Research Article

Detection of siderophore production from different cultural variables by CAS-agar plate assay

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Abstract

Background: Siderophores are low molecular weight, Fe ion specific chelating agents which have been elaborated by fungi growing under low Fe stress. *Trichoderma* sp use the surrounding soil environment for nutrients primarily by hyphal extension. In this mode, *Trichoderma* sp secrete siderophores into the soil to bind tightly Fe that is subsequently bring back into the cell by specific uptake mechanism. **Objective:** This study was aimed to detection of siderophores production form different cultural variable by Chrome Azurol S (CAS)-agar plate assay in reference the rate of colour change of CAS medium as a function of time (in days) when microorganism were grown in CAS ager plate and also evaluate the effect of the inoculum type, iron concentration and pH. **Material and methods:** Fourteen strains of *Trichoderma* strains as MP1, MP2 ... and MP14 were selected for study. Out of theses the most *Trichoderma* MP1, *Trichoderma* MP3 and *Trichoderma* MP 9 were proved efficient among all strains, which exhibit higher reaction rate in CAS. Blue Agar assay in qualitative measurement of siderophore production. **Results and conclusion:** In the present study it was found that siderophore production in *Trichoderma* strains is an environment dependent phenomenon which is affect by different variable as pH and Fe concentration, as proved in *in vitro* conditions. They also exhibit their confirmed its best siderophore elaboration at 1mM concentration of fe and at pH 4.5.

Keywords: Siderophore, Chrome Azurol S, *Trichoderma* strains, *in-vitro*, iron

Introduction

Siderophores are essential for the survival and growth of fungi in the soil and environments (Guerinot, 1994). *Trichoderma* spp. is a group of beneficial microorganisms in rhizosphere which have been considered as plant growth promoter due to nutrient uptake as well as plant disease manager because of phenomenal antimicrobial activity. *Trichoderma* spp. functions as bio-control agent by stingy the pathogen from Fe nutrition, thus resultant in improved yield of any beneficial crop (O' Sullivan and O'Gara, 1992). In soil, plant and roots relationship normally coexist with fungi and bacteria which may generate siderophores production capable of sequestering the available soluble iron. Plant root might be capable of taking up ions complexes of siderophore and using these as sources of iron. Fe requirement by microorganisms and plants have evolve specific mechanism to chelate insoluble iron through the release of siderophores, which are low molecular weight with high affinity and specific for iron and plant both will consume Fe-siderophore complexes (Weizhen Qi and Lei Zhao, 2012). About 500 siderophores are reported from selected microorganisms till date. In general, they are classified as hydroxamates, catecholates, salicylates and carboxylates and more recently with new group polycarboxylates (Kannahi, 2014; Renshaw et al., 2002).

A verity of different technique has been developed to detect siderophores, including use of mass spectrometry (Dimkpa et al., 2008a, b). However, the most universal detection method for siderophore production is the chrome azurol S (Carrillo-Castañeda et al*.,* 2005) assay based on functional or biological properties to the color change around the microbial colony of CAS-Fe complex from blue to orange after chelation of the bound iron by siderophores (Schwyn and Neilands, 1987). The technique is based on competition for iron between the ferric complex of an indicator dye (Carrillo et al*.,* 2005) and a siderophore produced by any microorganisms. The CAS technique can be applied as a liquid test. Alternatively, the dye can be integrated in solid

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medium for detection of siderophore. The CAS technique in liquid supernatants has been quantitative. However, it is not possible to quantify the CAS reaction on the solid medium (Raaska et al., 1993). CAS-agar plate assay use the term strong or light and symbols (++) or (+) to evaluate the diameter of coloured halo around the any microbial gowning colony (Manninen and Mattila-Sandholm, 1994).

This study was to detection of siderophore production from different cultural variables by CAS agar plate assay in reference the rate of colour change of CAS medium (in millimetres) as a function of time (in days) when microorganisms were grown in CAS agar plates and also evaluate the effect of the inoculum type, iron concentration and pH

Materials and methods

Fungal species

In the present paper, around 14 strains of *Trichoderma* as MP1, MP2 ... and MP14 were used and strains were maintained on 2 per cent (w/v) Potato Dextrose Ager slants at 4°C.

Determination of siderophore production in *Trichoderma* Strains

Ability of *Trichoderma* strains as MP1, MP2 ... and MP14 to produce iron-binding compounds of siderophore were detected by universal CAS assay in solid medium as per Schwyn and Neilands (1987).

