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SYNTHESIS OF SIDEROPHORES BY SOIL BACTERIA OF THE GENUS *Pseudomonas* UNDER VARIOUS CULTURE CONDITIONS

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Abstract. The ability of six strains belonging to the genus *Pseudomonas* isolated from the rhizosphere of wheat to produce pyoverdin was examined. The studied strains demonstrated a varied level of production of the siderophore, depending on the culture conditions. The highest level of pyoverdin was determined after 72 hours of growth at 20-25°C in iron-free medium supplemented with succinate. The synthesis of pyoverdin by all the strains studied was strongly repressed by the addition of iron ions (III) to the growth medium. Calcium, cadmium and magnesium ions stimulated the synthesis of the siderophore examined, whereas zinc and lead ions partially decreased its level. Enrichment of the growth medium in cobalt ions completely inhibited the synthesis of siderophores as well as growth of the bacteria.

Key words: siderophore, pyoverdin, iron limitation, *Pseudomonas*, soil bacteria

INTRODUCTION

Most strains of bacteria, including actinomycetes, and also fungi produce siderophores under iron limitation conditions. Siderophores are non-porphyrin, non-protein compounds that bind iron and their synthesis is repressed when this element is abundant [Neilands 1995]. Siderophores enable bacteria to take up iron under conditions of limited availability of the element in the environment. They are responsible for the dissolution, chelation and transport of iron (III) into the cell. Although iron accounts for about 4% of the total content of minerals in the earth's crust, under aerobic conditions or in alkaline or neutral environment it occurs in the form of complexes that are refractory to solubilization, which makes the element little available for organisms. The requirement of bacterial cells is not high and averages about 3×10^{-7} M, but the rhizosphere does not contain sufficient free iron (III) ions to allow their survival [Budzikiewicz 1993, 1997]. It, therefore, seems that in aerated soils, with pH above 7,

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the availability of iron is related to a high degree to the presence of siderophores. These chelators, secreted by microorganisms, also play a particularly important role in regulating the amount of assimilable iron in the rhizosphere of plants, by increasing the concentration of available iron in the immediate vicinity of the plant roots. Siderophores secreted by bacteria of the genus *Pseudomonas* are the focus of particularly intense studies. It is thought that the synthesis of siderophores by these bacteria is one of the main factors inhibiting the growth and development of bacterial and fungal pathogens [Leong 1986, Sharma and Johri 2003a, Bano and Musarrat 2004]. Fluorescing strains of this bacterium secrete pyoverdin, which is also known as pseudobactin, a yellow-green pigment that is capable of chelating iron. *Pseudomonas* strains can also secrete other siderophores, the best known of which is pyochelin, a siderophore with lower affinity for iron (III) ions than pyoverdin and probably has no biological activity with regard to plant pathogens. In terms of structure, pyochelins are derivatives of salicylic acid [Cornelis and Matthijs 2002]. Pyoverdins comprise a group of siderophores with similar structure, which contain a cyclic or linear oligopeptide linked to dihydroxychinone chromophore and dicarboxylic acid or amide. Differentiation within this group of compounds involves the peptide component of a siderophore. Pyoverdins differ from other siderophores in exceptionally strong affinity for iron (III) ions and high stability of the complexes formed [Meyer 2000, Bultreys *et al.* 2001, Meyer *et al.* 2002]. The literature indicates that the secretion of siderophores can be regulated by a number of factors, including carbon source in the growth medium and temperature [Duffy and Defago 1999, Djibaoui and Bensoltane 2005]. However, the results of studies carried out so far point to the homogeneity of the mechanisms determining the level of pyoverdin secreted by bacteria belonging to the genus *Pseudomonas*. In the soil, the natural habitat of these bacteria, there are several variable factors that can modify the level of released pyoverdin. For this reason the objective of these studies was to compare the ability of six different strains of *Pseudomonas* bacteria isolated from the rhizosphere of winter wheat to synthesize siderophores under various culture conditions. In recent years growing interest in the agriculture has been observed in non-pathogenic rhizospheric strains of bacteria with properties that would allow their use as biopesticides [Handelsman and Stabb 1996, Raupach and Kloepper 1998]. Biopesticides can be an excellent alternative for the plant protection chemicals, that are both costly and damaging for the environment. The research demonstrates that their efficiency is very high. Particularly useful as the biospecimen are the natural, non-pathogenic rhizospheric microorganisms capable of secondary metabolite synthesis, including the siderophores, which have a favorable influence on the plants. Especially great attention is paid to the *Pseudomonas* strains, which synthesize the pyoverdine, because of its significant biological activity [Nagarajkumar *et al.* 2004]. The results achieved in this research will help to prepare the material to purify and characterize the pyoverdine synthesized by the bacteria strains used in the experiment. Studies focused on the ability of these bacteria to synthesize siderophores can contribute to a more complete elucidation of the favorable effect of most *Pseudomonas* strains on the growth and development of plants.

