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Full Length Research Paper

A simple double-layered chrome azurol S agar (SD-CASA) plate assay to optimize the production of siderophores by a potential biocontrol agent *Bacillus*

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Siderophores, a high-affinity chelating agents for ferric iron, is one of the mechanisms by which biocontrol agents' act in inhibiting the growth of phytopathogens. *Bacillus subtilis* QM3, a siderophore producer, is a potential biocontrol agent. In this study, a simple double-layered chrome azurol S agar (SD-CASA) plate assay was developed. We further studied optimal conditions for detection siderophore production by *B. subtilis* QM3 using various history cultures, pH value, amino acid, and different carbon-nitrogen ratio as four variables. After incubating different history cultures we found out that the LB medium presented strong significant CAS reaction even for 12 h, and with the help of changing variables on SD-CASA plate assisting paper-disc diffusion, strain QM3 from LB medium with Fe^{3+} (10 µmol/L) showed difference interestingly. In addition, CAS reaction and growth reaction also increased with pH 7.0, tryptophan 10 µmol/L and carbon-nitrogen ratio 5:1. The findings on solid medium were similar as that in liquid medium. The SD-CASA assay should be a useful tool for screening potential biocontrol microorganisms by siderophore production.

Key words: A simple double-layered CAS agar assay, paper-disc diffusion, siderophores, *Bacillus subtilis* QM3.

INTRODUCTION

Under iron-limited conditions, most prokaryotic cells, certain fungi and plants elaborate multiple low-molecular-weight (often < 1000 Da), high-affinity chelating agents (although some siderophores have lower affinities), which solubilizes ferric iron in the environment and transport it into the cell (Crosa and Walsh, 2002; Ong and Neilands, 1979; Pérez-Miranda et al., 2007). These compounds are generically known as siderophores and they are typically found in iron-deficient cultures (Dimkpa et al., 2009).

Siderophores production by bacteria is considered as an important component of bacterial machinery for iron sufficiency and likely to be more important for survival and growth in the competitive soil environment, which is usually deficient in soluble iron (Khan et al., 2006). Siderophores exhibit considerable structural variability and affinity for iron, which determines the growth of a microorganism under competitive conditions when availability is a limiting factor (Dimkpa et al., 2009).

Heterologous siderophores or its producer organism may bring about various responses in other target bacterial species that are present within the same niche. Growth of some species may be inhibited and this has been attributed to be one of the mechanisms by which biocontrol agents' act in inhibiting the growth of pathogens in the rhizosphere (Kiyohara et al., 1982).

Bacillus subtilis QM3, a siderophore producer, investigated in this paper was illuminated to be a potential biocontrol agent (Hu et al., 2008). According to our preliminary results, the antibiotic produced by the strain QM3 which has bactericidal and probably fungicidal activity was confirmed an antagonistic mechanism against pathogens, including both spatial or nutritional competition and production of antibiotic metabolites. The nutritional competition, ferric (III) competition, will influence the antagonistic response.

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A variety of techniques has been developed to detect siderophores, including use of modern techniques such as mass spectrometry (Dimkpa et al., 2008a, b). To date, however, the most universal detection method for siderophore production is the chrome azurol S (Carrillo-Castañeda et al., 2005) assay based on biological or functional properties, recourse to the color change (colored halo) around the microbial colony of CAS-iron complex from blue to orange after chelation of the bound iron by siderophores (Schwyn and Neilands, 1987). This assay is based on a competition for iron between the ferric complex of an indicator dye (Carrillo et al., 2005) and a siderophore produced by microorganisms. However, it is difficult to grow fastidious microorganisms on the CAS agar plate and some ingredients of the CAS agar have innate antibacterial activity (Shin et al., 2001). The detergent hexadecyltrimetyl ammonium bromide (HDTMA) used in original CAS agar plate assay by Schwyn et al. (1987) and the modified methods (Calvente et al., 2001; Shin et al., 2001; Machuca and Milagres, 2003; Carrillo-Castañeda et al., 2005; Pérez-Miranda et al., 2007) proved to be toxic to some bacteria and fungi, moreover, the original CAS agar plate assay and some modified methods cannot quantify siderophore production (Shin et al., 2001). In particular, a simple optimized method for detection of siderophore production on CAS agar plate has been paid little attention.

