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# Detection of siderophores in growing cultures of *Pseudomonas* spp.

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## SUMMARY

The siderophores produced by *Pseudomonas fluorescens* and *P. chlororaphis* were detected from the culture supernatants in MM9 and modified King's medium by the universal CAS assay at wavelengths 620–690 nm. The CAS assay was applied to detect *Pseudomonas* siderophores directly in situ, during their production phase, in modified King's medium. Optimum results were detected with a final CAS concentration of 0.025 mM and an iron concentration of 1.25 μM. The problems of the method are discussed with respect to the absorbance spectrum, the toxicity of the HDTMA detergent, the influence of the iron concentration and the complexity of media for siderophore production.

## INTRODUCTION

Iron is an essential element for all bacteria except lactobasillae [1]. At low iron concentrations many aerobic and facultatively anaerobic bacteria as well as fungi are known to produce iron chelators, siderophores, for ensuring their iron availability. Siderophores are relatively low-molecular weight ferric-specific compounds. Most siderophores belong to the category of hydroxamates or catechols [5–7].

There are two general approaches to siderophore determination, based on chemical or microbiological methods [7]. Hydroxamate and catechol types of siderophores can be measured by chemical methods. However, other siderophores also exist, which cannot be included in these categories. The chemical methods suffer often from the lack of sensitivity and specificity [7]. Bioassays, on the other hand, are more sensitive and the existence of siderophores is detected by the influence of siderophores on the growth of test organisms. Bioassays, however, need viable stocks for the use of appropriate test organisms.

In general, most assays for siderophore detection are qualitative [7]. Today the most common detection method for siderophore production is the universal assay of Schwyn and Neilands [11]. The assay is based on the competitive exchange of iron (III) from an indicator dye, chrome azurol S (CAS). The affinity of CAS for iron (III) seems to be slightly lower than that of most siderophores, and hence the metal ion is quantitatively released to a competing ligand. The assay has been reported to be applicable to many Gram-positive organisms. However, the detergent HDTMA used in the assay to improve the transfer of iron may be toxic to some Gram-negative strains [11].

*Pseudomonas* spp. have been known for their siderophore production for many years and therefore many reports on the isolation and characterization of these siderophores have been published [2,3,9]. *Pseudomonas* species have been utilized in agriculture by using these siderophore-positive organisms against organisms causing plant disease [4,8,12]. The list of well-characterized *Pseudomonas* spp. siderophores include compounds like pyochelin, pseudobactins and pyoverdines [7].

The aim of this study was to develop a quantitative in situ method for the production of siderophores by *Pseudomonas*, based on the CAS method of Schwyn and Neilands [11].

## MATERIALS AND METHODS

### *Bacterial strains*

*Pseudomonas fluorescens* ELI105 and *Pseudomonas chlororaphis* ELI106 from the culture collection of VTT, Food Research Laboratory, were used in the experiments and maintained as lyophilized. The strains were isolated from natural wood logs of alder (*Alnus incana*) which had been inoculated with shiitake (*Lentinula edodes*) mycelia for mushroom cultivation [10].

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*Culture media and growth conditions for siderophore production*

*P. fluorescens* and *P. chlororaphis* were grown in three liquid media for siderophore production: King's Medium B without phosphate, KB (magnesium sulphate 1.5 g/l, Merck, tryptone 10 g/l, DIFCO, proteose peptone 10 g/l, DIFCO, glycerol 10 ml/l, BDH), Tryptone Soy Broth, TSB (BBL) and MM9 medium. MM9 was prepared as described by Neilands [5] for *Escherichia coli* except for 0.1 M Pipes (Sigma) which was added for pH adjustment (*P. chlororaphis* pH 6.0, *P. fluorescens* pH 6.8). 8-Hydroxyquinoline (Merck Suchardt) was used in the deferration of succinic acid (Sigma) and casamino acids (DIFCO). The glassware was acid-washed.

