The Detection and Determination of Siderophores from Plant Growth Promoting and Plant Disease Bacteria

การหาและการตรวจวัดไซเดอโรฟอร์จากแบคทีเรียที่ส่งเสริมการเจริญเติบโตและแบคทีเรียที่ก่อโรคพืช

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ABSTRACT

Seven strains of all plant growth promoting and plant disease bacteria isolated from difference source (soil and plant) were tested siderophore production by a well-known and universal chemical method using Chrome Azurol S (CAS) agar plates. Six strains of these produced siderophores in agar media but only three strains produced siderophores in several liquid media at neutral pH and fluorescent under UV light. The siderophore-iron complex (brown) also appeared immediately after adding iron solution. In addition, siderophore-producing strains from *P. putida and P. tolaasii* were tested with the Csaky test and Triphenyltetrazolium chloride test, indicating the hydroxamate type.

บทคัดย่อ

ทดสอบการผลิตไซเดอโรฟอร์จากแบคทีเรียที่ส่งเสริมการเจริญเติบโตและแบคทีเรียที่ก่อโรคพืช 7 สายพันธุ์จากแหล่งที่มาต่างกัน (ดินและพืช) ด้วยวิธีมาตรฐานที่นิยมอย่างแพร่หลายในอาหารวุ้นชนิดโครมอะซูรอลเอส(CAS) ในจานเพาะเชื้อ พบว่ามี 6 สายพันธุ์ที่สามารถผลิตไซเดอโรฟอร์ใน CAS ได้ แต่มีเพียง 3 สายพันธุ์เท่านั้นที่สามารถผลิตไซเดอโรฟอร์ได้ในอาหารเหลวต่าง ๆ ณ pH ที่เป็นกลาง เรืองแสงได้ภายใต้แสงอัลตราไวโอเลตและเกิดสารประกอบเชิงซ้อนกับเหล็กได้สารละลายสีน้ำตาลทันทีเมื่อเติมสารละลายเหล็กลงไป นอกจากนี้พบว่าแบคทีเรีย 2 สายพันธุ์คือ *P. putida* และ *P. tolaasii* นำมาทดสอบกับ Csaky test และ Triphenyltetrazolium chloride test พบว่าเป็นไซเดอโรฟอร์ประเภทไฮดรอกซาเมต

Keywords: Siderophores, Chrome Azurol S, Hydroxamate type
คำสำคัญ: ไซเดอโรฟอร์ โครมอะซูรอลเอส ประเภทไฮดรอกซาเมต

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Introduction

Iron has played an essential role in the evolution of every form of life on earth initial microorganisms until human. Nevertheless, under aerobic conditions at physiological pH, the solubility of iron in soil very low due to the low dissociation constant of the various oxide hydrates of Fe^{3+} about 10^{-38} M (Gram, L., 1994) Thus the concentration of free Fe^{3+} in the soil is at best about 10^{-17} M (Budzikiewicz, H. *et al*, 1997). This is too low to support the growth of microorganisms, which generally need concentrations approaching 10^{-6} M for normal growth. Consequently, to survive in such environments most of the aerobic and facultative anaerobic microorganisms synthesize siderophores, which are extracellular, low molecular weight (300-2,000 daltons), water-soluble molecules with a high affinity for ferric ions. These high-affinity iron(III) transport molecules scavenge ferric ions and deliver them to the cell and released for use in the organism's metabolic functions. Siderophores are currently of great interest because of their essential role in microorganisms, plants, theirs potential applications in the treatment of iron overload disease, as part of siderophore antibiotics, as selective growth factor for nutrient media and biocontrol agents.

Siderophore production has been reported in various microorganisms especially species of rhizobacteria including plant growth promoting and plant disease bacteria. Siderophores have been shown to be related to enhance the growth and yield of production of some plants. Whereas, the pathogenicity of bacteria has been associated with their abilities to synthesize siderophores for pathogenicity factor affection germination of spores and/or colonization of plants by microbial pathogen.