Preparation of the CAS (Chrome Azurol S) Blue agar

One litre of CAS blue agar was prepared using 60.5 mg CAS dissolved in 50 ml distilled water and mixed with 10 ml iron (III) solution (1 mM Fe Cl_3.6 H_2O , 10 mM HCI). Under stirring, this solution was slowly added to 72.9 mg H.D.T.M.A. (Hexa decyl tri methyl-ammonium bromide) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved for 20 min and also autoclaved a mixture of 750 ml water, 15 g agar, 30.24 g Pipes and 12 g of a solution of 50% (w/w) NaOH to raise the pH to the pKa of Pipes (6.8). The dye solution was finally poured along the glass wall and agitated with enough care to avoid foaming. Petri dishes (9.5 cm in diameter) were prepared with 30 ml of appropriate medium for culturing *Trichoderma* strains as MP1, MP2 ... and MP14. After solidification, the medium was cut into halves, one of which was replaced by CAS. blue agar (15 ml). The halves containing culture medium were inoculated with 5 mm discs of 7th day's old culture of *Trichoderma* strains grown on PDA medium. The inoculum was placed as far as possible, from the borderline between the two media. The plates were incubated at growth temperature (27 ± 2°C) of *Trichoderma* strains as MP1, MP2 ... and MP14. The CAS reaction rate was determined by measuring the intensity of color-change in the CAS-blue agar, starting from the borderline between the two media. The CAS-agar colour changed from blue to purple or dark purplish- red (magenta). The control plates of CAS-agar uninoculated were incubated under the same conditions CAS as described above and no color change in the CAS-blue agar was observed, even after long incubation periods (10-15 days). The experiment was carried out in triplicates.

Determination of the pH medium on siderophore production by *Trichoderma* strains

On the above objective, PDA medium was buffered with sodium acetate 0.1 M at pH 4.5 and 5.5. And the plates were inoculated and incubated as described above. Microbial growth of species and CAS reaction rate were examined day by day and compared with the non-buffered PDA medium (pH 6.5).

Determination of the iron medium on siderophore production by *Trichoderma* strains

On the above objective, PDA medium were prepared with iron (1mM to 4mM) .The plates were inoculated with 5 mm diameter culture discs of 14 different *Trichoderma* strains and incubated at 28±2°C for 7 days. Microbial growths of species and CAS reaction were examined day by day.

Statistical Analysis

All the experiments were carried out in triplicates. Data were expressed as means of five replicates and their Statistical analysis by Microsoft excel 2007. Statistical analysis of data were considered Significant at $P<0.05$. ANOVA was made based on the diameter of the radial growth among *Trichoderma* strains.

Results and discussion

Siderophore type compounds were produced by the different microorganisms and diffused through the CAS blue agar producing a colour change from blue to purple or pink. Our Results were qualitatively described in terms of colour change of CAS reaction as blue to purple or pink was observed (Table 1). All Strains of *Trichoderma* grew rapidly in the appropriate plate half containing PDA medium but did not grow at all in the plate half containing CAS blue agar. Colour change was observed in the CAS-blue agar when *Trichoderma* strains started to cover half of the Petri plate. siderophores' production by *Trichoderma* strains in solid medium was evaluated as the CAS reaction rate and expressed in mm day by day to measuring radial diameter. Results indicate, *Trichoderma* spp. had shown good production of siderophores but the potential of each strain to produce siderophores in the same conditions was different. *Trichoderma* MP1, MP3 and MP9 had shown fastest reaction of colour change was observed whereas other showed comparatively slow reaction indicated the

moderate reaction rate. Results concluded as *Trichoderma* MP1, MP3 and MP9 has given the fastest reaction of colour change which means *Trichoderma* MP1, MP3 and MP9 are a good type of siderophores producer instead of other strains only recognized as moderate siderophores producer. In this manner, we can correlate the production of siderophores in solid medium evaluated as CAS-reaction rate (mm/day), which can provide us the intensity of siderophores production in respective of test fungus.

Table 1. Efficacy of *Trichoderma* strains for siderophore's production

BCAs (strains)	CAS reaction	CAS blue Ager colour change
Trichoderma MP 1	+++	Blue to purple to dark mangeta
Trichoderma MP 2	+	Blue to purple to light mangeta
Trichoderma MP 3	+++	Blue to purple to dark mangeta
Trichoderma MP 4	++	Blue to purple to light mangeta
Trichoderma MP 5	+	Blue to purple to light mangeta
Trichoderma MP 6	-	Blue
Trichoderma MP 7	++	Blue to purple to light mangeta
Trichoderma MP 8	-	Blue
Trichoderma MP 9	+++	Blue to purple to dark mangeta
Trichoderma MP 10	+	Blue to purple to light mangeta
Trichoderma MP 11	--	Blue
Trichoderma MP 12	--	Blue
Trichoderma MP 13	-	Blue
Trichoderma MP 14	-	Blue
Control	-	Blue

Each value is mean of three replicates and CAS reaction for: -, No reaction; +, Slow reaction; ++, Moderate reaction; +++, Fast reaction

pH in siderophore production

Effect of different pH on the siderophore's production both in buffered PDA medium (pH 5.5 and 4.5) and non-buffered PDA medium (pH 6.5) are evaluated. Radial diameter of test fungi were measured day by day (Figugre 1). The most significant influences were observed with *Trichoderma* MP1, MP3 and MP9 during CAS-reaction. The reaction rate was altered because of different pH conditions. Maximum radial growth was noticed in *Trichoderma* strains at pH 4.5 whereas a notable decrease was found at pH 5.5.

