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Affinity Purification of a Siderophore That Exhibits an Antagonistic Effect against Soft Rot Bacterium

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Abstract—Bacterial colonies were isolated from different Egyptian soil samples. From these isolates, one bacterial species was found to produce siderophore. Using classical and biochemical identification methods, the siderophore producing isolate was identified as *Pseudomonas fluorescens*. Based on the affinity of siderophores for metal ions, an affinity chromatography system was designed for the purification of the siderophore in one step. It was possible to isolate 25 mg siderophore per liter of culture media. The purified siderophore was found to exist in two forms of approximately 30 and 90 kD. They are believed to be polymers of several siderophore molecules. Both forms were found to be active against the pathogen *Erwinia carotovora* var. *carotovora*, the causal bacteria of soft rot disease on potato tubers. The advantage of this method over other purification methods is that it uses metal ion so it can be applied for the purification of the known types of siderophores. Moreover, the purification is based on affinity chromatography, so the siderophore purity state permits several biotechnological applications without further treatments.

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Siderophores (from the Greek "iron carriers") are defined as relatively low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress. The role of these compounds is to scavenge iron from the environment and to make the mineral, which is an essential metal, available to the microbial cell [1]. According to the iron-coordinating functional groups, siderophores fall into three types. These types are hydroxamates (mycobactin and exochelin), catechols (enterobactin and vibriobactin), and thiazolines (pyochelin and yersiniabactin) [2]. Most aerobic microorganisms produce at least one siderophore, and in some cases, a single bacterial strain can produce two or more [3-5].

For several fluorescent *Pseudomonades*, it has been suggested that the siderophore-mediated competition for iron with soil borne pathogens is an important mechanism for biological control [6]. *Pseudomonas fluorescens* has been successfully applied on many types of crops to control a wide variety of soil-borne pathogens, including fungi, bacteria, viruses, and nematodes. *Pseudomonas fluorescens* can be considered as a biocontrol agent of the future to inhibit the growth of root disease pathogens. It can significantly reduce the infection by a great number of bacteria, such as *Ralstonia solanacearum* in potato, *Pseudomonas solanacearum* in tobacco, *Fusarium oxyporums* in tomato, and *Erwinia amylovora* (responsible for fire blight disease) on peach and apple [7, 8].

The mode of action of siderophores in the biocontrol of plant pathogens appears to be through the induction of systemic resistance, which depends on iron-regulated metabolites. Moreover, purified siderophores have been demonstrated to induce systemic resistance in different species. Thus, the role of siderophores in the suppression of plant disease in hydroponics systems may be highly effective. First, the pathogen is weakened in the rhizosphere by a lack of iron, and subsequently it encounters a host plant that has an enhanced defensive capacity [5, 8, 9].

Due to the important biotechnological applications of siderophores [10], this study aimed to establish a new, fast,

Abbreviations: AU) arbitrary units; BPYG) agar medium containing beef (0.3%), peptone (0.5%), yeast extract (0.5%), and glucose (2%); CFU) colony forming units.

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and simple purification method. Using affinity chromatography and iron as a ligand, it was possible to purify siderophore from *P. fluorescens* in one step. According to the concentration of siderophore in the solution, it can exist in a form of approximately 30 kD and a form of approximately 90 kD. The presence of different forms does not influence the activity of the purified siderophore. According to this study, the following results are presented.

MATERIALS AND METHODS

Detection of siderophore using tooth picking technique. Tooth picking technique is considered as a typing technique used for screening bacterial isolates for their ability to produce antagonistic effect against the indicator bacteria [11]. Two BPYG (agar medium containing beef (0.3%), peptone (0.5%), yeast extract (0.5%), and glucose (2%)) agar plates were used in this test, one of them was freshly inoculated with 1% of 24-h-old culture of tested bacteria (10^8 colony forming units (CFU) per ml), using inoculation pour technique. The other plate was used as template without being inoculated with the indicator strain. BPYG agar plates were stabbed consequently using sterile clean toothpick with single colony of each of the tested bacteria. The un-inoculated BPYG agar plate was first stabbed, and then the inoculated BPYG agar plate with the indicator bacterial strain (*Erwinia carotovora* var. *carotovora*), was stabbed with the same toothpick. The previous step was repeated with different single bacterial colony using the same previous BPYG agar plate. After stabbing all the test bacteria, the two agar plates were covered and incubated at 30°C for 24 to 48 h.

