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Isolation of Iron Chelating Bacteria and Characterization of Catecholate type of Siderophore by HPLC Analysis

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Abstract

Iron is one of the essential elements for a proper plant development. Microbial siderophores provide iron to plants to enhance their growth when the bioavailability of Fe is low in the soil. Iron chelating bacteria isolated from rhizospheric soil of leguminous plants. Further, those isolates were screened for siderophore production on a chrome-azurol S agar plate and also confirmed by quantitative siderophore assay. Total four isolates were capable of producing siderophore production in the range 7mm to 21mm. Whereas, one isolate was capable of producing catecholate type of siderophore production. Extraction of siderophore by ethyl acetate and their TLC analysis was performed for the same. Rf value of extracted catecholate type of siderophore was 0.73. Further, catecholate type of siderophore characterized by HPLC analysis using methanol / phosphoric acid as solvent system. Retention time of extracted siderophore was 7.2 min. Identification of isolates through 16s rRNA was done and their phylogenetic tree analysis was performed by MEGA 4.1 software. Identified siderophore producing iron chelating bacteria MSP3 was *Bacillus licheniformis*.

Key words: Iron Chelating Bacteria, Catecholate, Siderophore, HPLC

Introduction

Iron bioavailability in soil habitats and plant surfaces has been reported to be low. Under iron restricted condition many bacteria produced iron chelating molecules called siderophore. Low solubility of external iron presents a problem for microorganisms living in an aerobic environment and having an absolute requirement for iron. To combat this low solubility, most microorganisms have high-affinity iron transport systems composed of secreted low-molecular weight carriers termed siderophores, which bind iron and a membrane transport system recognizing the iron-siderophore complex (Neilands, 1984). Siderophores are produced by microorganisms in response to iron derivation and are able to convert insoluble ferric hydroxide polymers into soluble chelates which are substrates for high affinity transport mechanisms. Siderophores produced by rhizospheric

bacteria may enhance plant growth by increasing the availability of Fe near the root or by inhibiting the colonization of roots by other harmful microbes. Aim of the present study was to isolate and identify iron chelating bacteria and characterize catecholate type of siderophore produce by the bacteria.

Material and Methods

Isolation of microbes were carried out on different media's viz., Nutrient agar medium, Yeast extract mannitol agar medium (YEM), King's B medium, etc.Isolated bacterial strains were screened for siderophore production on Chrome-azurol S (CAS) agar medium(Schwyn et al., 1987). It was further confirmed by quantitative siderophoreArnow'sassay for catecholate typea spectrophotometric method (Arnow., 1937). Extraction of siderophore of MSP3 isolate was done by ethyl acetate. The ethyl acetate extract was dried and reconstitute it in methanol. The thin layer chromatography was carried out using the solvent system n-butanol: acetic acid: water (60:15:25) (Xie et al., 2006). The spots which appeared on the plate was calculated as R_f Value. Catecholate type of siderophore further characterized by HPLC analysis using methanol / 0.1 % phosphoric acid (1:1) as solvent system (Berner et al., 1991).Identification of isolates through 16s rRNA was done by automated DNA sequencer at Chromous Biotech Pvt. Ltd., Bangalore, India. Molecular phylogenetic tree analysis was done with MEGA 4.1 software (Tamura et al., 2007).

Results and Discussion

Total 18 isolates were obtained from rhizospheric soil of leguminous plant. Out of 18 isolates 4 isolates were showed siderophore production. These bacteria were plated on Chrome-azurol S (CAS) agar medium and showed the formation of yellow colour zone which was detected as production of siderophore (Figure 1).

Figure 1: Zone of siderophore production by MSP3 isolate

Out of those four isolates MSP1, MSP2 and MSP4 were capable of hydroxymate type of siderophore production with zone size of 13 mm, 18 mm and 9 mm, respectively.Whereas, MSP3 was showed a positive result for catecholate type of siderophore production activity with zone size of 21 mm (Table 1) and hence was selected for further study.

Table 1: Types and zone size of Siderophore produce by the isolates

Isolates	Hydroxymate	Catecholate	Zone size (mm)
MSP1	+	-	13
MSP2	+	-	18
MSP3	-	+	21
MSP4	+	-	9

Catecholate type of siderophore was detected as following, take 1 ml of culture supernatant, add 1 ml of 0.5 N HCl, add 1 ml of 10% nitrite molybdate reagent which produce yellow colour then after 5 min add 1ml of 1 NNaOH which produce colour change from yellow to red colour was stable for half an hour and measured absorbance at 510 nm by spectrophotometer. Quantitative analysis of MSP3 culture supernatant was gave strong Arnow's positive test as maximum production observed 31 µg/mlat 72 h after that production was decreased (Chart 1).

