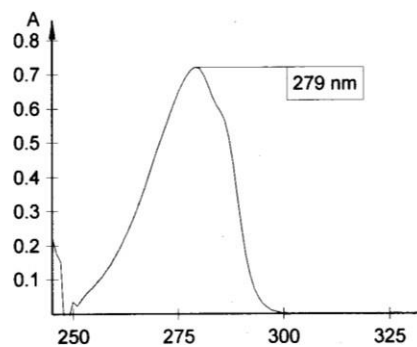


Figure 2: UV scan of the purified siderophore with a single peak at 279 nm indicating only one type of siderophore production by *Bacillus sp.*

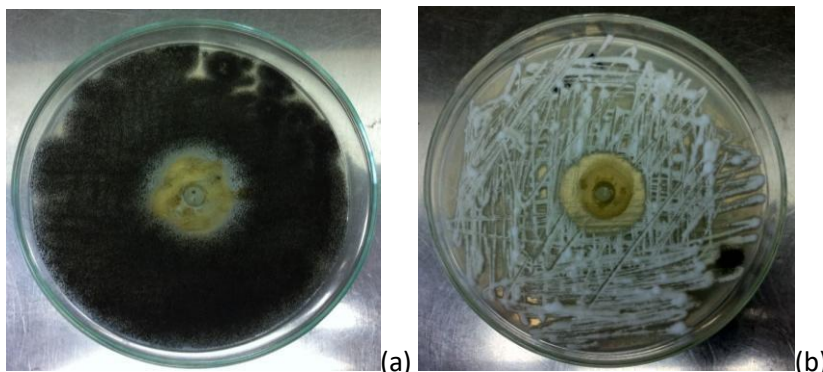


Figure 3: Purified siderophore from *Bacillus* suppress the growth of a) *Aspergillus niger* and b) *Fusarium oxysporium* on potato dextrose agar plate

show positive reaction and strong positive Arnow's assay revealed a catechol type of siderophore. Siderophore was extracted in ethyl acetate and purified in powder form. One liter of culture filtrate yielded 200 mg/L of pure siderophore upon drying by vacuum in a rota vapor (Buchi R-124, Switzerland). Both the standard catechol compound (2,3 dihydrobenzoic acid) and purified siderophore showed R_f value of 0.91 when subjected to analysis by thin layer chromatography using the solvent system butanol : acetic acid : water (12:3:5) as mobile phase (Sayyed R *et al.*, 2006). The purified siderophore was studied for its property to inhibit fungal pathogens and was found to suppress the growth of phytopathogens, *Fusarium oxysporium* and *Aspergillus niger* (Figure 3).

CONCLUSION

From our observations we conclude that *Bacillus sp.* isolated from rhizosphere of alfalfa produces a higher concentration of siderophores and has antifungal activity against certain plant deleterious fungi. Thus, this organism has the potential to act as an efficient siderophore based biocontrol agent. Further studies can help to exploit this isolate as a promising bio-inoculant in agriculture.

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