Detection of siderophore using the well-cut diffusion technique [12]. One micromole of $FeCl_3$ was added to 100 ml of agar medium BPYG. The medium was then seeded with the indicator strain. Several wells were punched out in the agar and then 0.1 ml of the supernatant of the suspected siderophore-producing bacteria was transferred into the well. The plates were incubated at 30°C for 24 to 48 h. The same experiment was repeated in the absence of $FeCl_3$ and was used as control [13]. The supernatant of the siderophore-producing bacteria will give clear zones in the control plates, whereas this clear zone will disappear in the $FeCl_3$ containing media.

Identification of siderophore-producing bacteria. The bacterial isolate proved to have the ability to inhibit the indicator strain was identified according to the standard identification methods. Morphological examinations including cell shape, Gram test, spore, and acid fast staining were performed; in addition, preliminary tests including motility, growth at pH 6-8, growth at 50°C, starch hydrolysis, citrate utilization, nitrate reduction, and catalase were also carried out [14]. Biochemical tests, using api-20E kit, were performed as a confirmatory identification [15].

Growth curve and kinetics of siderophore production. The growth curve was determined by measuring the absorbance of the bacterial strain grown in BPYG broth medium. Two flasks each containing 50 ml of sterile BPYG broth were inoculated with 0.5 ml of 24-h-old culture of bacterial strains growing in BPYG broth. Directly after inoculation, the absorbance was measured at 550 nm. The bacterial culture was incubated at 30°C with agitation at 1006*g* in a shaking incubator. After 1-h interval, the siderophore activity was assayed using the agar well-cut diffusion technique. The absorbance and the inhibitory activity for each sample were plotted against time using a semi-log chart.

The agar well diffusion technique used in this study depends on the diffusion of substance radially through an agar layer from circular cup cut out from the agar gel. In this method, Petri dishes (9 cm in diameter) were poured with 50 ml of BPYG agar medium inoculated with 1% (v/v) of indicator bacteria. Several wells were punched out of the agar medium by using clean sterile cork borer (5 mm in diameter). The base of each hole was sealed with a drop of melted sterile water agar. A certain volume (0.1 ml) of cell free supernatant was transferred into each well, and then the plates were incubated at 30°C for 24 h to allow diffusion of supernatant into the medium. After the incubation period, the inhibition zone appearing around each well was measured in mm. The antagonistic effect A expressed in arbitrary units (AU) was calculated according to the following equation:

$$A\ (\mathrm{AU}) = r_z^2/r_w^2,$$

where r_z (mm) is the radius of the inhibition zone and r_w (mm) is the radius of the well. Specific siderophore activity was determined on the bases of number of AU per CFU/ml of bacterial culture.

Acetone precipitation. Four volumes of cold (−20°C) acetone were added to one sample volume. Tubes were vortexed and then incubated for 60 min at −20°C. They were then centrifuged for 10 min at 4025*g*. The pellet, after discarding the acetone layer, was dissolved in 0.02 M acetate buffer at pH 3 (sample buffer).

Affinity column. Hi-Trap chelating HP column (Amersham Pharmacia Biotech, Germany) of 1 ml was used. The column was washed with 5 ml of de-ionized water. Then 0.5 ml of 0.1 M ferric sulfate solution was loaded. The column was again washed with 5 ml of de-ionized water. Then it was washed with five column volumes of sample buffer. The protein sample was applied. The column was eluted with five column volumes of sample buffer. Then it was eluted with the stripping buffer (sample buffer containing 0.5 M EDTA). Fractions were collected and were measured at 280 nm. Fractions were then assayed for protein content using the Bradford method [16], and for the siderophore inhibitory activity using the striping buffer as control.

SDS polyacrylamide gel electrophoresis. Gel electrophoresis was carried out by 12% SDS-PAGE according to the Laemmli [17] method using protein samples containing 15 μg of protein. Before adding the samples onto the gel, they were either boiled with sample buffer containing β-mercaptoethanol (reducing conditions) or boiled with buffer not containing β-mercaptoethanol (non-reducing conditions). Then the gels were revealed using Coomassie brilliant blue R-250 [18] and silver stain [19].