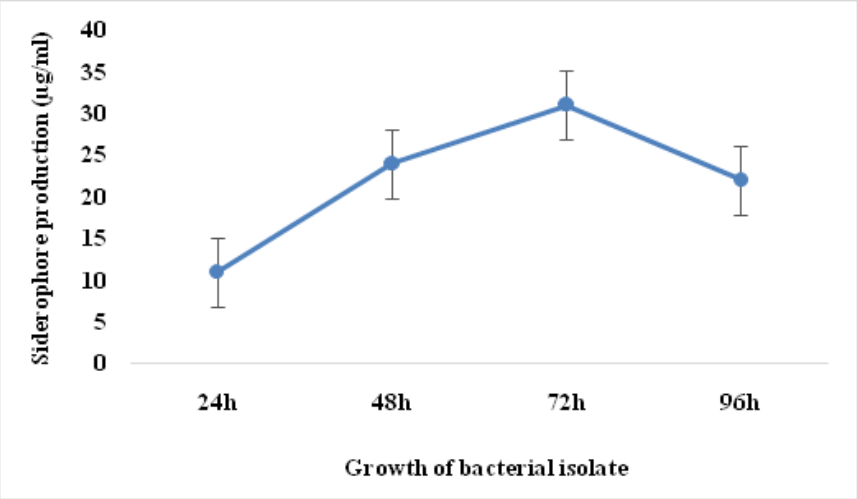

Chart 1: Quantitative analysis of siderophore production by MSP3 isolate

Purpose of TLC analysis of siderophore, extraction of siderophore from MSP3 was donethrough ethyl acetate followed by was dried with rotary evaporator and reconstitute it in methanol.TLC was carried out using n-butanol: acetic acid: water as solvent system.The dried plate was sprayed with 0.1 M $FeCl_3$in 0.1 N HClto detect iron binding compounds.Formation of dark grey spot indicates production of catecholate type of siderophore. Siderophore extract of MSP3 had one spot on TLC plate with Rf value of 0.73. Further characterization of catecholate was confirm through HPLC analysis using methanol / 0.1% phosphoric acid (1:1) as solvent system and flow

rate was 1 ml / min. The peak was seen in the sample at 5.3, 7.2 and 12.03 minutes which was very similar with Dihydroxybenzoic acid (DHBA) as standard for catecholate type of siderophore (Chart 2).Most catecholate type ofsiderophores,including bacillibactin and enterobactinuse 2,3-DHBA as a biosyntheticsubunitfor efficient chelation of the iron (Pfleger et al., 2008).Culture extracts of *Bacillus subtilis* was reported to produce bacillibactin as catecholate type of siderophore (Hotta et al., 2010).*Bacillus anthracis* and *Bacillus cereus* were also reported as excrete two catecholate siderophores petrobactin and bacillibactin (Wilson et al., 2006).

