Revista Latinoamericana de Microbiología

Volumen *Volume* **44** **Número** *Number* **3-4** **Julio-Diciembre** *July-December* **2002**

Artículo:

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Revista Latinoamericana de MICROBIOLOGÍA
ALAM

Vol. 44, No. 3-4
July - September. 2002
October - December. 2002
pp. 112 - 117

ORIGINAL ARTICLE

Evaluation of the siderophores production by *Pseudomonas aeruginosa* PSS

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ABSTRACT. Siderophores are compounds secreted under low iron stress, that act as a specific ferric iron chelate agents and due to their potentialities in the biological control of phytophatogenic fungi and bacteria their study have been stimulated in recent years.
Siderophores produced by different *Pseudomonas* species have been widely studied as biological agents and it is an alternative to take into account in the control of phytopathogenic microorganisms in agriculture. The purpose of this paper was the study the influence of some culture medium, and the iron concentration in the production of this metabolite.
The experiments were carried out in a conventional batch system in succinate, glucose and glutamic medium. The highest metabolite concentration was obtained in glucose and glutamic medium.
The increase of Fe(III) concentration, had a negative effect in siderophores production, specially above 10 μM.
The evaluation of the studied medium lead to the conclusion that it is possible to increase the production of this metabolite by the strain of *Pseudomonas aeruginosa* PSS, in a glutamic medium without iron addition.

Key words: *Pseudomonas aeruginosa, siderophores, pyoverdin,* iron.

RESUMEN. Los sideróforos son compuestos que se producen en condiciones de hierro limitante que actúan como agentes quelantes específicos del ion férrico, y debido a las potencialidades que tienen en el control biológico, de hongos y bacterias fitopatógenas, han despertado gran interés en los últimos años.
La producción de sideróforos por *Pseudomonas* spp., es una alternativa a tener en cuenta para la preparación de productos biológicos, en el control de microorganismos fitopatógenos en la agricultura, de ahí que el propósito fundamental de este trabajo, fuera el estudio de diferentes medios que influyen en la producción de este metabolito, y la concentración de hierro para la producción de sideróforos por la *Pseudomonas aeruginosa* PSS.
Las experiencias se llevaron a cabo en un sistema batch convencional en los medios: succinato, glucosa y glutámico, encontrándose que las mayores concentraciones finales del metabolito se obtienen en los medios glucosa y glutámico.
La adición de concentraciones crecientes de Fe (III) al medio, provocó un efecto negativo en la producción de sideróforos, el cual se acentúa a partir de 10 μM.
La evaluación de los medios antes mencionados nos permitió llegar a la conclusión de que es posible incrementar la producción de este metabolito por la cepa de *Pseudomonas aeruginosa* PSS empleando el medio glutámico sin adiciones de hierro.

Palabras clave: *Pseudomonas aeruginosa, sideróforos, pioverdina, hierro.*

INTRODUCTION

The introduction of biotechnology products into agriculture have been improved in order to increase yields and crop quality, extremely important for developing countries.[4]

Recently there has been an increasing interest in the use of biological control and siderophores produced by several of the fluorescent pseudomonas, as an alternative to take into account due to the fact that they reduce the rhizospheric population of phytopathogenic fungi and bacteria.[2,8]

Siderophores are thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth.[5,6,17]

Siderophores production by strains of *Pseudomonas* spp., as a constituent of biological products, for plant disease control, is of great interest because its possibilities in the substitution of chemical pesticides.[12]

The increase and eventual commercialization of fluorescent pseudomonads as biocontrol agents depend on in part to the understanding of the mechanism involved in the antagonist interactions between bacteria, pathogen and host plant.[19]

Pseudomonas spp. have been employed efficiently as biocontrol agents and present time there are some commercial products in the market,[20] nevertheless, the applications of purified siderophores, as bacteriostatic or fungistatic agents in combination with other antibacterial factors will certainly raise a great interest.[18]

In previous research, we have selected the strain *Pseudomonas aeruginosa* PSS, due to its higher siderophores production . In this paper, our purpose was to study the influence of some culture medium and iron concentration in the production of this metabolite by *Pseudomonas aeruginosa* PSS.

MATERIALS AND METHODS

Bacterial strain

Pseudomonas aeruginosa PSS from the culture collection of Cuban Institute for Research on Sugar Cane by-

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Products, was used in the experiments and maintained as lyophilized powder.