RESULTS

Detection and identification of the siderophore-producing bacteria. Four bacterial isolates out of 52 bacteria, proved to induce antagonistic effect against *Erwinia carotovora* var. *carotovora* using the tooth pick technique, were subjected to investigations to determine which of them produce siderophore. They were tested for their ability to produce the inhibitory substance in liquid culture in the presence of ferric chloride (Table 1). Three of the tested isolates (1, 4, and 15) showed a positive inhibitory effect when their supernatants were tested against the indicator bacterium in the presence or absence of $FeCl_3$. Meanwhile, only one isolate (isolate 6) showed a negative inhibitory effect in the presence of $FeCl_3$ (Table 1).

Table 1. Sensitivity of *Erwinia carotovora* var. *carotovora* to antimicrobial agents produced by four isolates using the well-cut diffusion technique

Candidate bacterial isolates	Inhibition zone, mm	
	without $FeCl_3$	with $FeCl_3$
1	37.2 ± 3.61	29.1 ± 5.8
4	13.0 ± 7.8	10.9 ± 2.2
15	18.4 ± 1.8	17.1 ± 4.9
6	9.2 ± 3.1	0

Table 2. Bacterial isolate identification using conventional and biochemical tests

Test	Siderophore producer
Cell shape	short rod
Gram stain	–
Spore forming	–
Acid fast	–
Motility	+
Growth at pH 6-8	+
Growth at 7.5% NaCl	–
Growth at 50°C	–
Starch hydrolysis	+
Citrate utilization	–
Nitrate reduction	–
Catalase	+
Confirmatory kits	api-20E kit [15]
Nomenclature	*Pseudomonas fluorescens*

Identification results of the siderophore-producing bacterium are presented in Table 2. Results show that it is a motile, Gram-negative, short rod shape bacterium. The bacterium gave positive results for starch hydrolysis, catalase, oxidase, and Vogues–Proskauer reaction, while it was negative for nitrate, indole, citrate, and methyl red. From the classical and biochemical experiments, the siderophore producer was identified as *Pseudomonas fluorescens* (coded PFS6).

Growth curve and kinetics of siderophore production. The production/activity of siderophore in BPYG broth was relatively attributed to the growth phase of bacterial cells in culture (Fig. 1a). The siderophore-producing bacterium *Pseudomonas fluorescens* reached the stationary phase after 12 h with siderophore activity of 21.2 ± 0.8 AU. Early detection of activity was 1 h after bacterial inoculation. Figure 1b shows that the siderophore activity reached its maximum after 5 h (mid-log phase).

Isolation and purification of siderophore. Siderophore in culture medium was precipitated with acetone. The precipitate was dissolved in the sample buffer (0.02 M acetate buffer at pH 3). Protein sample was then applied to the Hi-Trap affinity column at flow rate 1 ml/min. Two protein peaks were obtained from the column (Fig. 2, solid line curve). The first peak was eluted with the sample buffer. Meanwhile the second peak was eluted with the same buffer containing 0.5 M EDTA, and it contained all the inhibitory activity (Fig. 2, dashed line curve). The second peak fractions were then subjected to gel electrophoresis.

Determination of molecular weight of the siderophore. According to the results obtained in gel electrophoresis under non-reducing conditions, three types of fractions were obtained (Fig. 3, lanes *2*, *4*, and *5*). The molecular masses of the bands were calculated from the relation between the molecular mass of the marker and the relative mobility of the separated bands. Their molecular masses were found to be approximately 30 and 90 kD. Fractions corresponding to the protein band in lane *4* were analyzed under reducing conditions (Fig. 3, lane *3*). The same two bands were obtained at approximately 90 and 30 kD.

Inhibitory activity of affinity chromatography fractions. Three fraction types were tested for their inhibitory activity based on the gel electrophoresis pattern (Fig. 3).