*P. fluorescens* and *P. chlororaphis* were grown in KB and TSB media in shake flask cultures (96 rpm) for 6 days at 25 °C in the dark. The bacterial inocula were prepared by suspending a loopful of an actively growing culture in saline. The inoculum level was calibrated to give a turbidity of 0.04 (which corresponds to $5 \times 10^7$ bacteria/ml) at 620 nm by a Multiskan MCC microplate reader (Labsystems Oy, Finland). Three hundred $\mu$l of this inocula was suspended in 30 ml of media. Samples taken daily were centrifuged at $10000 \times g$ for 10 min and filter-sterilized (0.20 $\mu$m, Millipore). Samples were concentrated by lyophilization and stored at −20 °C for subsequent siderophore analysis.

The *Pseudomonas* strains were grown in MM9 medium according to Schwyn and Neilands [11] for 6 (*P. fluorescens*) and 10 (*P. chlororaphis*) days at 25 °C in the dark. The samples were concentrated by lyophilization and stored at −20 °C for subsequent siderophore analysis.

*Turbidity measurements*

From the above experiments before the centrifugation step the cell density in MM9 and KB media were monitored daily by Multiskan MCC microplate reader at 620 nm in order to define the growth phase. Changes in medium pH were detected daily.

*Siderophore detection in culture supernatants*

Siderophore detection from supernatants was performed according to the method of Schwyn and Neilands [11]. Before performing the analysis the absorbance spectra of the CAS reagent (Fluka Chemical) was measured with various Deferriferrioxamine B (Desferal methanesulfonate, Ciba-Geigy Corporation) concentrations (3.0 $\mu$g/l, 80 $\mu$g/l which corresponds to 0.1 mM or 2.8 mM, respectively) after an incubation period of 24 h at room temperature. The siderophore production of the culture supernatants in KB, TSB and MM9 media were measured by CAS reagent (Fluka Chemical) at 420 nm, 620–630 nm and 690 nm. The samples were suspended in water to one-fifth of original volume and incubated for 24 h at room temperature before each measurement.

*Detection of siderophore production in situ*

*P. fluorescens* was grown in modified KB supplemented with MM9 salts [11]. The medium pH was adjusted with 0.1 M Pipes to pH 6.8. CAS-assay solution was prepared according to the method of Schwyn and Neilands [11] except for different concentrations of the CAS reagent and $FeCl_3$ which were added in the growth media of *P. fluorescens*. The stock solutions were sterilized separately at 121 °C for 15 min. The final concentrations of CAS, HDTMA (hexadecyltrimethylammonium bromide, Fluka Chemical) and iron in the growth medium were: (i) 0.025 mM CAS + 0.05 mM HDTMA with 0.625, 1.25, 2.5, 5.0 or 10.0 $\mu$M iron; (ii) 0.05 mM CAS + 0.1 mM HDTMA with 1.25, 2.5, 5.0 or 10.0 $\mu$M iron; (iii) 0.1 mM CAS + 0.2 mM HDTMA with 2.5, 5.0 or 10.0 $\mu$M iron.

*P. fluorescens* was grown in Euro jars, 30 ml (A. Ahlström, Finland) with agitating (96 rpm) at 25 °C in the dark for 9 days. The bacterial inocula were prepared as previously. Serial samples were taken daily, filter-sterilized (0.20 $\mu$m, Millipore) and the siderophore production was directly detected at 420, 620 and 690 nm. During the growth period the cell density was monitored at 620 nm and the medium pH was measured.

## RESULTS

*Absorbance spectra of the CAS reagent*

The changes in the absorbance spectra of the CAS reagent after the release of iron were detected with deferriferrioxamine B. The spectrum of the CAS reagent clearly showed two absorbance peaks, a sharp one decreasing at 690 nm and another wider one increasing at 430–440 nm as a function of iron removal from CAS. The release of iron from the CAS reagent, which is accompanied by a colour change from blue to orange, was most accurately detected at 690 nm (data not shown).