Fermentation conditions

To initiate growth of Pseudomonas aeruginosa PSS a lyophilized culture was placed onto sterile Kings Medium B agar[10] and incubated for 24 hours at 30°C. The culture was transferred to seed broth (200 mL of Kings Medium B) contained in a 500 mL Erlenmeyer flask and incubated at 30°C on a rotary shaker (175 rpm) for 6-8 hours.

A 500 mL Erlenmeyer flask containing 200 mL of the same seed medium was incubated as specified above.

The seed culture was transferred to a 5 liter fermenter containing each one 3.5 liter of the three liquid media described in Table 1 (pH 7).

The effect of iron concentration in the medium on siderophores production was studied by adding $FeCl_3$ in increasing amounts of 1,10, 100 and 248 µM to the glutamic medium.

All glassware was cleaned in 6M HCL to remove residual iron and rinsed in deionized water.

Assay in liquid cultures

Bacterial growth was estimated directly by spectrophotometric measurement of the OD600 (Amax) using a PM 2A spectrophotometer and dry biomass concentration (bmax) Changes in medium pH were monitored simultaneously.

Succinic acid was determined by HPLC with HYPERSIL 50 DS column in water at flow rate of 0.6 mL/min pH 2.5 at OD_{210}; glutamic acid was determined by procedure of Greenstein and Winitz;[9] and glucose by the procedure of 3,5 dinitrosalicilic acid.[15]

Siderophores assay

The amount of siderophores excreted into the culture medium was determined by spectrophotometry. Concentration was calculated using absorption maximum and the molar absorption coefficient (λ_{max}= 400 nm and ε= 20 000 $M^{-1}cm^{-1}$) according to the method of Meyer and Abdallah.[14]

Bioassay of cell free supernatant for activity against Sclerotium rolfsii in vitro.

A 5 mm diameter mycelial disk taken from an actively growing colony of *Sclerotium rolfsii* (grown on Sabouroud maltosa agar) placed in the center of a 9 cm diameter petri dish containing Sabouroud Maltose Agar and 15 mL of sterile filtered supernatant. Dishes were incubated at 300C and micelial diameter was measured for 7 days. In the control sample the sterile filtered supernatant was replaced by equal volume of sterile water.

Kinetic analysis

Biomass/substrate Yield (Yx/s) was calculated as gram of dry biomass/gram of substrate; product/substrate yield (Y p/s) as µmoles of siderophores/g substrate, and productivity (P) as siderophores concentration/time interval between inoculation and the end of the growth period.

Table 1. Composition of culture media.

Compounds gL-1	Media			
	King B	Succinate	Glucose	Glutamic
K_2HPO4	—	6	0.56	
KH_2PO4	—	3	—	
$(NH_4)_2 SO_4$	—	1	—	
$Mg SO_4 . 7 H_2 0$	—	0.2	—	
Sodium succinate	—	4	—	
Glycerin	10	—	—	
Proteose -Peptone	20	—	—	
$Mg SO_4$	1.5	—	—	
Glucose	—	—	10	
Urea	—	—	0.85	
Glutamic acid	—	—	—1	
$(NH_4)_2 NO_3$	—	—	—	1
Na_2SO_4	—	—	—	0.02
NaCl	—	—	—	0.02

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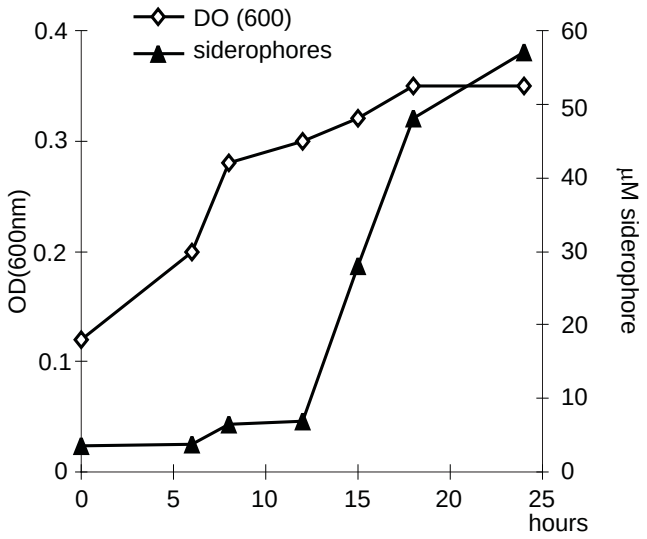

Figure 1. *Growth and production of siderophores by* Pseudomonas aeruginosa *PSS in succinate medium.*