In view of this fact, we developed a modified method named 'simple double-layered CAS agar (SD-CASA) assay' with a paper-disc agar diffusion for inoculating a certain amount of bacteria suspension as a quantitative, low cost methodology for siderophore production when microorganisms were grown in CAS agar plates. Besides, by employing this methodology, it is convenient and scientific to optimize the culture conditions to evaluate the effect of pH, carbon-nitrogen ratio and type of exogenous amino acids of the media on siderophore production by *B. subtilis* QM3 and growth of strain QM3. To our knowledge, this is the first paper using a simple method for the development of antagonistic mechanisms by which biocontrol agent act in inhibiting the growth of phytopathogens.

MATERIALS AND METHODS

Bacterial strain

B. subtilis QM3 used in the present study was the first isolation from Qinghai yak dung in China of a potential plant growth-promoting bacterium and a significant biocontrol agent with a broad pathogen-inhibition spectrum against 11 phytopathogenic fungi especially to *Alternaria solani* (Hu et al., 2008). The organisms was maintained on nutrient agar (NA) slants and stored at 4°C. The slants were freshly made once a month.

Media

Three commonly used broth media in this investigation were the nutrient broth (NB) (Curran and Evans, 1937) containing 1.0% peptone, 1.0% beef extract, 0.5% sodium chloride, pH 7.0; Luria-Bertani (LB) (Kiyohara et al., 1982) contained 1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2; Sucrose-Asparagine (SA) (Scher and Baker, 1982) containing 20 g sucrose, 2 g L-asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ to 1 L of water (pH 7.0). Three media will become solid by adding agar (1.5%, W/V). Distilled and deionized water was used for preparing the culture media.

Preparation of glassware

All glassware used was cleaned with 3 mol/L HCl to remove iron and rinsed in deionized water without exception (Cabaj and Kosakowska, 2007).

SD-CASA plate assay

Petri dishes (9 cm in diameter) were prepared with CAS-blue agar (dye solution 10 ml) as bottom agar plate. After solidifying, overlays of the appropriate nutrient medium (6 mL) were applied over those agar plates in order to be tested for siderophore production. Overnight plates at 32°C were used for subsequent experiments.

CAS-blue agar was prepared according to Schwyn and Neilands (Shin et al., 2001) using 60.5 mg Chrome azurol S (CAS) dissolved in 50 mL water distilled, deionized, and mixed with 10 mL iron (III) solution (1 mmol/L $FeCl_3 .6H_20$, 10 mmol/L HCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 mL water. The resultant dark blue mixture was diluted 20-fold and autoclaved at 121°C for 15 min. Agar (2%, w/v) was used as gelling agent.

Conventional paper-disc agar diffusion assays were used for investigating the effect of pH, carbon-nitrogen ratio and type of exogenous amino acids so as to offset the effects of inoculums. Sterilized filter paper discs, 10 mm in diameter, were placed on overnight agar plate in sterile conditions. A 10 µL supernatant achieved by centrifuging of fermentation broth of strain QM3 at 11,000 g for 15 min was diffused onto a paper disc. The uninoculated plates of CAS-agar as control were incubated under the same conditions as described previously. All these experiments were made at least three times with three replicates for each one.

The CAS reaction was determined by measuring the position or distance of the advancing color-change front (in centimeter) in the CAS-blue agar, starting from the center of the paper-disc after incubation times. The clone diameter (CD) of strain QM3 (growth reaction) and orange halo diameter (HD) (CAS reaction) were determined when the effect of pH, carbon-nitrogen ratio and type of exogenous amino acids in the appropriate LB medium were evaluated.

Effect of pH

To study the effect of culture pH on siderophore production of strain QM3, LB broth medium plus iron (10 µmol/L $FeCl_3$) was adjusted with 6 mol/L HCl or 5 mol/L NaOH for pH 5.0 to 11.0. A 100 mL LB with iron in a 250 mL flask was inoculated by adding 2 mL fermentation broth of strain QM3 (1.0×10^8 cfu/mL) and incubated for 12 h with agitation (200 rpm) at 32°C. The bacterial cells were then pelleted by centrifugation at 11,000 g for 15 min at 4°C and clear supernatants were utilized for assay on corresponding CAS-blue agar plates as described previously.