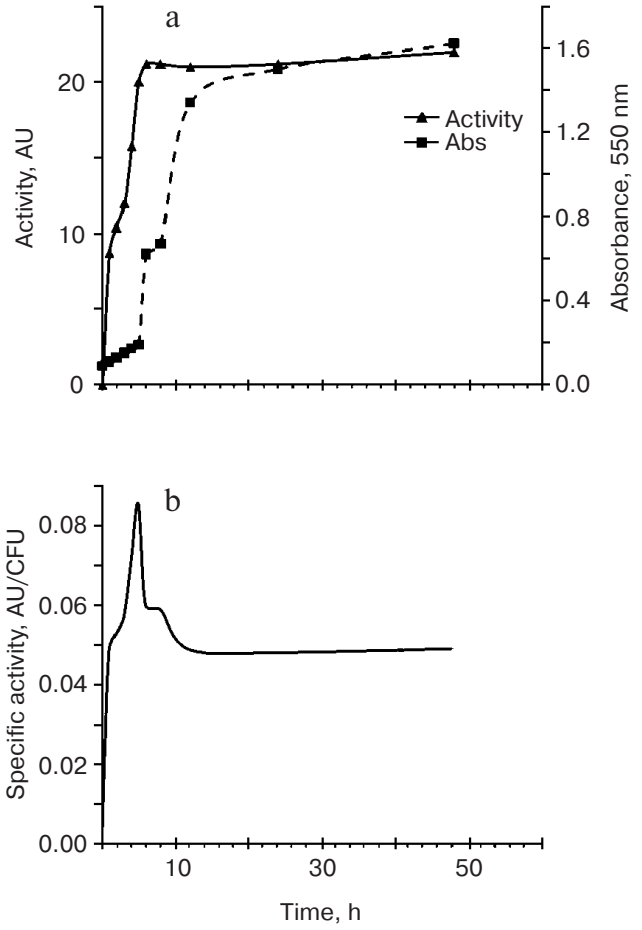

Fig. 1. Bacterial growth rate and specific siderophore activity. a) The bacterial growth, measured as absorbance at 550 nm, and its corresponding siderophore activity were plotted against time (in hours). b) The specific siderophore activity of the same bacterial growth expressed as AU/CFU is plotted against the growth period in hours.

These fractions were those containing protein bands of 90 kD, 30 kD, and fractions containing both protein bands (90 and 30 kD). The inhibitory activity test results are reported in Fig. 4. Both protein bands of 90 and 30 kD were found to be active against the control pathogen as judged from the inhibition zone (Fig. 4, wells *2* and *5*, respectively). Also, the protein fraction containing both protein bands (Fig. 4, well *4*) was also found to be active against the control pathogen. At the same time, these data were comparable to those of the total protein extract, which was used as a control (Fig. 4, well *1*).

DISCUSSION

The four bacterial isolates that produced a considerable inhibition zone against *Erwinia carotovora* var. *carotovora* were isolated from different soils infested with *E. carotovora* var. *carotovora*. The characterization of the antagonistic substance produced by the four isolates revealed that isolate 6 showed a negative inhibitory effect in the presence of $FeCl_3$ (Table 1). This criterion indicates that isolate PFS6 is a siderophore producer [20, 21]. It was defined as the Gram-negative bacterium *P. fluorescens* as shown in Table 2. The siderophore pro-

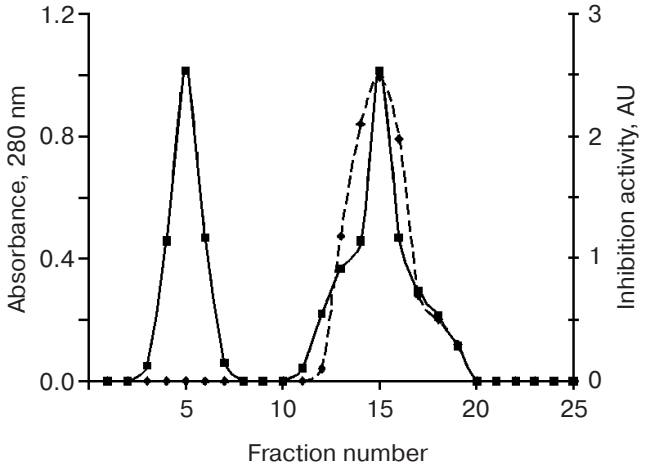

Fig. 2. Hi-Trap chelating HP affinity column for siderophore purification. Siderophore protein content (solid curve) and the inhibition activity against the indicator strain *E. carotovora* var. *carotovora* (dashed curve) were determined for each fraction obtained from the Hi-Trap chelating HP column.