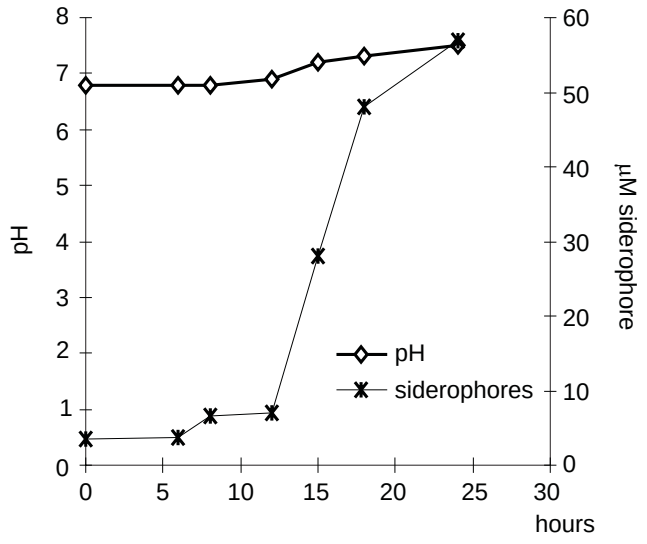

Figure 2. *pH changes and siderophores production in succinate medium during growth period of* Pseudomonas aeruginosa *PSS.*

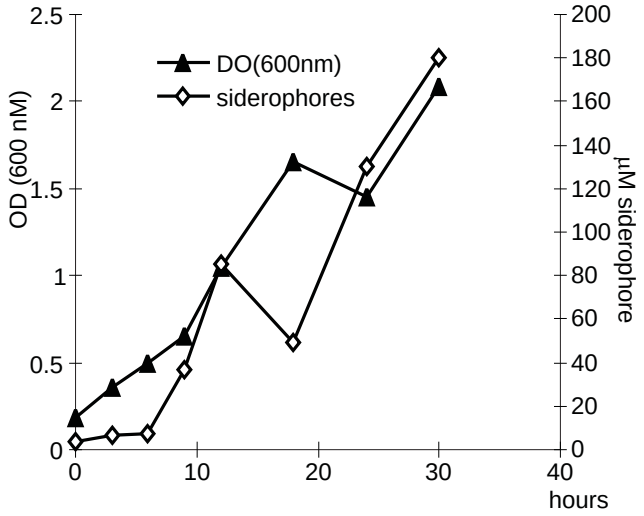

Figure 3. *Growth and production of siderophores by* Pseudomonas aeruginosa *PSS in glucose medium.*

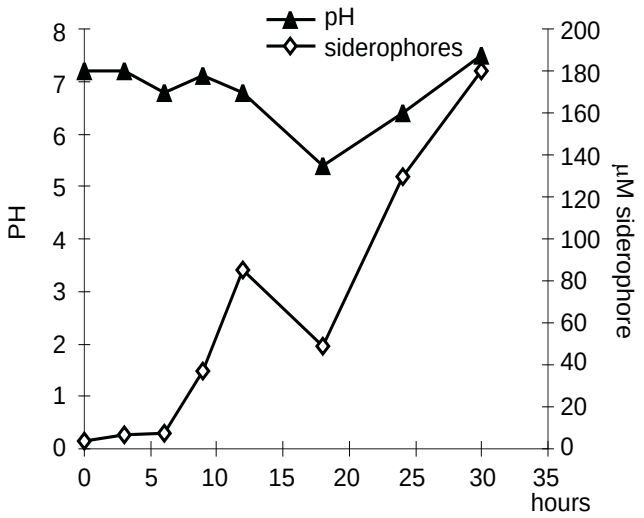

Figure 4. *pH changes and siderophores production in glucose medium during growth period of* Pseudomonas aeruginosa *PSS.*

Results and discussion

The clearest siderophores production was detected in succinate medium and continue during the logarithmic phase in parallel with growth (Fig.1). Final siderophore concentration achieved was almost 60 µM.

In this medium specific growth rate (µ) was 0.07 h-1, lower than that reported by Champomier-Verges et al[5] with *Pseudomonas aeruginosa* PA01 with maximal growth (OD_{650}) of 0.650 h-1, suggesting that succinate was used by *Pseudomonas aeruginosa* PSS more efficiently in siderophore synthesis than in growth, presumably due to the influence of succinate in the synthesis of this metabolite. This proposal is based on the structure of pyoverdine, in which the 3-amino moiety of the chromophore is substituted with various acyl groups derived from succinate, malate o α-ketoglutarate.[11,7]

The change in succinate medium pH during the growth period on succinate medium is shown in Fig. 2. The pH of the medium increase a little bit from 7 to 7.5 although siderophores production was increased during the growth period.