Effect of carbon-nitrogen ratio

To study the effect of carbon-nitrogen ratio on siderophore production by the strain QM3, LB broth medium (pH 7.0) were

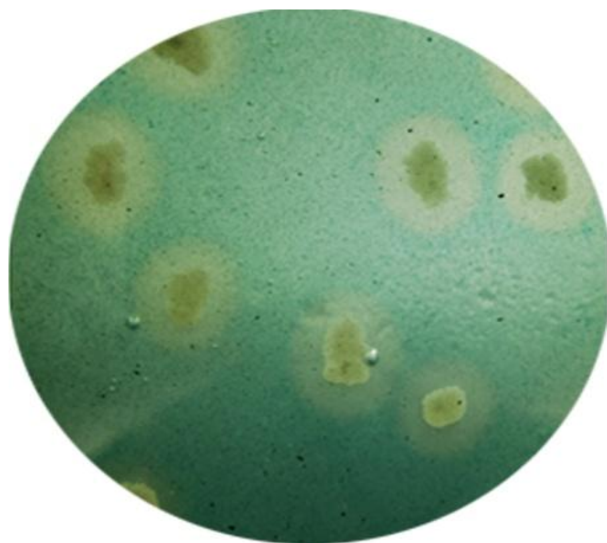

Figure 1. A simple double-layered CAS agar (SD-CASA) assay of *B. subtilis* QM3. Orange halos around the colonies on blue agar are indicative of siderophore excretion.

prepared with different carbon-nitrogen ratio (2:1, 3:1, 5:1, 8:1, 10:1) through appropriate glucose added. Other protocols were similar as mentioned earlier. Each was done in duplicate in three sets of experiments.

Effect of type of exogenous amino acids

The study of the effect of type of exogenous amino acids was performed with three types of amino acids: tyrosine, tryptophan and aspartate. Each of them all was added to the LB broth medium (pH 7.0, carbon-nitrogen ratio 5:1) at concentration of 10 μmol/L. Practically, the following steps were similar to the aforementioned steps.

Siderophores detection in liquid medium

From overnight cultures of strain QM3 in glass flasks (50 mL), a sample of 10 μL of cell suspension was inoculated using a new LB medium (pH 7.0, carbon-nitrogen ratio 5:1) supplemented with iron (10 μmol/L $FeCl_3$) and tryptophan (10 μmol/L), and another sample was used for to another new LB (pH 7.2, 10 μmol/L $FeCl_3$) without any exogenous amino acids. After 48 h of incubation with agitation, the bacterial cells were then pelleted by centrifugation at 11,000 g for 15 min at 4°C and the clear supernatants were diluted to 10 in deionized water to determine their absorbance spectra from 200 to 800 nm using a UV-Visible spectrophotometer (1601-Shimadzu, Japan) in 1.0 cm cells (Carrillo-Castañeda et al., 2005), against LB medium without inoculation blank. Assays in solid and liquid media were carried out by triplicate. Experiments were repeated at least twice.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) and the least significant difference test at the 5% level was used for comparison of the means.

RESULTS

Siderophore production on the SD-CASA assay

Using inoculation loop the microorganisms were inoculated on SD-CASA plate with LB medium overlay. The ability of strain QM3 to produce siderophores on CAS-blue agar plate was shown in Figure 1. Strain QM3 produced a strong reaction using the SD-CASA assay. After incubating for a period of 12 h in the dark at 32°C, a change in color from dark blue to orange (as reported for microorganisms that produce hydroxamates) was observed in the bottom agar plate, exclusively surrounding producer microorganisms, and no color change in the CAS-blue agar was observed, even after long incubation periods (Figure 1).

CAS reactions of strain QM3 were affected by the previous culture history of the cells. Those that had been grown previously in LB medium showed marked differences in color than in NB and SA media (Figure 2). The color change from dark blue (CK means without inoculation) to emerging orange on CAS agar was 12 h for cells grown in LB medium and compared with 36 h in SA and NB. In contrast, the range of color showed only small differences in NB and SA media. Generally, the change of color in LB increased with the extension of the incubating time. As a rule slowly growing cultures make smaller zones than faster growing ones (Figure 2).