*Detection of iron-binding activity of P. fluorescens and P. chlororaphis culture supernatants*

*P. fluorescens* and *P. chlororaphis* were grown in KB, TSB and MM9 media in order to detect siderophores in these media. The results of siderophore production monitored as absorbance change at 690 nm are illustrated in Fig. 1a. Siderophores were detected in all other media except TSB, which contains highest concentrations of nutrients of the media used (results not shown). The clearest siderophore production was detected in MM9 medium with *P. fluorescens* (Fig. 1a) although the growth was very poor (Fig. 1b). Furthermore, siderophore production was

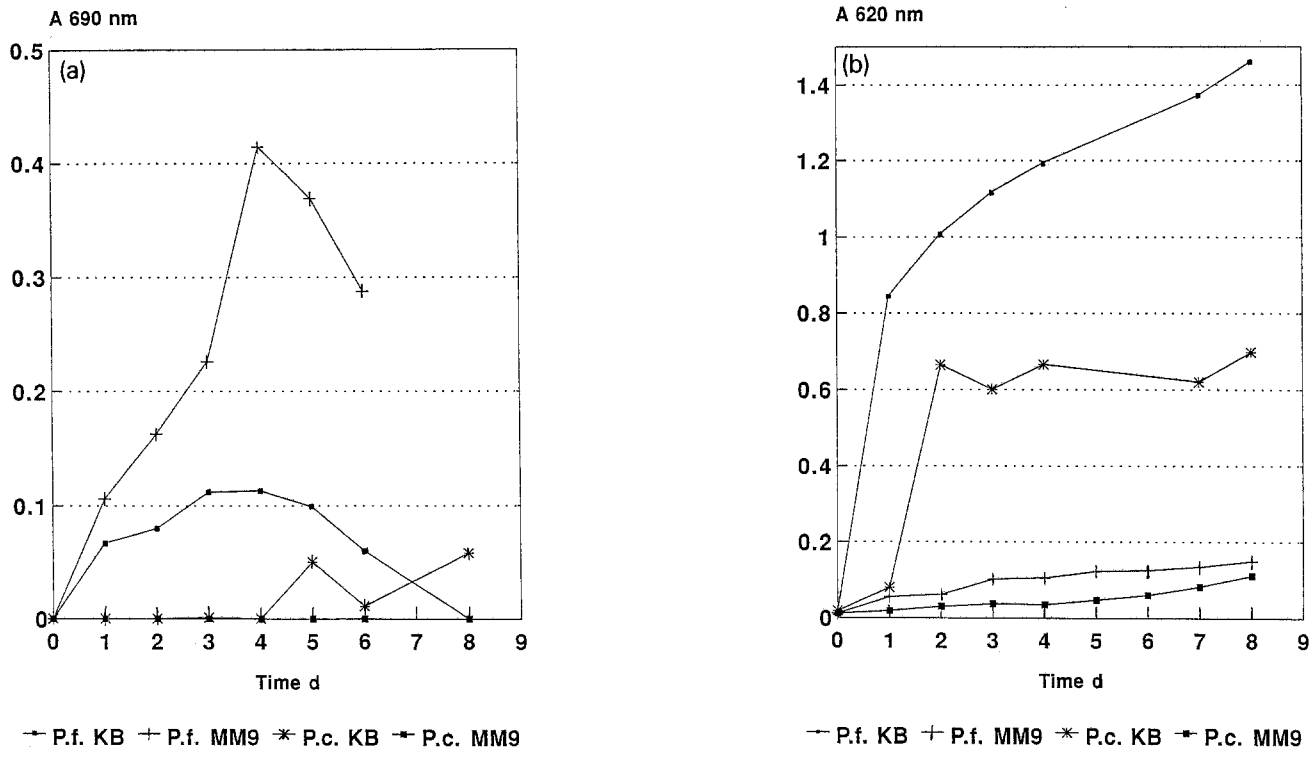


Fig. 1. (a) Siderophore production of *P. fluorescens* and *P. chlororaphis* in KB and MM9 media measured at 690 nm with CAS-reagent from the culture supernatants. (b) The growth of *P. fluorescens* and *P. chlororaphis* in KB and MM9 media measured as culture turbidity at 620 nm.