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In glucose medium, growth increase until 30 hours of incubation (Fig.3) with an specific growth rate (μ) of 0.1394 higher than in succinate medium, presumably because glucose is a simple sugar capable to entry in metabolic pathway.

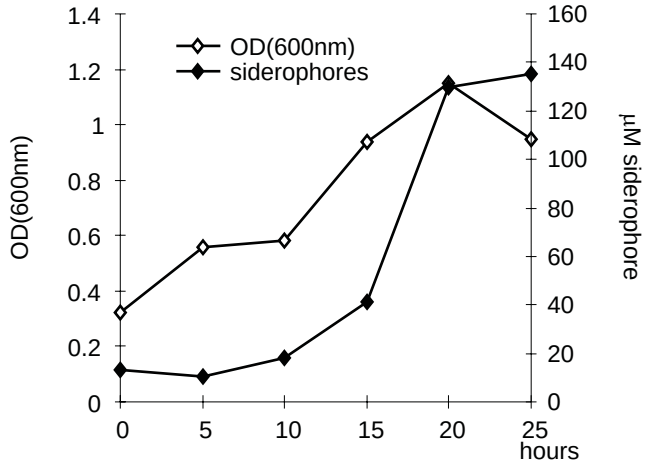

Figure 5. *Growth and production of siderophores by Pseudomonas aeruginosa* PSS in glutamic medium.

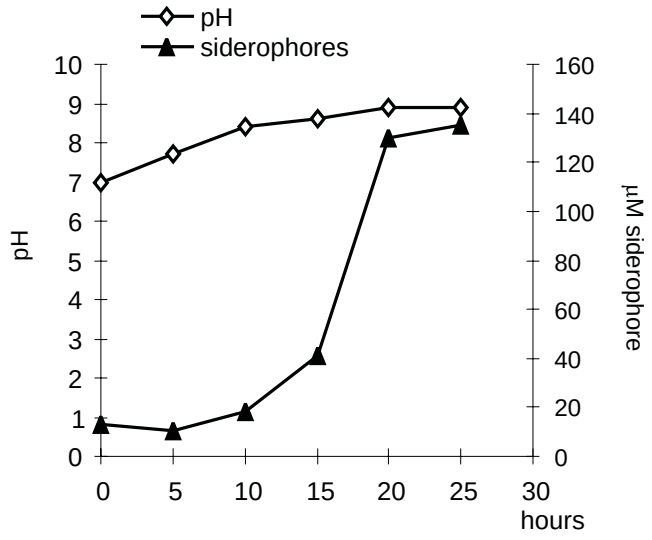

Figure 6. *pH changes and siderophores production in glutamic medium during growth period of* Pseudomonas aeruginosa *PSS.*

Table 2. Yield of the biomass formation (Y x/s) and siderophores (Y p/s) respect to substrate consume and productivity (P μmolesL^{-1}h-^{1}).

Medium	Y x/s	Y p/s	P μML^{-1}h-
Succinate medium	0.057	3.37	2.37
Glucose medium	0.74	10..3	7.5
Glutamic medium	0.65	128	5.75

The change in pH glucose medium during the growth period was also measured (Fig. 4). The pH of medium decreased from 7 to 5.5 in 18 hours in accordance with siderophores concentration which was lowered from 80 μM to 50 μM. After that time, the pH medium shifted from 5.5 to 7, which correlated well with a high level of siderophores concentration of 180 μM at the end of the growth period.

We interpreted the changes of pH as a result of glucose metabolism and the lowered pH as a consequence of acidic pH due to the destruction of the compound, in correspondence to Budzikiewicz[1] who pointed out that pyoverdins are rather labile especially in the presence of acid or O_2.

Growth and siderophores production in glutamic medium, are shown in Fig. 5. Siderophores were produced in parallel to growth and were less than in glucose medium with an specific growth rate (μ) of 0.064 consistently with a glutamic minimum medium with glutamic acid as a sole carbon source in a low concentration of 1 gL-1. Maximum siderophores value of 140 μM was achieved after 25 hours.

The pH of the medium increased from 7 to 8.5 during the growth period in accordance with the siderophores concentration (Fig. 6), which suggest that alkalinity is important to avoid siderophore destruction, as has been previously stated.[1]

The comparison between biomass yield and siderophores productivity are shown in Table 2.