Effect of pH

Growth reaction and CAS reaction were also affected by pH in the growth medium (Figure 3A). The most significant influences were observed in the experiments with strain QM3 growing at pH 7.0. In this case, strain QM3 had the highest growth yield and hydroxamate production. Alternatively, cells grown on pH 5.0 and 11.0 had significantly less growth reaction and CAS reaction. In general, the best pH of pre-medium for QM3 was 7.0 (Figure 3).

Effect of types of exogenous amino acids

Both growth reactions and CAS reactions produced by QM3 were similar for all the types of amino acids tested (Figure 3B). Hydroxamte synthesis was less affected by exogenous amino acids adding to LB (10 μmol/L $FeCl_3$) in the growth medium contrasted with CK and LB in Figure 2. On the other hand, strain QM3 produced stronger growth reaction even when exogenous amino acids (10 μmol/L) were present at LB medium. Compared to three amino acids, tryptophan was selected for the following experiments.

Effect of ratio of carbon and nitrogen

The effects of different ratio of carbon and nitrogen (C/N)

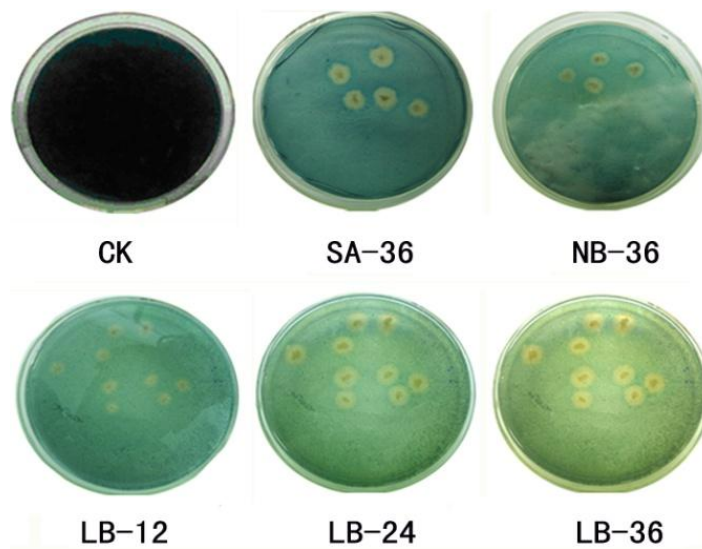

Figure 2. CAS-blue agar plate and the appropriate nutrient medium inoculated with *B. subtilis* QM3 at 32°C. CK, without inoculation; SA-36, SA nutrient medium for 36 h; NB-36, NB nutrient medium for 36 h; LB-12, LB nutrient medium for 12 h; LB-24, LB nutrient medium for 12 h; LB-36, LB nutrient medium for 36 h.

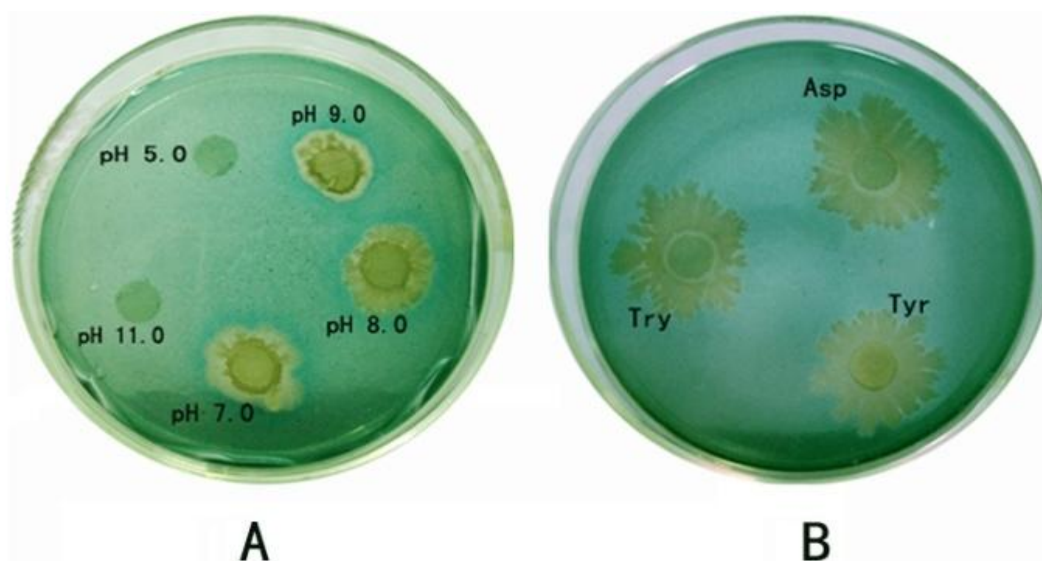

Figure 3. Effect of pH in the growth LB medium (A) and types of exogenous amino acids on siderophore production of *B. subtilis* QM3 for 12 h (B). The paper-disc diffusion was used in order to offset influence of inoculation. Asp, aspartate, Try, tryptophan, Tyr, tyrosine.

