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Novel strains of *Bacillus cereus* Wah1 and *Enterobacter cloacae* Wkh with high potential for production of siderophores

Mahmood Maleki*
Assistant Professor of Plant Breeding, Department of Biotechnology, Institute of Science and High Technology and Environmental Science, Graduate University of Advanced Technology, Kerman, Iran, maleki.li@gmail.com
Sedigheh Norouzpour
MSc student of Plant Breeding, Graduate University of Advanced Technology, Kerman, Iran, sedighe.poorahmadi@yahoo.com
Elham Rezvannejad
Assistant Professor of Animal Breeding, Department of Biotechnology, Institute of Science and High Technology and Environmental Science, Graduate University of Advanced Technology, Kerman, Iran, rezvannejad2002@yahoo.com
Shahryar Shakeri
Assistant Professor of Microbiology, Department of Biotechnology, Institute of Science and High Technology and Environmental Science, Graduate University of Advanced Technology, Kerman, Iran, shahryar.shakeri@yahoo.com

Abstract

Introduction: Siderophores are relatively low-weight molecules (500 to 1000 Dalton), which are produced by many microorganisms as the chelating ligands of iron under iron deficiency. Plants can absorb the siderophore-iron complex to meet their needs for iron. The aim of this study was isolation and identification of siderophore producing bacteria.

Materials and methods: In this study, we used liquid chrome azurol S (CAS) assay for the isolation of siderophore producing plant growth promoting rhizobacteria (PGPRs). Then, different kinds of the produced siderophores by the best strains were determined using overlaid chrome azurol S (O-CAS) assay. Finally, two best siderophores producing strains were identified using the 16S rDNA gene sequencing analysis.

Results: The results showed that 69.3 percent of the isolated bacteria could produce siderophores. The lowest and highst levels of siderophores belonged to the strains Cke1 (17.58 percent siderophore unit) and Wah1 (97.76 percent siderophore unit) respectively. Based on the O-CAS assay method, these strains produced catchol and hydroxamat types of siderophores. Two strains Wah1 and Wkh were identified using 16S rDNA gene sequencing analysis as *Bacillus cereus* and *Enterobacter cloacae*, respectively.

Discussion and conclusion: Wah1 and Wkh produced more siderophores (as siderophore unit) than other strains, which were studied using the same method until now. Therefore, they could be valuable strains to study their potential as biological fertilizers and plant protection against the soil born pathogens.

Key words: Bacteria, Liquid CAS Assay, O-CAS Assay, Siderophore

*Corresponding Author

Introduction

Iron is the fourth most abundant element in the soil, but a small amount of it is available for the microorganisms and plants due to the very low solubility of iron minerals, especially in the arid areas, (1- 4). Plants have the ability to absorb iron under iron deficiency conditions by using two different strategies, lowering pH of rhizospher by the release of the protons or production of the siderophores (5, 6). Nevertheless, iron deficiency in calcareus and dry soils is a common phenomenon leading to a significant reduction in the crop yields (5, 7).

This deficiency is compensated by using the chemical fertilizers (chelated iron) that are not eco-friendly and have high cost. In addition, the production or selection of the tolerant cultivares to Fe deficiency are extremely complex due to the non-uniform nature of soils and environmental conditions. Thus, plant breeding programs have slow progress (5).

Therfore, the use of microorganisms such as PGPR to improve the availability of nutrients for plants is an important and necessary factor for agriculture (8) to reduce risks for the human health and the environment (9, 10).

PGPRs are useful for the plant growth. These rhizobacteria stimulate the plant growth with both direct and indirect mechanisms (11, 12). Direct mechanisms include production of the phytohormons like auxin, ACC deaminase, siderophores and improvment of the solubilization of insoluble phosphate (13- 16). Indirect mechanisms also will begin when PGPR acts like biocontrol agents and reduce the disease (11).

Various works have indicated that many PGPRs have the ability for siderophore production (17- 22). Siderophores are low-weight molecules (500 to 1000 Dalton) that could solubilize Fe^{+3} by chelating them. Then, palnts and microorganisms can absorb Fe^{+3}-siderophore combination and improve their growth (23- 26). The growth factors of plants would be increased using siderophore-producing rhizobacteria (27).

The purpose of this study was the isolation and identification of the best siderophore-producing rhizobacteria originated from Iran.