MATERIAL AND METHODS

The studies involved a total of six strains representing the genus *Pseudomonas*, isolated from the rhizosphere of winter wheat. The strains were identified based on morphological and biochemical characteristics, with the use of Microgen (UK) tests that include 24 biochemical traits. The isolated and identified strains were maintained and stored on KING B medium supplemented with nitrofurantoin. The strains used in the experiments were *Pseudomonas putida*: P1, P2 and P3 and *P. fluorescens*: F1, F2 and F3.

All the laboratory glassware used in the experiments was left for 24h in a 2M solution of hydrochloric acid in order to remove all iron ions and then washed a few times in deionized water. All culture media were also prepared using deionized water.

The bacteria were grown in iron-deficient succinate medium, pH 7,0, as described elsewhere [Meyer and Abdallah 1978] which consists of 6 $g \cdot dm^{-3}$ K_2HPO_4, 3 $g \cdot dm^{-3}$ KH_2PO_4, 1 $g \cdot dm^{-3}$ $(NH_4)_2SO_4$, 0.2 $g \cdot dm^{-3}$ $MgSO_4 \times 7H_2O$, 4 $g \cdot dm^{-3}$ succinic acid. The cultures were maintained on the same medium in agar plates. In the experiment researching the influence of the carbon source on the effectiveness of pyoverdine secretion succinic acid was replaced by glycerol or mannitol.

The cultures were grown for 72 hours at 25ºC in a rotary shaker. The growth of the bacteria was monitored by measuring the optical density of the cultures at 550 nm.

Siderophores were detected using the universal Chrome Azurol S test [Schwyn and Neilands 1986]. Quantitative determinations of pyoverdin were made photometrically at 400 nm according to Meyer and Abdallah [1978]. The productivity of pyoverdin was determined as the ratio A_{400}/A_{550}.

RESULTS

The universal Chrome Azurol S test detected the presence of siderophores in the case of all the strains tested. The effect of the medium composition on the level of siderophore synthesis by the *Pseudomonas* strains covered by the study was examined (Table 1, Fig. 1).

Table 1. Effect of the composition of culture medium on the amount of pyoverdin secreted by the strains studied
Tabela 1. Wpływ składu podłoża hodowlanego na ilość wydzielanej przez badane szczepy piowerdyny

Strain Szczep	Medium supplemented with 0.4% – Pożywka wzbogacona o 0,4%					
	Succinate – Bursztynian		Glycerol – Glicerol		Mannitol	
	A400	A550	A400	A550	A400	A550
P1	0.20	0.50	0.10	0.85	0.05	0.55
P2	4.15	1.10	1.50	1.36	0.76	1.31
P3	5.65	1.30	2.00	1.40	0.54	1.48
F1	2.20	0.94	0.90	0.99	0.93	1.12
F2	1.50	0.70	0.70	0.75	0.80	0.99
F3	1.00	0.62	0.35	0.51	0.72	0.92