on the siderophore production and growth yield are shown in Figure 4. As can be seen, growth reaction (CD values) was significantly affected by ratio of carbon and nitrogen ($p < 0.05$ for all the ratio values tested), whereas with CAS reaction (HD values) ratio value 5:1 was observed, the higher significance than others was tested. Again, ratio value 5:1 showed the higher values of growth reaction (Figure 4).

Siderophore detection in liquid medium

To attempt a correlation between siderophore production in solid and in liquid media, the strain QM3 was inoculated in LB liquid and solid respectively (with and without pH 7.0, tryptophan 10 μmol/L and C/N 5:1). In solid medium, the siderophore production was evaluated by the modified CAS-blue agar while absorbance was

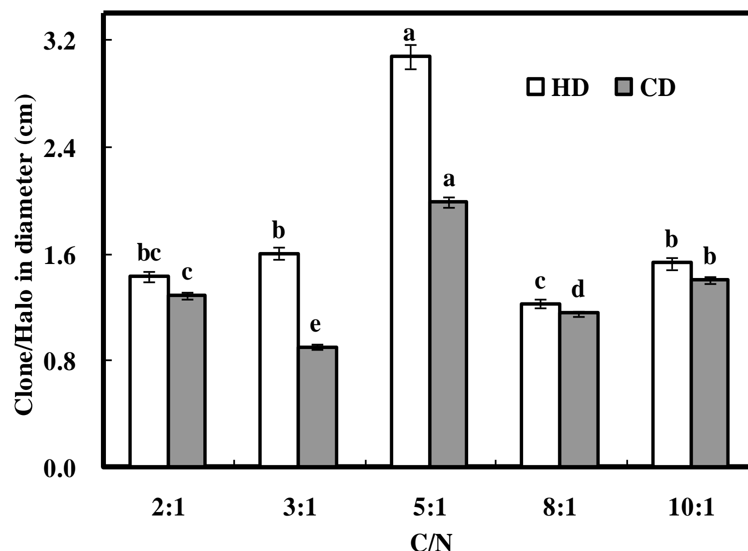

Figure 4. Effect of carbon-nitrogen ratio (C/N) in the growth LB medium on siderophore production of *B. subtilis* QM3 for 12 h using the SD-CASA assay. Each was done in duplicate in three sets of experiments. Different lowercase letters show the least significant difference at the 5% level for comparison of the means. HD (□), means diameters of halos; CD (■), means diameters of clones.