The detection of cultures for siderophores production was done using the universal chemical assay described by Schwyn and Neilands (1987), referred to as the Chrome Azurol S (CAS) assay, provides a very powerful detection method to demonstrate production of uncharacterized siderophores. This assay is based on a competition for iron between the ferric complex of a indicator dye, Chrome Azurol S (CAS), and a chelator or siderophore produced by microorganisms. The iron is removed from CAS by the siderophore, which apparently has a higher affinity for iron (III). The positive reaction results in a color change of CAS reagent (usually from blue to orange) (Adriane, M. F. *et al.*, 1999).

Siderophores are generally classified according to their main chelating groups as hydroxamates, phenol-catecholates and carboxylate. Consequently, the most sensitive and specific of the hydroxamate assay was developed by Csaky to detect bound hydroxamine. In these assay, a samples is heat in sulfuric acid to release free hydroxylamine, which is stable under acidic conditions. Samples containing hydroxamates will appear red to violet in color. In addition, triphenyltetrazolium Chloride Test was a technique for determination of hydroxamime derivatives based on the reduction of alkaline triphenyltetrazolium chloride and the reduction is temperature dependent. The positive result will appear orange color. Whereas the Arnow is convenient and very specific for co-planar *cis*-diols such as catechol. These assay based on nitrosation of the ring and its subsequent conversion to the molybdate complex, which is yellow in acid and red in alkaline media (Ishimaru, C.A., and Loper, J. E., 1993). Therefore, the objective of this research will be detected and determined the siderophores from plant growth and plant disease bacteria including study on type of siderophores.

Methodology

Microorganisms

Plant Growth Promoting Bacteria (*Pseudomonas putida*) was bought from Thailand Institute of Scientific and Technological Research whereas all strains of Plant Disease Bacteria (*Xantomonas* spp. , *Erwinia* spp. and *Pseudomonas tolaasii*) were kindly supplied by Plant Pathology and Microbiology Division, Department of Agriculture Information Service.

Materials

CAS agar plate was modified by Schwyn and Neilands (1987). 5% ferric citrate solution from Fluka, Switzerland was used in A.R. grade. All reagents for

study on type of siderophore were used in A.R. grade. Reagents for Csaky Test and Triphenyltetrazolium Chloride Test were followed by Gillam, A. H. *et al.* (1981) and another for Arnow assay followed from Ishimaru, C. A. and Loper, J. E. (1993). Both Siderophores supernatant from *P. putida and P. tolaasii* were studied in our work. Deionized water from Osma Orion was used for all purposed. The optimal formula of liquid medium (No.2 and No. 5) were modified by Budzikiewicz, H. *et al.* (1995).

Experimental procedures

- *Detection of siderophore*

For determination of siderophores, CAS assay was employed by spotted three positions of the growing bacterial cultures on CAS agar plates. The radius of any clearing zones surrounding the colonies were then measured after incubated for 3 days at 37°C.

- *Determination of siderophore*

The production of siderophores in eight formula of liquid medium were investigated after the producing siderophores cultures on CAS agar, by inoculate the cultures in each liquid media which were adjusted pH to 6.5-7.0 after autoclaved. Siderophore production was then detected under UV light and formed ferric complex with added 5% ferric citrate solution.

- *Study on type of siderophore*

Siderophores production obtained from *P. putida* and *P. tolaasii* were studied on type of siderophore using well-known method of Csaky test, Triphenyltetrazolium chloride test (for hydroxamate) and Arnow assay (for catecholate).

Results and Discussion

Detection of siderophore

All strains of bacteria produced siderophores were developing orange zone around colonies on CAS agar medium (except the strain of *Erwinia chrysanthemi* pv *zeae*) because of the iron is removed from CAS by siderophore and the positive reaction results in a color change from blue to orange. In addition, *P. putida* has the most radius of appearing zone that rather than *P. tolaasii* and others (Fig. 1). It showed that *P. putida* was the best of the siderophore producer in CAS agar.