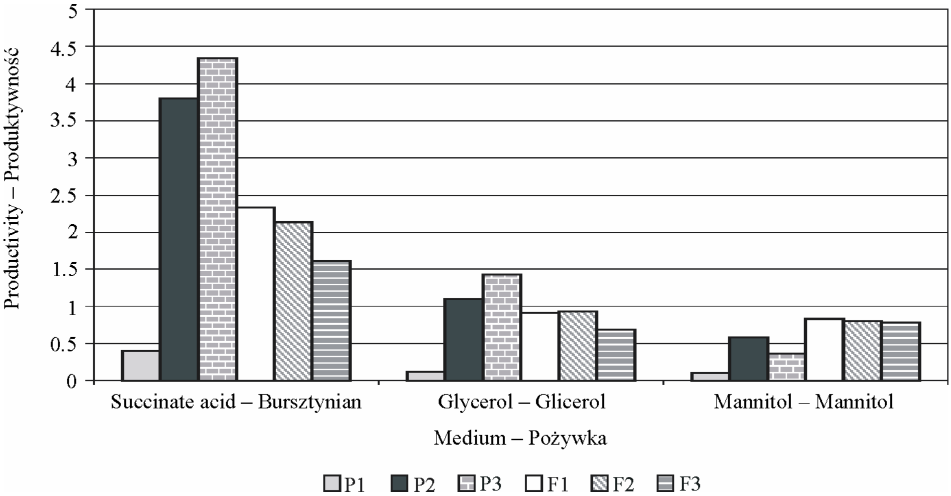

Fig. 1. Effect of the composition of culture medium on the amount of pyoverdin secreted by the strains studied
Rys. 1. Wpływ składu podłoża hodowlanego na ilość wydzielanej przez badane szczepy piowerdyny

In these experiments the highest productivity of pyoverdins was found in the media supplemented with succinic acid. The growth rate of the bacteria was higher in media with glycerol or mannitol but the cells secreted far fewer siderophores. Regardless of the type of the medium, two of the strains, *Pseudomonas putida* P2 and P3, were characterized by the highest siderophore synthesizing capacity. The intensity of pyoverdin synthesis by these strains was a dozen or so times higher than for the *P. putida* strain P1. The intensity of pyoverdin synthesis by the strains studied also depended on the length of the bacterial culture (Table 2, Fig. 2). Intensive secretion of the pyoverdine was observed on the third day of bacterial breeding. The strains showed systematic increase in siderophore productivity between 24 and 72 hours of culture, followed by a slight drop of the amount of the siderophores studied in the culture medium. This tendency was visible for all the six strains studied.

Table 2. Effect of bacteria cultivation period length on the amount of secreted pyoverdin
Tabela 2. Wpływ czasu hodowli bakterii na ilość wydzielanej piowerdyny

Strain Szczep	Time – Czas, h							
	24		48		72		120	
	A 400	A550	A400	A550	A400	A550	A400	A550
P1	0	0.01	0.04	0.2	0.22	0.45	0.28	0.42
P2	0.36	0.345	3.51	0.9	4.25	1.10	4.1	1.0
P3	0.29	0.25	3.23	0.85	6.41	1.30	5.22	1.35
F1	0.05	0.1	1.56	0.78	2.88	0.90	2.8	0.84
F2	0.04	0.15	0.97	0.57	2.45	0.70	2.00	0.63
F3	0.01	0.15	0.40	0.41	1.14	0.60	1.00	0.6

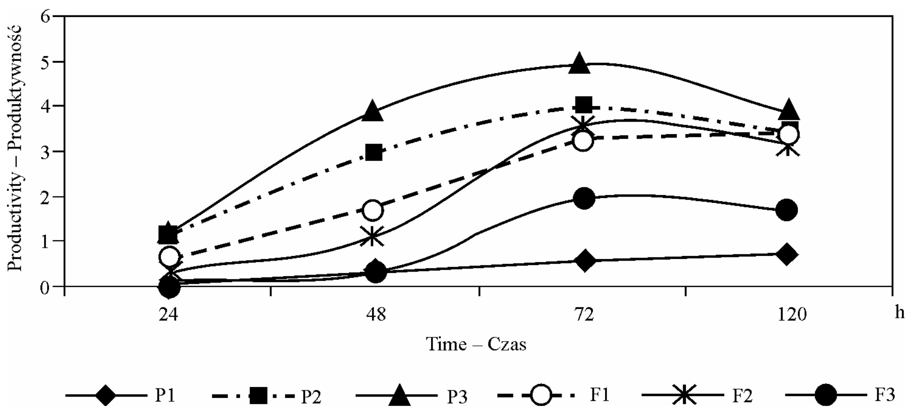

Fig. 2. Effect of bacteria cultivation period length on the amount of secreted pyoverdin
Rys. 2. Wpływ czasu hodowli bakterii na ilość wydzielanej piowerdyny