Materials and methods

General Isolation of rhizobacteria: The soil samples collected from the rhizospher of different plants from different provinces of Iran. Soil samples were placed in the plastic bags, and transported to the laboratory. Soil was crushed with a mortar thoroughly, then 1 gram of the crushed soils was used for preparation of the serial dilution of 10^{-1} – 10^{-6} in the physiological serum. After that 100 µl from 10^{-6} dilution was added to the nutrient agar plates. Inoculated cultures were incubated at 30ºC during 72 hours. After appearance of colonies, single colony was isolated and prepared for more analysis (13, 28).

Specific isolation of bacteria containing siderophore

Cultivation of rhizobacteria: In this stage, siderophore producing rhizobacteria were screened using the specific medium of liquid CAS assay (29). Bacterial strains were inoculated into M9 minimal medium. The M9 medium was prepared as follow: KH_2PO_4 3 gr/L, NaCl 0.5 gr/L, $CaCl_2$ 0.025 gr/L, $MgSO_4$ 0.088 gr/L, Glucose 2gr/L and NH4Cl 1gr/L. After inoculation, the samples were incubated four days at 30° C and 100 rpm. After four days, 1 ml of liquid medium centrifuged for 10 minutes at 5000 rpm and after centrifugation, 0.5 ml of each sample was transferred to the new vial.

Chrome Azurol S Liquid Assay: Reagents were prepared as follow:

CAS assay solution:

2 mM CAS stock solution: 0.121g CAS in 100 ml water,

1 mM Fe stock solution: 1 mM FeCl3.6H2O in 10 mM HCL,

Piperazine buffer: dissolved 4.307 g piperazine in 30 ml water. Add 6.75 ml concentrated HCl to bring the pH to 5.6,

Hexadecyltrimetyl ammonium bromide (HDTMA): dissolved 0.0219 g HDTMA in 50 ml water in a 100 ml mixing cylinder.

We mixed 1.5 ml Fe solution with 7.5 ml CAS solution and add to the HDTMA in the mixing cylinder. Piperazine-1, 4-bis(2-ethanesulfonic acid) (PIPES) solution was added to the mixing cylinder and brought volume up to 100 ml with water.

Shuttle solution was 0.2 M 5-Sulfosalicylic acid. We added 0.5 ml CAS assay solution to 0.5 ml culture supernatant and mixed. Then 10 µl of the shuttle solution was added and mixed. After a few minutes, Siderophores, if present, will remove iron from the dye complex, resulting in a reduction in blue color of the solution. The absorbance (A_{630}) for the loss of blue color was measured. For A_{630} measurements, we used the minimal medium as a blank, and used the minimal medium plus CAS assay solution plus shuttle as a reference (r). The sample (s) should have a lower reading than the reference. Siderophore units are defined as $\left[\frac{(Ar-As)}{Ar}\right] 100 = \%$ siderophore units.

Statistical analysis: All experiments were done based on completely randomized design (CRD) and all data were analyzed using excel and SAS softwares.

O-CAS assay

Cultivation of rhizobacteria: First, all bacteria were grown in the nutrient broth medium for 24 hours. After that 1 ml of the bacterial suspensions was centrifuged and bacterial pellet were washed by NaCl (8% w/v) solution. Then, the washed pellet was used for inoculation of solid M9 medium. It should be mentioned that solid M9 medium has the same combination like M9 medium discussed above plus agar 15 gr/L.

Preparation of CAS medium: The medium for 100 ml was as follows: CAS 6.05 mg, HDTMA 7.29 mg, PIPES 3.024 g, and 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 1 mL. Agarose (0.9%, w/v) was used as gelling agent (30).

Determination of produced siderophores: Ten ml of the CAS medium was applied over those mediums containing cultivated rhizobacteria to test the siderophore production. After 30 min, a change in the color will be appeared in the overlaid medium from blue to purple (for siderophores of the catechol type) or from blue to orange (for hydroxamates type) (30).

PCR amplification of bacterial 16S rDNA genes and electrophoretic analysis: Genomic DNA of isolated bacteria was extracted according to the method of Ausubel et al. (13, 31). The 16S rDNA gene was PCR amplified from genomic DNA isolated from the best siderophore producing rhizobacteria following standard PCR protocols (32). Primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used to amplify the 16S ribosomal gene. The PCR products were separated by their electrophoresis in a 1% agarose gel for 2 h. After that, staining was done using the ethidium bromide. Amplification products (1531 bp in size) were stored at -20°C. The clean PCR product was subjected to cycle sequencing in forward directions.