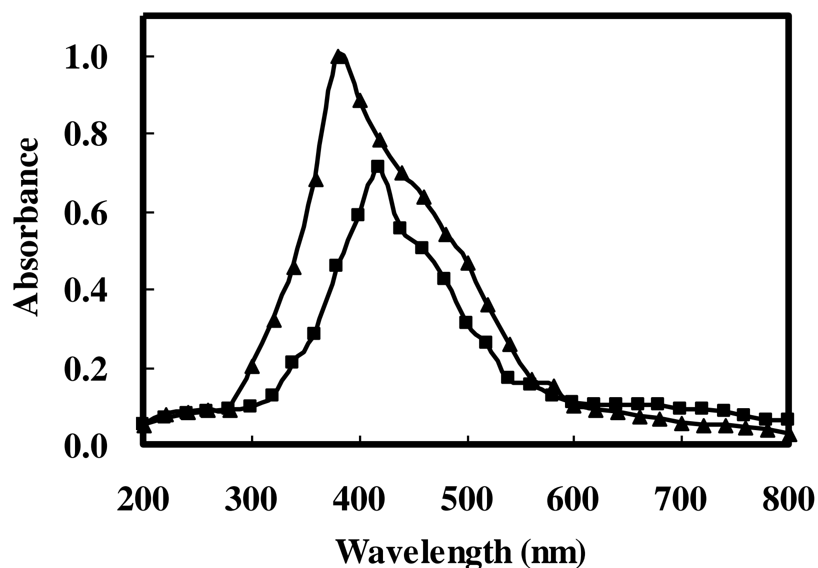

Figure 5. An absorption spectrum (200 to 800 nm) of clear supernatants from cultures of the *B. subtilis* QM3 grown in the indicated liquid medium. Assays were carried out by triplicate. Experiments were repeated at least twice. History growth medium: (■) LB medium (pH 7.2, 10 μmol/L $FeCl_3$) without any exogenous amino acids; (▲) LB medium (pH 7.0, carbon-nitrogen ratio 5:1) supplemented with iron (10 μmol/L $FeCl_3$) and tryptophan (10 μmol/L).

surveyed in liquid medium (Figure 5). The siderophore production by QM3 in solid medium supplemented with pH 7.0, tryptophan 10 μmol/L and C/N 5:1 showed the same profile as in liquid. In other words, the CAS reaction presented a significant increase or advance both in solid and in liquid (Figure 5).

DISCUSSION

In an earlier work the CAS-agar plate was modified to allow for the growth of different microorganisms without problems of inhibition caused by the toxicity of HDTMA used in the CAS reagent preparation (Milagres et al., 1999). Whereas SD-CASA assay developed in this paper is a simpler and cheaper method, even for siderophore production of a potential biocontrol agent. On the other hand, when compared with most of the methods used to determine siderophores which are carried out on the microorganism culture supernatant because siderophores are found there, SD-CASA is performed on both the microorganism culture supernatant as inducer and the microorganism themselves as main producers. This implied, in certain cases, addition of heterologous siderophores results in a growth stimulatory effect (Khan et al., 2006).

Siderophore-producing bacteria have been used as biocontrol agents to combat plant pathogens (Gram, 1996). This is particularly important when evaluating the potential of a strain for biocontrol (Manninen and Mattila-Sandholm, 1994). *B. subtilis* QM3, a spore-forming bacterium commonly used in commercial and research bio-control products to control a variety of plant pathogens (Hu et al., 2008), was proved to be a siderophore producer using universal CAS assay and presented distinct orange halos on SD-CASA assay. This indicated an iron chelator removed iron from the blue CAS complex thus causing its color to change to orange.

In the majority of studies, siderophore production of a particular organism has been studied in defined media, for example, minimal medium supplemented with glucose and/or amino acids, which have suggested that siderophore production is substrate dependent (Gram, 1996). In this study we have also shown that the substrate is a crucial factor when studying siderophore production. Strain QM3 used in this study on CAS-agar was siderophore positive when cultured on more nutrient rich substrates, like LB medium. But adding exogenous amino acids in LB siderophore production of QM3 was no a significant increase even though the paper-disc diffusion was used, which is different from the view that siderophores require a peptide backbone on which the chelating groups are bound, and depending on the bio-synthetic capabilities of the organism, it may be required that some of the amino acid are supplied by the substrate (Gram, 1996). Others, exogenous amino acids may improve growth action of strain QM3. It has been shown that the nitrogen requirement for growth of QM3 is essential.

Generally a concentration low enough, only micromolar quantities of iron, to induce siderophore production, and with increasingly stronger siderophore produced in response to increasingly insoluble iron sources (Liermann et al., 2000). From our results, growth reaction and CAS reaction of strain QM3 were significantly affected by the pH and carbon-nitrogen ratio of the LB. The standard LB medium with a 10 μmol/L $FeCl_3$ is very important. When Fe concentrations are approximately a micro-molar order of magnitude, siderophore-dependent, high affinity Fe uptake systems are sufficient for obtaining iron from the environment (Liermann et al., 2000). This could be because of extra requirements for siderophore synthesis.

The siderophore production by QM3 both in optimized LB and un-optimized (original) LB has certain correlation between solid and liquid medium. Especially a strong absorption peak was observed for optimization at 380 nm in contrast to 420 nm for un-optimization, and peak area of optimization is much bigger than of un-optimization (Figure 5). The importance of advance in wavelength and increases in peak area due to siderophore activity has generally been underemphasized, and it deserves further study.