Another factor determining the level of secretion of siderophores by the strains studied was the pH of the culture medium used (Table 3, Fig. 3). All the strains synthesized the most siderophores in media with pH 6.5 and 7.0. Strains P1, P2, P3 and F3 demonstrated the greatest pyoverdin production at pH 7.0 and a somewhat lower level at pH 6.5, whereas the secretion of siderophores by strains F1 and F2 was the highest at pH 6.5 and slightly lower at 7.0. The amount of pyoverdin in culture media with pH 5.5 and 8.5 was the lowest. The changes in the level of pyoverdin secreted by strain P1 were the slightest, since this particular strain synthesized scant amounts of the siderophore under all the experimental conditions examined.

Table 3. Effect of pH of culture medium on the amount of pyoverdin secreted by the strains studied
Tabela 3. Wpływ pH podłoża hodowlanego na ilość wydzielanej przez badane szczepy piowerdyny

Strain Szczep	pH of medium – pH pożywki									
	5.5		6.5		7.0		7.5		8.5	
	A400	A550	A400	A550	A400	A550	A400	A550	A400	A550
P1	0.02	0.100	0.16	0.39	0.22	0.45	0.10	0.43	0.01	0.10
P2	0.64	0.65	4.03	1.10	4.98	1.27	2.9	1.28	1.15	0.85
P3	0.70	0.53	5.68	1.3	5.89	1.31	4.20	1.15	1.21	0.99
F1	0.10	0.580	2.24	0.87	2.31	1.04	1.17	0.65	0.5	0.560
F2	0.19	0.280	2.05	0.76	2.18	0.900	1.26	0.629	0.25	0.450
F3	0.28	0.410	1.044	0.65	1.30	0.720	0.85	0.58	0.21	0.354

In experiments in which the effect of culture temperature on the level of siderophore synthesis was examined, it was determined that most of the investigated strains synthesized the highest amount of siderophores at the temperature of 25°C (Table 4, Fig. 4). In the temperature range 30-35°C a lower level of siderophore synthesis was observed in spite of intensive bacterial growth. Also it is noteworthy that high productivity of pyoverdin was also determined at 20°C. Strains F1 and F2 did not demonstrate greater differences between the productivity of pyoverdins at the growth temperatures of 20 and 25°C. Cultures of strain F2, as opposed to all the remaining ones, did not show any significant decrease in the amount of pyoverdin secreted at 35°C as compared to the lower temperatures.

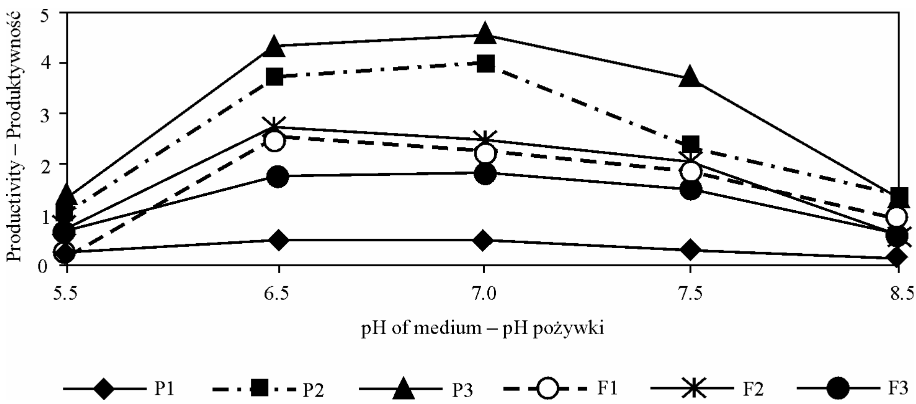

Fig. 3. Effect of pH of culture medium on the amount of pyoverdin secreted by the strains studied
Rys. 3. Wpływ pH podłoża hodowlanego na ilość wydzielanej przez badane szczepy piowerdyny

Table 4. Effect of bacterial culture temperature on the amount of pyoverdin secreted by the strains studied
Tabela 4. Wpływ temperatury hodowli bakterii na ilość wydzielanej przez badane szczepy piowerdyny