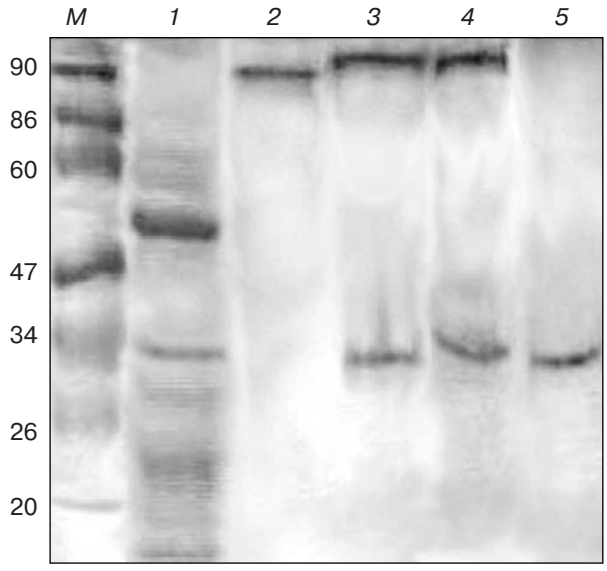

Fig. 3. SDS-PAGE of the siderophore. Samples were either boiled with sample buffer containing β-mercaptoethanol (reducing conditions) or boiled with buffer not containing β-mercaptoethanol (non-reducing conditions). Lanes: *1*) total bacterial cell culture proteins; *2*, *4*, *5*) siderophore fractions electrophoresed under non-reducing conditions; *3*) the same protein fraction as in lane *4*, but electrophoresed under reducing conditions; M) molecular weight markers.

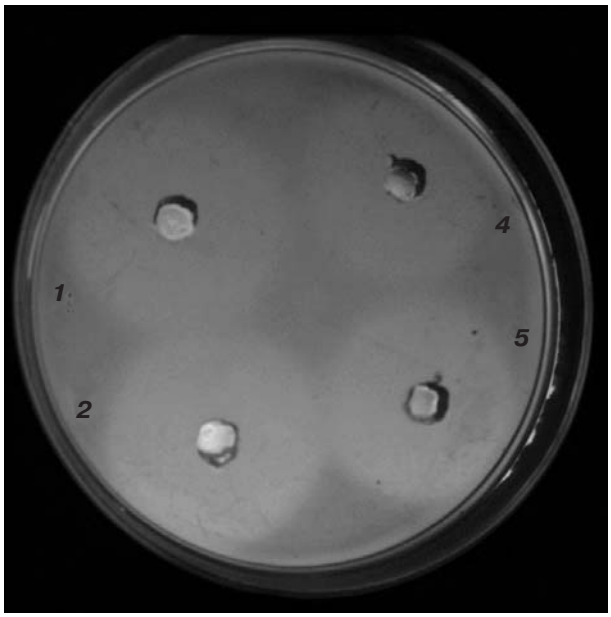

Fig. 4. Inhibitory effect of the second peak fractions obtained from the Hi-Trap chelating HP column against the indicator strain *Erwinia carotovora* var. *carotovora* using the well-cut diffusion technique. Figure numbering is equivalent to the lanes in the gel electrophoresis of Fig. 3.

duced by PFS6 was principally secreted during the log phase of cell growth (Fig. 1a) with a maximum specific activity of 0.09 AU after 5 h at mid-log phase (Fig. 1b). Its production in liquid culture facilitates its purification, and avoids further pretreatment needed to elaborate siderophore [22]. Based on these data and knowing that *P. fluorescens* siderophore nature does not change with the change of medium conditions [23], the isolated siderophore is believed to be a hydroxamate siderophore (pyoverdine).

Several methods have been proposed for the purification of siderophore. Some of these methods are based on the use of ammonium sulfate precipitation followed by chromatography techniques [24]. Others are based on the use of boronate affinity chromatography [25]. As stated previously, the aim is to find a simple and fast technique for the purification of siderophore to be suitable for biotechnological applications. The choice was made on affinity chromatography for the purification of the siderophore. This choice was based on the following considerations. First, affinity chromatography is highly specific. So the purified protein will have a high degree of purity and purification will be carried out in the least number of steps, which will reduce the cost and time of purification. Second, the purification is based on the binding of the protein of interest to its ligand. So, the rate of binding is limited by the amount of ligand accessible on the affinity matrix and not on the amount of the affinity matrix itself. Hence, with the least amount of affinity matrix, large amounts of protein can be purified [26]. According to these parameters, the choice was made on a Hi-Trap affinity column. Siderophores are known for their affinity to iron [27]. Even, siderophore-producing bacteria were found to grow in heavy metal containing media as mercury, lead [28], and cadmium [29]. Based on the high affinity of siderophores for iron, the later was selected to accomplish this step of purification (Fig. 2).