evident in modified KB medium. *P. chlororaphis* grew slower in KB medium than *P. fluorescens* and also its siderophore production was very slow and remained at low level in both media tested.

*Siderophore detection with CAS reagent in situ*

The siderophore production in situ was measured by *P. fluorescens* grown in modified KB medium added with the CAS-assay solutions of various concentrations. The results of the siderophore production of *P. fluorescens* in KB medium during 5 days of growth are shown in Fig. 2. The combination of CAS at concentration of 0.025 mM and HDTMA at 0.05 mM with 1.25 $\mu$M iron proved to be most advantageous for siderophore production and detection in situ. This combination provided the highest and fastest siderophore production in the modified KB medium (results not shown). At CAS + HDTMA concentration of 0.05 mM (CAS) and 0.1 mM (HDTMA) with 10.0 $\mu$M iron a good siderophore production could also be achieved but the growth of *P. fluorescens* was delayed (results not shown). In the media with the highest concentration of CAS + HDTMA the growth was totally inhibited and siderophores could not be detected during the incubation period (Fig. 2).

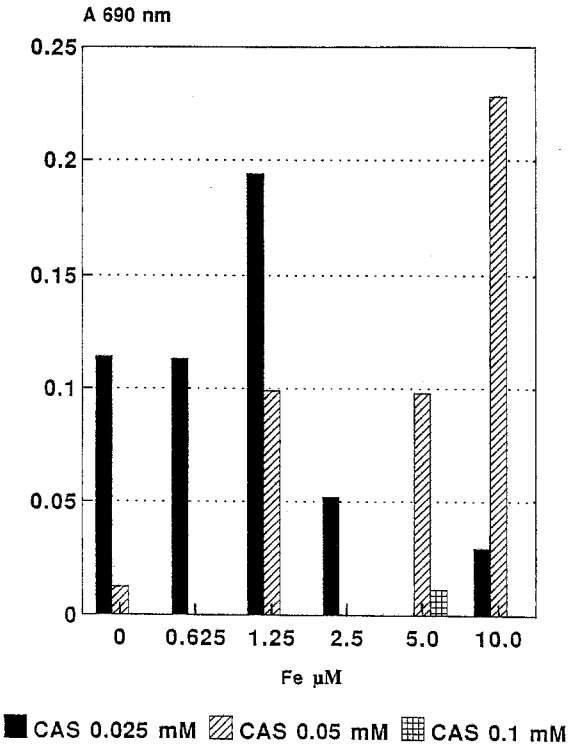


Fig. 2. Production and detection of siderophores in situ in modified KB medium at different concentrations of CAS and iron.