Iron in siderophore production

Siderophore's production was affect by Fe concentration in growth medium. As the iron concentration was increased, siderophores's production was repressed in medium. So that it was fully clear that increasing iron concentration has stopped the siderophores's concentration by decreasing radial growth of *Trichoderma* strains. According to our data 1mM concentration was proved to be most suitable dose of iron concentration which can enhance siderophore reaction by *Trichoderma* strains. We have proved in our data that, *Trichoderma* MP1, MP3 and MP9 (Figure 1 and 2) which was observed by evaluating the radial growth of fungus in mm/day in *invitro* condition. Same methodology was followed in respect of iron requirement of micro-organism, In iron deficient condition, *Trichoderma* MP1, MP3 and MP9 have demonstrated higher reaction rate at 1mM iron concentration which was lowest among all provided iron dose (Figure 2) that was also proved in context of *Pseudomonas, N. crassa, F. dimerum* and *Mucor* sp, siderophores production was repressed as at 3 µm Fe (III).

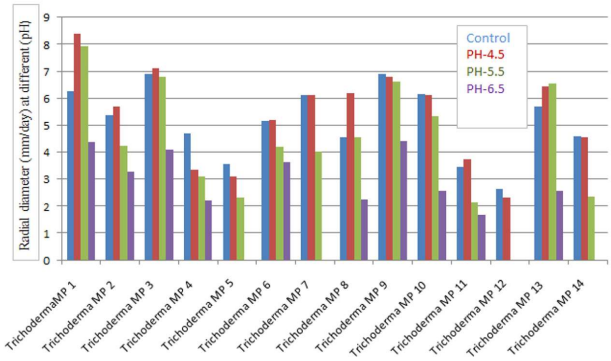

Figure 1. Effect of the pH medium on the siderophore production by *Trichoderma* strains growing on CAS-agar plates modified

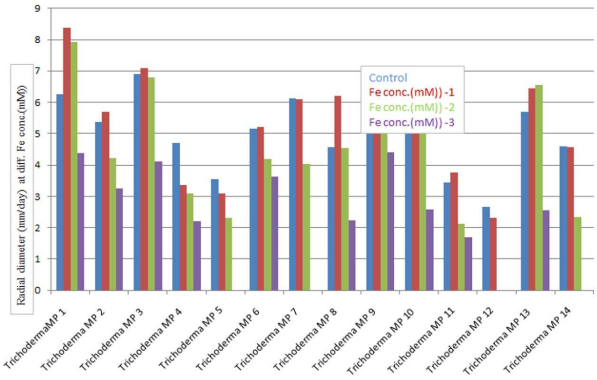

Figure 2. Effect of different iron concentration on siderophore production by *Trichoderma* strains growing on CAS-agar plates modified

Conclusion

The technique used by us for express the CAS -reaction rate (mm per day) can be measured as a quantitative measurement of siderophore's production by different microorganisms in solid medium, on the other hand as mentioned in literature that it's not possible on the agar plate assay (Raaska et al., 1993). Besides, this technique allowed to study that the effect of different variables (e.g. iron concentration, inoculum-type and pH of the medium) on the siderophore's production by *Trichoderma* fungi (Srivastava

et al., 2013). It is also known that the bio-synthesis of siderophores is regulated by iron content of the medium (Neilands, 1993). The require of regulations for towering iron concentrations as the one observed for *A. niger* suggested the presence of a non-typical siderophore which also reacted with CAS reagent. As we know *A. niger* is good producer of citric and oxalic acids (Roehr et al., 1992), probably these organic acids could be reacting with CAS at high iron concentrations. Siderophores also have been shown to be valuable source as a drug when administered to patients combating iron-overload diseases. Iron-overload diseases, known as thalassemia, is major problem in the world, affecting hundreds of thousands of people each year (Brianne, 2004).

The ecological and environmental advantage of these experiments is that *Trichoderma* sp is constantly has been considered good bio-control. It should be inoculated in soil at certain pH condition would be beneficial for soil environment as well as iron in an aerated environment. It is exists in the ferric form and highly insoluble in neutral or alkaline soil (Shenker et al., 1995), So, *Trichoderma* MP1, MP3 and MP9 would be able to utilize a high affinity Fe transport system, would produce proficient siderophores which would be able to battle iron deficient situation and also with dangerous pathogens.

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