To verify the purity of the purified siderophore, gel electrophoresis was carried out using 12% gel and the gel was stained with silver stain. This staining method offers the greatest sensitivity in non-radioactive protein detection. The use of this staining method permits the detection of minor bands (as low as 5 ng per band), so it will confirm the high purity of the purified protein. Surprisingly, in non-reducing conditions (Fig. 3, lanes *2*, *4*, and *5*) some fractions showed one band of approximately 90 or 30 kD (Fig. 3, lanes *2* and *5*, respectively). Others showed two bands of approximately 90 and 30 kD (Fig. 3, lane *4*). The presence of these two bands raised the question about the presence of either one protein of 30 kD that can also exist in a polymerized form of approximately 90 kD or the presence of two distinguished siderophores of different molecular masses. To answer this question, siderophore protein samples before and after passing over the affinity column were subjected to gel electrophoresis under reducing conditions (Fig. 3, lanes *1* and *3*, respectively). The siderophore protein band of 90 kD was not found in the samples before passing over the affinity column and only the band of 30 kD was observed (Fig. 3, lane *1*). However, the same two bands of 90 and 30 kD were found in reducing and non-reducing conditions (Fig. 3, lanes *3* and *4*, respectively). The presence of a high molecular mass protein band in the pure siderophore in reduced and non reduced conditions, whereas this band is completely absent from the protein samples before purification, indicates that this additional band is due to the purity state of the siderophore.

In some cases, siderophore was found to be dimerized [30]. In some other cases, proteins were found to interact together by hydrophobic interactions that cannot be simply abolished under reduced conditions [31]. Consequently, it is believed that the siderophore molecules (in this study) interact together by hydrophobic interactions to form polymers detectable in gel electrophoresis under reducing and non-reducing conditions. Even more, the fact that the 30 kD band has a molecular mass higher than that known for siderophores (<15 kD) [32] suggests that this band is already a polymer of several siderophore molecules.

The data raised the question about the effect of polymerization on the siderophore activity. So, protein fractions corresponding to lanes *2*, *4*, and *5* in Fig. 3 were analyzed for their inhibitory activity in comparison to the crude protein sample (Fig. 3, lane *1*). According to the

Table 3. Comparison of the siderophore purification procedure used in this study with those from the literature

Source	Media	Bacterial strain	Purification step	Protein content, mg/liter	References
Stock sample	succinate minimal media	*Alcaligenes faecalis*	solvent extraction Amberlite XAD-400 column Sep-Pak C_{18} column	 297 50	[32]
Barley rhizospheres	same	*Pseudomonas fluorescens*	diaion HP-20 column silica gel column partition chromatography	 7	[33]
Chickpea	»	*Pseudomonas putida*	size exclusion chromatography Dowex-50 RP-HLPC	 500 	[34]
Different plants	GASN media	*Pseudomonas syringae*	Sephadex-CM-25 column Sephadex-DEAE column HLPC	 100 	[35]
Soil	BPYG	*Pseudomonas fluorescens*	Hi-Trap chelating HP column	25	this study

results, the high-molecular-mass (i.e. 90 kD) polymerized state (Fig. 4, well *2*) had comparable activity to the low-molecular-mass (i.e. 30 kD) form (Fig. 4, well *5*) in comparison to the crude protein sample (Fig. 4, well *1*). Furthermore, the presence of the polymerized forms together (Fig. 4, well *4*) does not influence the siderophore inhibitory activity.

To establish the efficiency of the purification method in comparison to published data, the data from our purification method were compared to that of the literature (Table 3). It can be noted that the amount of siderophore produced in this study is comparable to that of *Alcaligenes faecalis* [32] and at the same time there was no need for several steps of purification. The difference in the amounts of the purified siderophore among the selected publications [33-35] can be, in part, accounted for by the differences in the selected bacteria and the conditions of growth in each study.

In conclusion, the work presented in this study shows a new method of siderophore purification based on the affinity of siderophores for metal ions. The described method is fast and simple and can be used for the preparation of large amounts of siderophore. Under the described experimental conditions, it was possible to purify approximately 25 mg siderophore per liter of culture medium. Since the purification is based on affinity chromatography, the purity state of the siderophore permits several biotechnological applications without further treatments. The advantage of this method over other affinity purification methods is that this method uses metal ion, so it can be applied for the three known types of siderophores. Further studies are being carried out to optimize the production and purification of siderophore of different bacterial species using this method.

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