Strain Szczep	Temperature – Temperatura, °C									
	15		20		25		30		35	
	A400	A550	A400	A 550	A400	A550	A400	A550	A400	A550
P1	0.0	0.08	0.02	0.100	0.16	0.39	0.28	0.78	0.045	0.5
P2	0.33	0.30	2.55	0.68	4.43	1.10	4.12	1.2	0.62	0.62
P3	0.08	0.15	3.07	0.79	5.88	1.3	5.16	1.8	0.424	0.48
F1	0.108	0.18	1.28	0.47	2.24	0.87	2.25	0.98	0.24	0.45
F2	0.055	0.11	0.950	0.26	2.05	0.66	3.24	0.90	1.08	0.42
F3	0.002	0.11	0.47	0.34	1.044	0.5	1.53	0.89	0.084	0.53

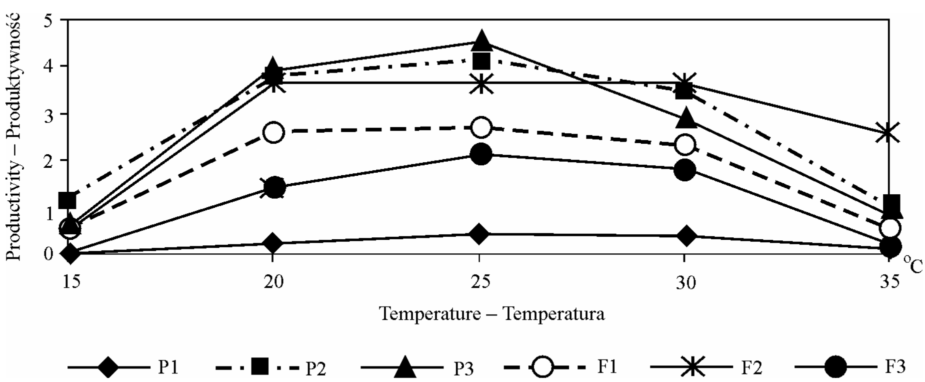

Fig. 4. Effect of bacterial culture temperature on the amount of pyoverdin secreted by the strains studied
Rys. 4. Wpływ temperatury hodowli bakterii na ilość wydzielanej przez badane szczepy piowerdyny

Enrichment of the culture medium in iron (III) ions even at low concentration (1 µM) in the case of all the examined strains resulted in a drop in the amount of siderophores synthesized from about 20 to 50% as compared to the control, not containing iron ions (Table 5, Fig. 5).

Table 5. Effect of the presence of iron ions in culture medium on the amount of pyoverdin secreted by the strains studied
Tabela 5. Wpływ obecności jonów żelaza w podłożu hodowlanym na ilość wydzielanej przez badane szczepy piowerdyny

Strain Szczep	Final concentration of Fe(III) ions – Stężenie końcowe jonów Fe(III), µM									
	Control – Kontrola		1		10		50		100	
	A 400	A550	A 400	A550	A 400	A550	A 400	A550	A 400	A550
P1	0.22	0.45	0.20	0.45	0.2	0.60	0.15	0.60	0.0	0.69
P2	4.75	1.2	4.00	1.38	3.2	1.56	1.2	1.60	0.90	1.70
P3	6.41	1.10	5.00	1.25	3.89	1.41	0.36	1.41	0.30	1.65
F1	2.88	0.82	1.20	0.98	1.3	0.98	1.10	1.10	0.50	1.25
F2	2.45	0.72	1.35	0.79	1.1	1.00	1.10	1.00	0.20	1.30
F3	1.14	0.65	0.80	0.90	0.8	1.00	0.80	1.10	0.52	1.25