Conclusion

In conclusion, the SD-CASA assay on solid medium can be a useful tool for screening siderophore producer microorganisms. Because its protocol is simpler and cheaper but can offset the effect of inoculation on siderophore by using paper-disc diffusion. We have shown that the choice of substrate is crucial for the evaluation of siderophore production of microorganisms. Particularly, strain QM3, a potential use in biocontrol, is evaluated to improve of siderophore production.

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REFERENCES

Cabaj A, Kosakowska A (2007). Iron-dependent growth of and siderophore production by two heterotrophic bacteria isolated from brackish water of the southern Baltic Sea. Microbiol. Res., In Press.

Calvente V, de Orellano ME, Sansone G, Benuzzi D, Sanz de Tosetti MI (2001). A simple agar plate assay for screening siderophore producer yeasts. J. Microbiol. Meth., 47(3):273-279.

Carrillo-Castañeda G, Muñoz JJ, Peralta-Videa JR(2005). A spectrophotometric method to determine the siderophore production by strains of *fluorescent Pseudomonas* in the presence of copper and iron. Microchem. J., 81(1):35-40.

Crosa JH, Walsh CT (2002). Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol. Mol. Biol. R., 66(2): 223-249.

Curran HR, Evans FR (1937). The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bacteriol., 34(2):179-189.

Dimkpa CO, Merten D, Svatoš A, Büchel G, Kothe E (2009). Metal-induced oxidative stress impacting plant growth in contaminated soil

is alleviated by microbial siderophores. Soil Biol. Biochem., 41(1):154-162.

Dimkpa CO, Svatoš A, Dabrowska P, Schmidt A, Boland W, Kothe E (2008a). Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* Spp. Chemosphere, 74(1):19-25.

Dimkpa CO, Svatoš A, Merten D, Büchel G, Kothe E (2008b). Hydroxamate siderophores produced by streptomyces acidiscabies E13 bind nickel and promote growth in cowpea (Vigna Unguiculata L.) under nickel stress. Can. J. Microbiol., 54(3):163-172.

Gram L (1996). The influence of substrate on siderophore production by fish spoilage bacteria. J. Microbiol. Meth., 25(3):199-205.

Hu Q-P, Xu J-G, Song P, Song J-N, Chen W-L (2008). Isolation and identification of a potential biocontrol agent *Bacillus subtilis* QM3 from Qinghai yak dung in China. World. J. Microb. Biot., 24(11):2451-2458.

Khan A, Geetha R, Akolkar A, Pandya A, Archana G, Desai AJ (2006). Differential cross-utilization of heterologous siderophores by nodule bacteria of *Cajanus Cajan* and its possible role in growth under iron-limited conditions. Appl. Soil. Ecol., 34(1):19-26.

Kiyohara H, Nagao K, Yana K (1982). Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microb., 43(2): 454-457.

Liermann LJ, Kalinowski BE, Brantley SL, Ferry JG (2000). Role of bacterial siderophores in dissolution of hornblende. Geochim. Cosmochim. Acta, 64(4): 587-602.

Machuca A, Milagres AM (2003). Use of CAS-agar plate modified to study the effect of different variables on the siderophore production by *Aspergillus*. Lett. Appl. Microbiol., 36(3):177-181.

Manninen M, Mattila-Sandholm T (1994). Methods for the detection of *Pseudomonas* siderophores. J. Microbiol. Meth., 19(3):223-234.

Milagres AMF, Machuca A, Napoleão D (1999). Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. J. Microbiol. Meth., 37(1):1-6.

Ong SA, Neilands JB (1979). Siderophores in microbially processed cheese. J. Agric. Food Chem., 27: 990-995.

Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera LS, Fernández FJ (2007). O-CAS, a fast and universal method for siderophore detection. J. Microbiol. Meth., 70(1): 127-131.

Scher FM, Baker R (1982). Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. Phytopathology, 72(12):1567-1573.

Schwyn B, Neilands JB (1987). Universal chemical assay for the detection and determination of siderophores. Anal. Biochem., 160(1): 47-56.

Shin SH, Lim Y, Lee SE, Yang NW, Rhee JH (2001). CAS agar diffusion assay for the measurement of siderophores in biological fluids. J. Microbiol. Meth., 44(1):89-95.