Determination of siderophore

Due to the maximum production of siderophores in all 8 formula of liquid medium were obtained from *P. putida* and *P. tolaasii*, therefore these strains were studied on type of siderophores for further (Table 1). The siderophores produced by *P. putida* (in No. 5) and *P. tolaasii* (in No. 2) in the optimal of liquid medium were yellow green pigment and fluorescence under ultraviolet light. Furthermore, the complexes of both had dark brown and red brown color, respectively (Fig. 2). It was indicated that both strains were found to be the best siderophore producer in liquid medium at neutral pH. However, *E. carotovora* pv *carotovora* produced some trace amounts and the other strains can not produce any siderophores.

Study on type of siderophore

The positive results in the Csaky test (red color) and Triphenyltetrazolium chloride test (orange color) are indicated that both strains produced hydroxamate type of siderophore and confirmed by negative results (yellow color) in the Arnow assay for catecholate of type (Fig. 3).

Conclusion

P. putida (Plant Growth Promoting Bacteria) was the best producer of siderophore in CAS agar media. In addition, these strains produce siderophore as well as *P. tolaasii* (Plant Disease Bacteria) and they are found to be the best siderophore producer in liquid medium at neutral pH. Both strains can also produce hydroxamate type of siderophores.

The knowledge of plant growth promoting and plant disease bacteria producing siderophore will contribute toward finding a novel siderophores. This approach is facilitating the development of new strains with modified traits for enhanced biocontrol efficacy.

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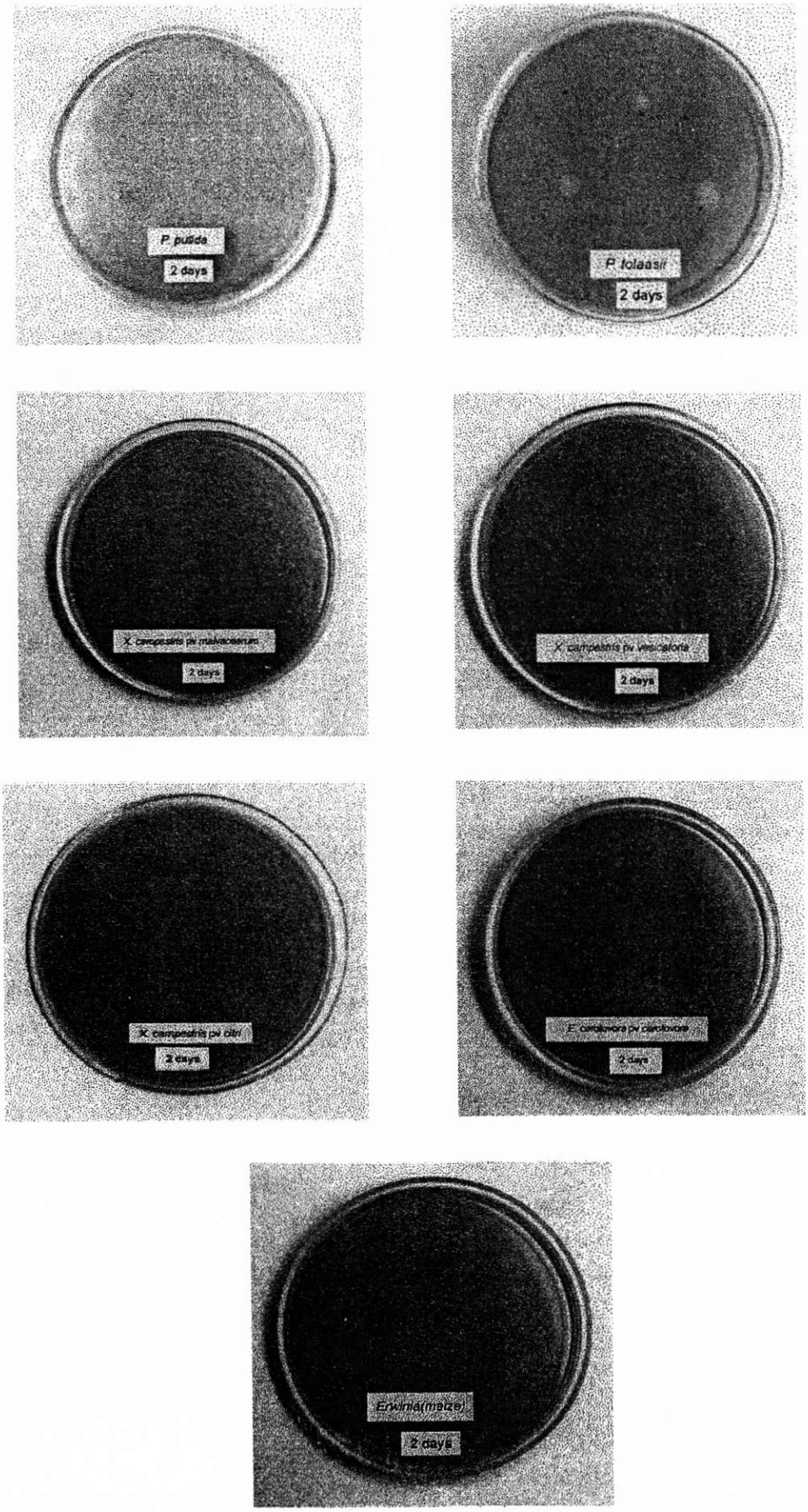
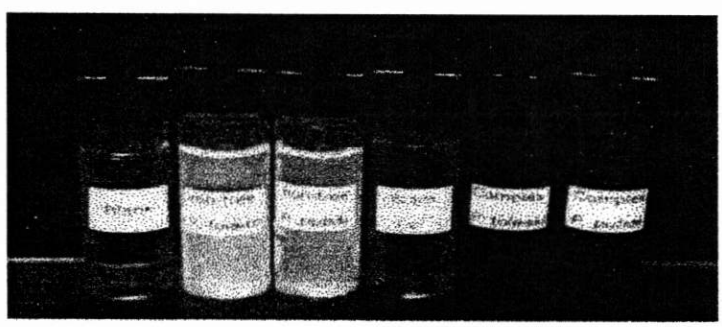