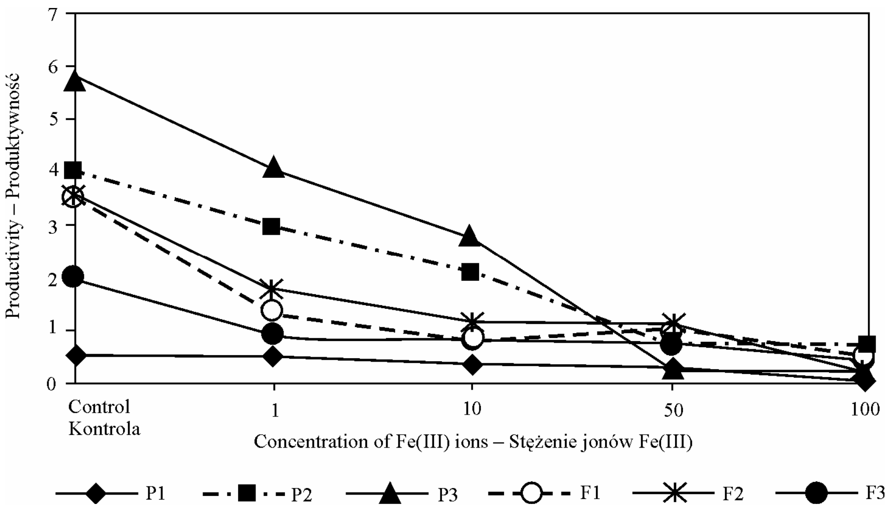

Fig. 5. Effect of the presence of iron ions in culture medium on the amount of pyoverdin secreted by the strains studied
Rys. 5. Wpływ obecności jonów żelaza w podłożu hodowlanym na ilość wydzielanej przez badane szczepy piowerdyny

A further increase in the concentration of Fe (III) ions resulted in almost complete inhibition of the secretion of siderophores by the bacterial cells. The highest, close to 100% inhibition of pyoverdin secretion in the presence of 50 µM concentration of iron ions, was observed for strain P3, which synthesized the largest amount of pyoverdins under the experimental conditions examined. A significant factor that determined the amount of pyoverdins secreted by all the six strains is the ions of selected metals in concentration 100 µM (Table 6, Fig. 6). It is noteworthy that in the case of all the strains, an increased productivity of siderophores in the presence of Ca^{2+} and Cd^{2+} and also to a somewhat lower extent in the presence of Mg^{2+} was determined. However, in the media supplemented with Zn^{2+} and Pb^{2+} a low level of siderophore synthesis, accompanied by an intense growth of bacteria, was observed. An exception were two strains, F1 and F3, in the case of which no significant changes in the production of siderophores in the presence of Pb^{2+} in the growth medium, compared to the control, were observed. In the case of media supplemented with Cu^{2+} ions, strains P1, F1, F2 and F3 fully ceased to synthesize siderophores and showed only slight growth. In media containing Co^{2+} ions both siderophore synthesis and growth of the studied strains were inhibited.

Tab. 6. Effect of metal ions on the amount of pyoverdin secreted by the strains studied
Tab. 6. Wpływ jonów metali na ilość wydzielanej przez badane szczepy piowerdyny

Strain Szczep	100 µM metal ions – 100 µM jonów metali													
	Control Kontrola		Ca		Mg		Cd		Zn		Pb		Cu	
	A400	A550	A400	A550	A400	A550	A400	A550	A400	A550	A400	A550	A400	A550
P1	0.21	0.38	0.21	0.51	0.26	0.41	0.2	0.29	0.01	0.30	0	0.10	0.00	0.00
P2	4.85	1.20	6.92	1.4	4.92	1.0	5.91	1.20	2.83	1.61	0.95	0.90	2.08	1.14
P3	5.81	1.15	7.15	1.30	6.32	1.22	6.9	1.20	2.98	1.41	2.8	1.00	2.50	1.03
F1	2.78	0.82	3.85	1.15	3.88	1.21	2.98	0.75	0.48	0.30	2.61	0.9	0.00	0.30
F2	2.31	0.70	2.30	1.00	2.71	1.10	3.10	0.71	0.36	0.28	1.9	1.00	0.00	0.10
F3	1.21	0.72	1.64	1.13	1.51	0.9	1.9	0.73	0.12	0.10	1.2	1.1	0.00	0.20