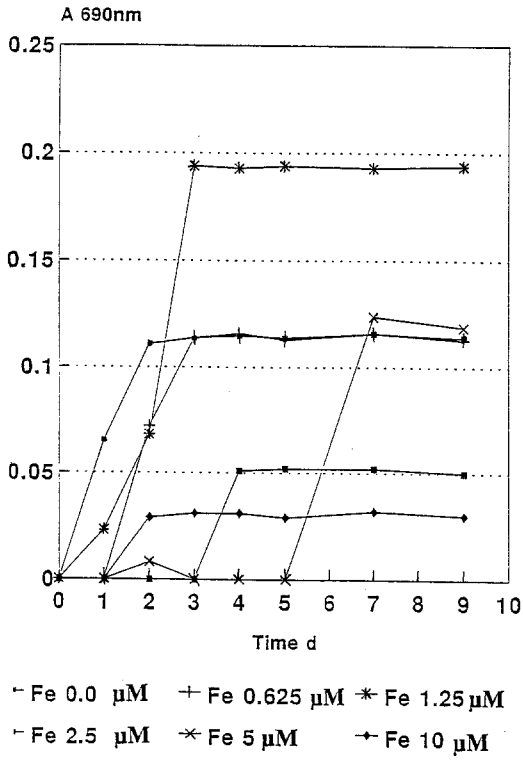


Fig. 3. The optimization of iron levels for siderophore production during different growth phases of *P. fluorescens* (0.025 mM CAS) in KB medium.

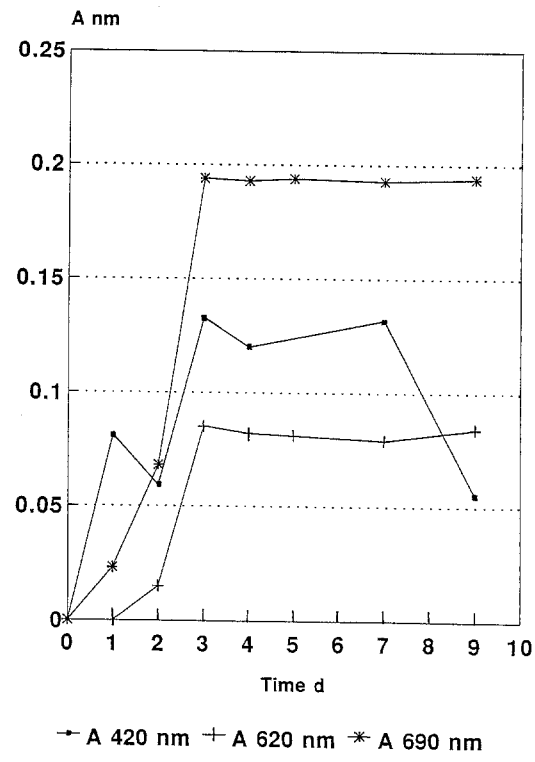


Fig. 4. The in situ detection of *P. fluorescens* siderophores by the CAS assay (0.025 mM CAS, 1.25 µM Fe) at different wavelengths.

Siderophores could be detected in all media which contained 0.025 mM CAS and 0.05 mM HDTMA, although the amount and appearance of the siderophore varied significantly (Fig. 3). The highest siderophore production was detected in a medium containing 1.25 µM iron after 3 days growth. Lower or higher amounts of iron resulted in to either late appearance or decreased amounts of siderophores. The present concentration of iron apparently ensured sufficient initial growth but did not prevent the production of siderophores.

The detection of siderophores was compared in the media containing 0.025 mM CAS, 0.05 mM HDTMA and 1.25 µM iron at different wavelengths to test the sensitivity of the measurement. The greatest change in absorbance for the CAS reagent was clearly observed at 690 nm. The absorbance differencies at 620 nm and 420 nm were essentially smaller than at 690 nm (Fig. 4).

## DISCUSSION

The chemical method of Schwyn and Neilands [11] to measure the existence of siderophores is based on an iron-chelating colour reagent, which changes the colour from blue to orange during iron removal [11]. The assay has become a universal tool for siderophore analysis and it is commonly utilized for the detection of new siderophores of biomedical and agricultural interest. The CAS assay of liquid supernates of cultures has been stated to be quantitative [11]. Also, the transfer rate of the ferric ion from the CAS complex to the siderophore gives important clues regarding the nature of the siderophore, hydroxamates being relatively slow compared to catechols [11]. Using catechols, enterobactin and vibriobactin, the colour change takes place in minutes, whereas with hydroxamates, such as desferrioxamine B, the process may take from one to several days.