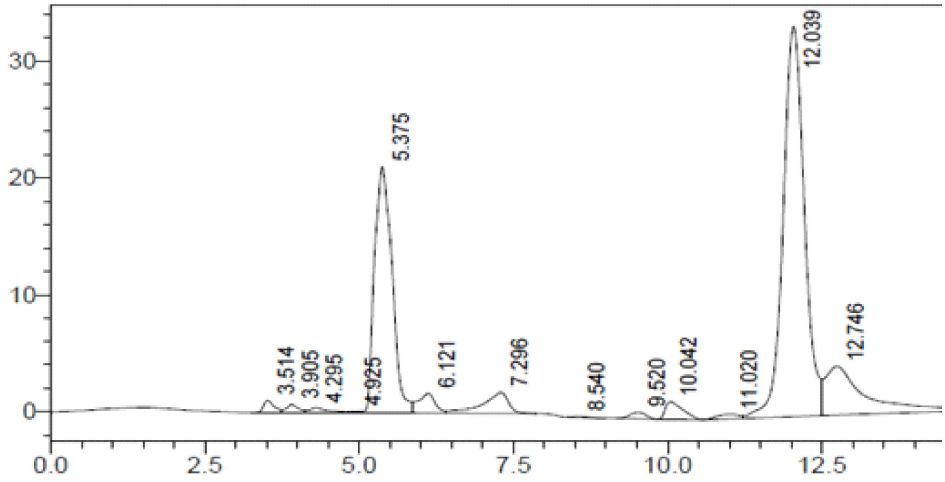

Chart 2: HPLC separation of catecholates from MSP3 isolate

The most ideal and suitable method for the identification, 16S rRNA gene sequencing identified strain MSP3 as *Bacillus licheniformis*.Neighbour-joining phylogenetic tree based on 16s rRNA gene sequencing constructed for the same isolate was showed comparative similarity to*Bacillus licheniformis*drawn by using MEGA 4.1 software(Chart 3).

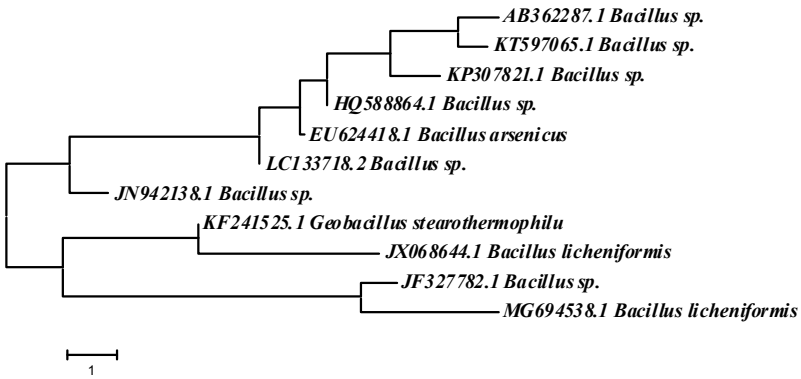

Chart 3: Phylogenetic tree analysis of MSP3 isolate

The *Bacillus licheniformis* siderophore producing strain was reported has potential of plant growth

promotion as multifunctional bioferilizer (Kayasth et al., 2013). *Bacillus licheniformis*was also reported as gram positive catecholate type of siderophore producing strain (Temirov et al., 2003; Maurya et al., 2015).

Conclusions

Present study suggested that *Bacillus licheniformis*is the most efficient iron chelating strain on the basis of their siderophore production activity and characterized as catecholate type of siderophore producing bacteria.This isolate may be useful for plant growth promotionof crop, grown under iron limiting conditions as siderophoresupplements.

References

Arnow., 1937.Colorimetric estimation of the components of 3, 4-hydroxyphenylalanine-tyrosine mixtures. Annual Review of Biochemistry, 50, 715-731.

Berner et al., 1991. Identification of enterobactin and linear dihydroxybenzoylserine compounds by HPLC and ion spray mass spectrometry (LC/MS and MS/MS). Biology of Metals 4: 113-118.

Hotta et al., 2010. Siderophore-mediated iron acquisition in *Bacillus anthracis* and related strains. Microbiology, 156, 1918-1925.

Kayasth et al., 2013.Exploring the potential of PGPR strain *Bacillus licheniformis* to be developed as multifunctional biofertilizer. Central European Journal of Experimental Biology, 2(1), 12-17.

Maurya et al., 2015.Extraction and Characterization of CatecholicSiderophores in *Bacillus* Sp. Isolated from the Mangrove Sediments. International Journal of Advanced Research in Chemical Sciences, 2(10), 42-46.

Neilands, 1984.Methodology of siderophores. In: Siderophores from Microorganisms and Plants. Structure and Bonding, 58.

Pfleger et al., 2008. Structural and functional analysis of AsbF: Origin of the stealth 3,4-dihydroxybenzoic acid subunit for petrobactin biosynthesis. PNAS, 105(44), 17133-17138.

Schwyn et al., 1987.Universal chemical assay for the detection and determination of siderophores. Annals of Biochemistry, 160, 47-56.

Tamura et al., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24(8), 1596-1599.

Temirov et al., 2003. A CatecholicSiderophore Produced by the Thermoresistant *Bacillus licheniformis* VK21 Strain. Russian Journal of Bioorganic Chemistry, 29(6), 597-604.

Wilson et al., 2006.Siderophores of *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis*. Biochemical and Biophysical Research Communications, 348, 320-325.

Xie et al., 2006. High-resolution analysis of catechol-type siderophores using polyamide thin layer chromatography. Journal of Microbiological Methods.

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