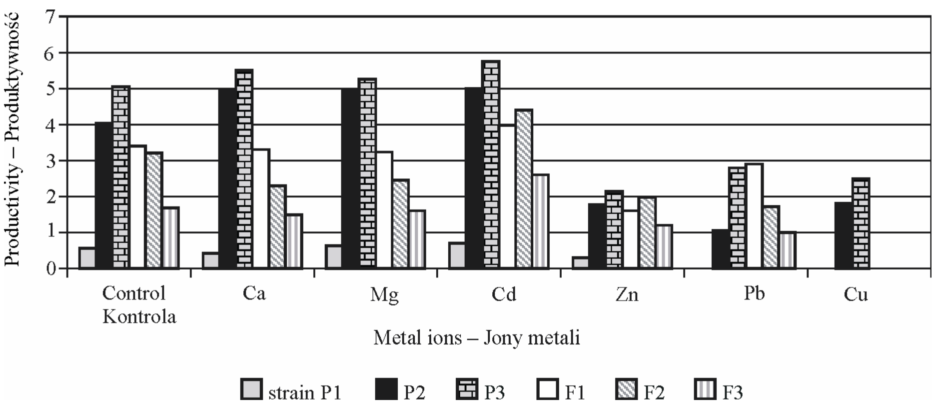

Fig. 6. Effect of metal ions on the amount of pyoverdin secreted by the strains studied
Rys. 6. Wpływ jonów metali na ilość wydzielanej przez badane szczepy piowerdyny

DISCUSSION

At low iron concentrations a fundamental role in the process of the assimilation of this element by microorganisms is played by a transport system that is highly specific for siderophores. Fluorescent bacteria of the genus *Pseudomonas* under iron-limitation conditions produce pyoverdin, a yellow-green pigment with particularly high affinity for iron ions [Budzikiewicz 1993, 1997]. The aim of the current study was to investigate the ability of six strains of bacteria representing the genus *Pseudomonas*, isolated from the rhizosphere of plants, to synthesize pyoverdin under a range of different culture conditions. All the strains selected for the study were able to synthesize pyoverdin under the culture conditions employed. However, considerable differences in the amounts of siderophores secreted by the individual studied strains were determined. The studies made indicate that the amount of pyoverdin produced by soil bacteria representing the genus *Pseudomonas* depends on a number of factors, e.g. source of carbon in the medium, temperature of the culture and presence of metal ions in the growth medium. The literature points to a significant dependence between the source of carbon in the medium and the level of pyoverdin synthesis. Succinic acid is regarded as a factor that stimulates the synthesis of this siderophore [Meyer and Abdallah 1978]. This was confirmed by the studies reported herein, since the productivity of pyoverdin in the media supplemented with succinic acid was 2-3 times higher than in the media with glycerol or mannitol. Similar observations have been made for *Pseudomonas sp*. strains by Sharma and Jorhi [2003b] and for *Pseudomonas fluorescens* by Djibaoui and Bensoltane [2005].

Intensive emission of the pyoverdine was observed on the third day of bacterial breeding. The latest research on the biosynthesis regulation of the pyoverdine by *Pseudomonas aeruginosa* and *Pseudomonas putida* shows dependency of the pyoverdine synthesis effectiveness on the bacteria culture density [Stintzi *et al*. 1998, Ren *et al*. 2005]. Coordination of the microorganisms behavior dependent on the culture density is described as quorum sensing. This phenomenon can supposedly also occur in the pyoverdine synthesis regulation by these strains *Pseudomonas.* The results of the research however, do not allow confirming or denying this statement.

The secretion of pyoverdin by the bacterial strains studied was also found to be dependent on the temperature of the culture. The optimal temperature at which the highest amounts of pyoverdin were determined was 20 or 25ºC, depending on the strain. This seems to be related to the adaptation of these microorganisms to the conditions in the soil, in which the temperature usually does not exceed 25ºC. However, the optimal pH of the medium was determined to be in the range 6.5-7.0. This phenomenon may be of particular importance in the natural environment of these bacteria in which the availability of iron in the form that is accessible to bacteria shows a considerable drop at a reaction approaching neutral values. The reduced level of pyoverdin in bacterial cultures in media with pH in excess of 7.5 can also be caused by the instability of the structure of the siderophore in alkaline solutions [Meyer and Abdallah 1978]. Similar observations have been made for *Pseudomonas sp*. strain 267 by Marek-Kozaczuk and Skorupska [1997].