The detection of siderophores from culture supernatants according to the method of Schwyn and Neilands [11] has been used for measuring the iron-binding capacity after the production of siderophores and not during their production in liquid cultures. In this work the production of siderophores was successfully carried out using a new quantitative in situ detection method. The detection of siderophores on agar media can be compared to the present method because it indicates the production of siderophores during growth by the colour change on the agar. The *Pseudomonas* strains used in these experiments were chosen because in the previous paper [10] they

strongly inhibited the mycelial growth of shiitake (*Lentinula edodes*) and also showed a positive reaction on the CAS agar test for siderophore production. In fact, the agar method can be easily used for screening purposes because the dye is incorporated into the agar medium and strains producing siderophores can be detected by orange-coloured halos [11]. However, quantitative measurements of siderophore production on the agar test is not possible.

The detergent HDTMA has been reported to play a crucial role in the interference of the CAS assay. Too low concentrations of the detergent led to precipitation of the dye and too high concentrations are toxic to many bacteria [11]. The detergent, however, is needed to disperse the triphenylmethane reagent [7]. The toxicity of HDTMA does not play any role in the detection of siderophores from liquid culture media because the reagents are added to the withdrawn supernatant. However, in the agar test and in the present application, the toxicity of reagents are of vital importance. Preliminary experiments showed that HDTMA was very toxic towards *P. chlororaphis*. Other detergents like zwitterionic 3-(dimethyl-dodecyl-ammonium)-propane-sulphonate have been proposed instead of HDTMA [7], and indeed, preliminary experiments with *P. chlororaphis* and this detergent have shown to be very promising (unpublished data).

The MM9 medium sets out certain limits to the assay mainly because several strains do not grow in this media or their growth is very slow. The basic of MM9 medium contains Tris buffer, casaminoacids, thiamine and succinate recommended to be further supplemented with other additives and, therefore, modifications may be necessary to perform [11]. The interference of KB medium with the CAS assay has also been observed [2,7]. KB medium is low in iron and contains a basic mixture of proteose peptone, magnesium sulphate and phosphate. However, phosphates may act as interfering chelates and precipitation agents [2,5]. Consequently, a modified KB medium without phosphate was used in this study. Furthermore, it has been stated that the stability of the blue indicator CAS is especially sensitive at a neutral pH range [11]. In the present study a pH range of 6.0–6.8 was used.

The right balance between iron concentrations and the CAS-complex were found to be important for optimal siderophore production and detection. Too high concentrations of iron reduced the need for siderophore production by the organism. Too low concentrations, on the other hand, slows down the initial growth of the organism. It has been reported that slowly growing cultures make smaller halos on the siderophore agar plates than fast growing ones [11]. It seems that the growth rate of the organism has to be secured by nutrients to be sufficiently rapid to result in deprivation of iron.

It is well known that the CAS assay is usually measured at 630 nm due to the fact that most siderophore types exhibit more on less linear absorbance at 630 nm vs. concentration of chelation [11]. The absorbance spectra of the CAS reagent was also analysed because in the present study the production of siderophores were studied in situ. Since the results clearly showed that the absorbance differences at 690 nm were greater than at 630 nm, this absorbance wavelength was chosen as the measurement point for detection of *Pseudomonas* siderophores in situ. Thus, the present results clearly indicates that the reactions with studied organisms was more sensitive at 690 nm and in future studies there seems to be a need for individual investigation for various organisms.

Applications of purified siderophores will have an important role in the future. The use of *Pseudomonas* bacteria for biocontrol purposes have already been utilized efficiently but isolated siderophores have much broader application areas. As an example, desferoxamine has for many years been successfully used in human medicine to treat patients with iron toxicity. The use of purified siderophores as bacteriostatic or fungistatic agents in combination with other antibacterial factors will certainly raise great interest. However, although the production steps of various siderophores have been reported much work still remains in the development of the detection methods, as well as optimized production and utilization of different siderophores.

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