Figure 1. Photographs of each culture on CAS agar plates.

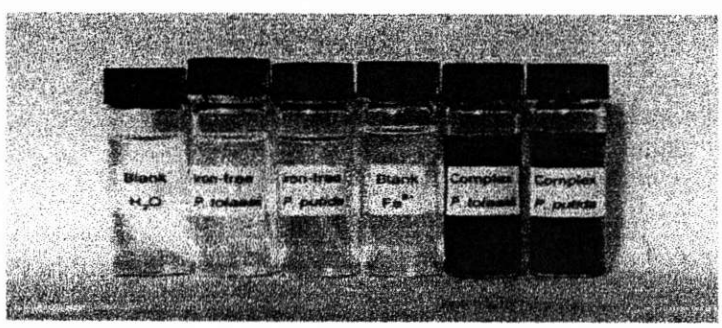
Table 1. Comparison of difference strains producing distinction of siderophores in optimal formula of liquid medium.

Strains	Optimal formula of liquid medium	The illumination under UV light	The complexation with ferric ion solution
Plant Growth Promoting Bacteria			
Pseudomonas putida	No. 5	green fluorescence	dark brown
Plant Disease Bacteria			
1. *Pseudomonas tolaasii*	No. 2	green fluorescence	red brown
2. *Xantomonas campestris* pv *malvacearum*	-	-	-
3. *Xantomonas campestris* pv *vesicatoria*	-	-	-
4. *Xantomonas campestris* pv *citri*	-	-	-
5. *Erwinia carotovora* pv *carotovora*	No. 6	light blue fluorescence	brown
6. *Erwinia chrysanthemi* pv *zeae*	-	-	-

Note : - not detected



a



b

Figure 2. Photographs showed a) llumination of siderophores under UV light. b) supernatant and complex