As indicated in the literature, the presence of iron ions brings about the repression of synthesis of siderophores in microorganisms. The biosynthesis of siderophores is negatively regulated by the presence of iron (II) ions inside the bacterial cell and of iron (III) ions in the environment it lives in. Under conditions of abundance of iron, bacterial cells do not secrete siderophores but take this element up by the so-called low affinity system, that is via free diffusion through the cellular membranes [Leong *et al*. 1991, Venturi *et al.* 1995]. In the experiments presented in this paper, inhibition of siderophore production was observed already on the addition of 1 µM Fe (III) to the culture medium.

All the strains showed increased production of siderophores in the presence of Ca^{2+} and Cd^{2+} ions and to a somewhat smaller extent in the presence of Mg^{2+}. Conversely, in the case of media supplemented with Zn^{2+}, Pb^{2+} and Cu^{2+} ions the secretion of siderophores by the bacterial cells was inhibited. The results presented by Dao *et al.* [1999] indicate that cadmium stimulates the synthesis of pyoverdins by *Pseudomonas aeruginosa*. On the other hand, Sharma and Johri [2003b] found that the production of siderophores by *Pseudomonas* strain GRP3A increased in the presence of Zn^{2+} and Cu^{2+}. Somewhat different dependences than those observed in the current study were found in the research carried out by Carrillo-Castaneda *et al.* [2005] in which the presence of $CuSO_4$ in the growth medium did not cause any changes in the amount of secreted pyoverdin. Cobalt ions, similarly to our observations, were also a strong inhibitor of bacterial growth and siderophore synthesis by *Pseudomonas* strain 267 [Marek-Kozaczuk and Skorupska 1997]. Worth note is the phenomenon of the stimulation of the synthesis of siderophores by certain *Pseudomonas* strains in the presence of cadmium, copper or zinc, which belong to more toxic factors contaminating the surface layers of the soil. This may confirm the theory that siderophores comprise part of the response of the bacterial cell to certain stress factors [Hofte *et al.* 1989, Dao *et al*. 1999].

CONCLUSIONS

The results obtained suggest that the amount of the pyoverdin synthesized by *Pseudomonas* strains depends on many environmental factors. The synthesis of pyoverdin in all the strains researched undergoes a typical repression in the presence of iron ions (III) in the medium. For the level of iron chelates secreted the source of carbon in the culture medium is very important. It was found that the presence of succinic acid in the medium stimulates the secretion of pyoverdin by the strains researched. The greatest amount of pyoverdin was synthesized by the strains researched at the temperature of 25ºC, whereas at 30ºC there was observed less pyoverdin in the culture media despite a more intensive bacterial growth. The presence of Ca^{2+} and Cd^{2+} ions in the culture medium intensified the secretion of siderophores, while in the media enriched with Zn^{2+}, Pb^{2+} and Cu^{2+} ions there was found an inhibition of siderophores (secreted) by bacterial cells.

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SYNTEZA SIDEROFORÓW PRZEZ GLEBOWE BAKTERIE Z RODZAJU *Pseudomonas* W ZMIENNYCH WARUNKACH HODOWLI

Streszczenie. Zbadano zdolność sześciu szczepów z rodzaju *Pseudomonas* wyizolowanych z ryzosfery pszenicy do produkcji piowerdyny (pyoverdin). Badane szczepy wykazywały zróżnicowany poziom produkcji tego sideroforu w zależności od warunków hodowli. Wykazano, że syntetyzowały one największą ilość piowerdyny w 72. godzinie hodowli i w temperaturze 20-25°C, na bezżelazowej bursztynianowej pożywce. Synteza piowerdyny u wszystkich badanych szczepów podlegała silnej represji po dodaniu do podłoża hodowlanego jonów żelaza (III). Jony wapnia, kadmu i magnezu w niewielkim stopniu stymulowały syntezę badanego sideroforu, natomiast jony cynku i ołowiu obniżały jej poziom. Wzbogacenie podłoża hodowlanego w jony kobaltu całkowicie zahamowało syntezę sideroforów i wzrost bakterii.

Słowa kluczowe: siderofory, piowerdyna, deficyt żelaza, *Pseudomonas*, bakterie glebowe

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