The highest Y x/s was obtained in glucose medium (0.74) while the highest Y p/s was obtained in glutamic medium presumable due to the presence of glutamic acids in a culture medium that induces siderophore production, as others amino acids in agreement with Casida.[3]

Taking into account that our main objective is the production of siderophores and the highest yield product/substrate was obtained in glutamic medium, we decided to carried out the study of the effect of iron over siderophores production in this medium.

Effect of iron in the siderophores production

Siderophores are iron-specific compounds which are secreted under low iron stress and which capture iron from the environment.[1] On the other hand, the biosynthesis and secretion of siderophores are strictly regulated by environmental factors, of which iron concentration is the most important.[13] Taking into account this factor, we carried out the study of increased additions of Fe(III) to the glutamic medium on the siderophore production.

As shown in Fig. 7 and Fig. 8, although cell growth reached a maximal value above 10 μM added Fe(III) siderophores biosynthesis was lowered at this concentration,

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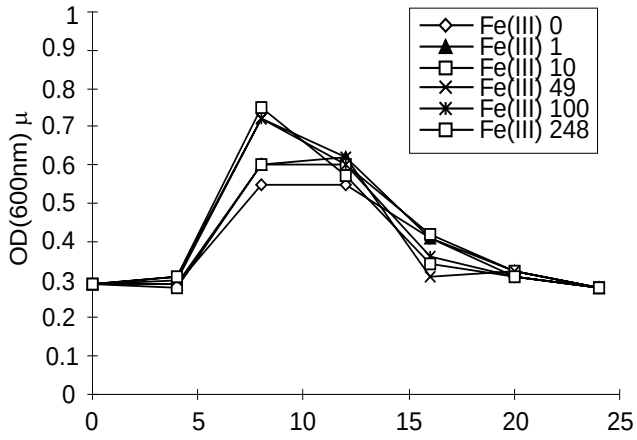

Figure 7. *Growth of* Pseudomonas aeruginosa *PSS as a function of Fe(III) added to the glutamic medium.*

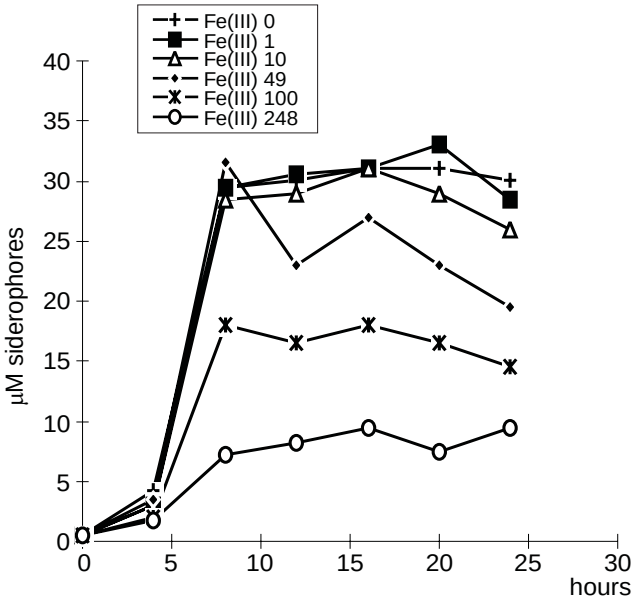

Figure 8. *Siderophores production as a function of Fe(III) added to the glutamic medium.*

Table 3. Influence of increasing Fe(III) concentration in glutamic medium on antifungal activity of cell free supernatant against Sclerotium rolfsii *in vitro*.

Concentration of Fe(III) (µ M)	Inhibition of micelial diameter (%) (24 hours)	Inhibition of micelial diameter (%) (48 hours)
0	35	27
1	20	20
10	20	13
49	17	15
100	17	17
248	20	15

since cell growth and the siderophores production are inversely proportional responses.

Iron concentration of 10 µM is considered to be high and generally results in excellent cell growth with only modest yields of siderophores.[16] Nevertheless, Manninen and Mattila-Sandholm,[13] reported siderophores production at an iron concentration of 50 µM. In our study, siderophores production was still evident at an iron concentration of 248 µM, while a maximum amount was obtained without the addition of Fe(III) according those results of Mattila-Sandholm.[13]

Antifungal activity

Antifungal activity of cell free supernatant against *Sclerotium rolfsii*, as a function of Fe(III) concentration in glutamic medium, are presented in Table 3. The antifungal metabolites inhibited the mycelial diameter of *Sclerotium rolfsii* without Fe(III) and with high Fe(III) concentration (248 µM), pointed out that this antifungal activity is not due to the production of siderophores.

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