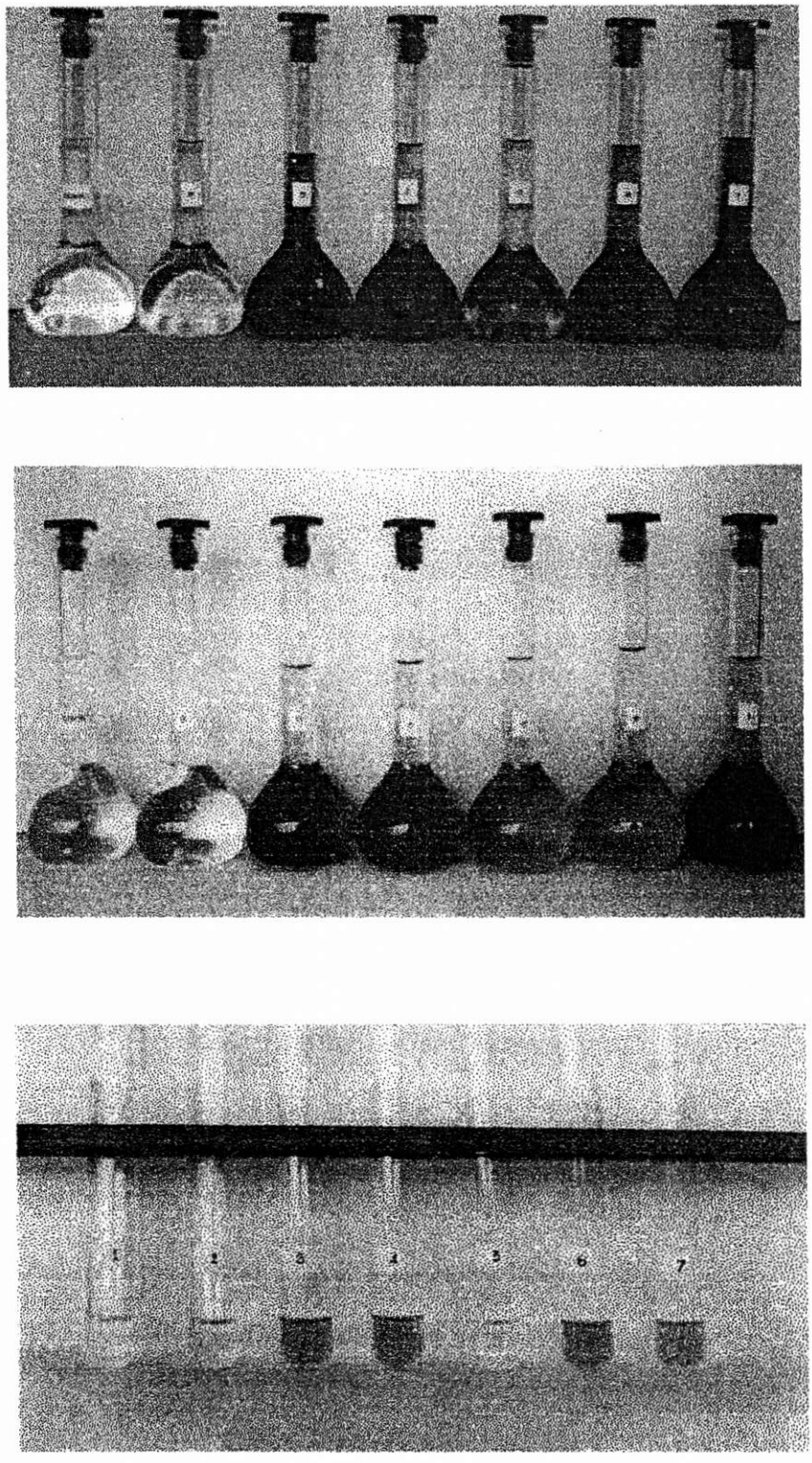


Figure 3. Photographs showed the results;
a) Csaky test b) Triphenyltetrazolium chloride test and c) Arnow assay.
Blank, 1 : deionized water
a, 2 : *P. tolaasii* - siderophore supernatant
b, 3 : *P. tolaasii* - siderophore crude
c, 4 : *P. tolaasii* - purified siderophore
d, 5 : *P. putida* - siderophore supernatant
e, 6 : *P. putida* - siderophore crude
f, 7 : *P. putida* - purified siderophore