Molecular identification and phylogenetic analysis: Sequence analysis was performed by using the algorithms BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple-sequence alignment was carried out using a freely available alignment program, Mega4.1. Bacterial identifications were based on the 16S rDNA gene sequence similarity. Neighbors joining phylogenetic trees were generated using the top alignment matches (13, 32).

Result

Isolation of PGPR: Soil samples were collected from different plant rhizosphere in different provinces of Iran. In the present work, we conducted an extensive screening procedure for the isolation of PGPRs. PGPRs were isolated on the nutrient agar medium. Out of large number of bacterial colonies appeared in the primary culture, seventy five colonies were selected based on their shapes and colors.

Siderophore Production: As mentioned above, liquid CAS assay was used based on completely random design with three replications to screen siderophore-producing PGPRs. The primary results showed that 52 strains had the ability to produce siderophores.

Data analysis indicated that there was a significant difference at 1% level between treatments (Table 1). It means that at least one strain has a significant difference with others.

In order to identify the best siderophore-producing strain, Duncan's Multiple Range Test was conducted between all the siderophore-producing PGPRs (Table 2). Based on the mean comparison, Wah1 and Wkh were the best strains for production of siderophores. These strains could produce 97.76 and 96.99 percent of siderophore unit, respectively. Cke1 had the lowest ability to produce siderophore (Table 2).

Table 1- Variance analysis of siderophore producing bacteria.

Sources of Variation	DF	Sum of Squares	Mean Square
Treatment	51	8.11	0.16**
Error	104	0.058	0.00056
total	155	8.17	

** Significant differences at 1% probability level

Table 2- Mean comparison between the siderophore producing PGPRs based on Duncan's Multiple Range Tests.

No.	Strains	Siderophore unit	No.	Strains	Siderophore unit	No.	Strains	Siderophore unit
1	Wah1	97.76 a	19	Ake4	63.35 jk	37	Hke5	42.52 rst
2	Wkh	96.99 ab	20	Gke3	63.19 jk	38	Ver19	41.84 st
3	Ver4	93.69 abc	21	Mho	62.47 k	39	Mke2	39.17 tu
4	Hke2	92.00 bc	22	Hke6	61.20 k	40	Hke1	36.21 uv
5	Hke3	90.59 c	23	Aes1	58.47 kl	41	Ake3	36.06 uv
6	Aes2	90.04 cd	24	Ama3	57.76 klm	42	Ver11	33.88 uvw
7	Wta2	88.74 cde	25	Ver2	55.74 lmn	43	Ake1	33.71 uvw
8	Lka4	88.70 edf	26	Sho4	54.09 lmno	44	Aes4	32.47 vwx
9	Hke8	84.88 def	27	Wha	53.58 lmno	45	Ama1	29.06 wxy
10	Ake7	83.56 efg	28	Lka7	52.59 mnop	46	Lka5	27.88 xyz
11	Hke4	81.98 fg	29	Ver6	52.03 nop	47	Sho1	27.78 xyz
12	Ake9	81.32 fg	30	Aes3	50.46 nopq	48	Ver15	26.35 yza
13	Hke7	78.47 gh	31	Ver13	49.47 opq	49	Cke2	23.41 zab
14	Wah2	73.53 hi	32	Ver17	49.44 opq	50	Min2	21.77 abc
15	Ver9	73.52 hi	33	Ver18	48.93 opq	51	Wta1	19.74 bc
16	Ver10	72.36 i	34	Ver16	47.80 pqr	52	Cke1	17.58 c
17	Gke4	68.46 ij	35	Ver14	46.12 qrs			
18	Ake2	68.03 ij	36	Cke3	45.74 qrs			

Among all the strains that were able to produce siderophore, 8 strains (15.38 %) produced less than 30 percent of siderophore unit, 14 (26.9 %) between 30 and 50%, 14 strains (26.9 %) between 50 and 70%, 10 strains (19.23 %) between 70 and 90 and 6 strains (11.5 %) more than 90 percent (Figure 1).

CAS Detection Method: We used O-CAS method (30) to determine the type of siderophores produced by the best siderophore-producing rhizobacteria. 16 strains were analyzed for the determination of the type of siderophores. The results showed that 10 strains changed the blue color of medium to purple (catechol-type siderophores), 4 strains changed the blue color to orange (hydroxamate-type siderophores) and 2 strains changed the blue color to orange-purple (catechol and hydroxamate) (Table 3, Figures 2 and 3).

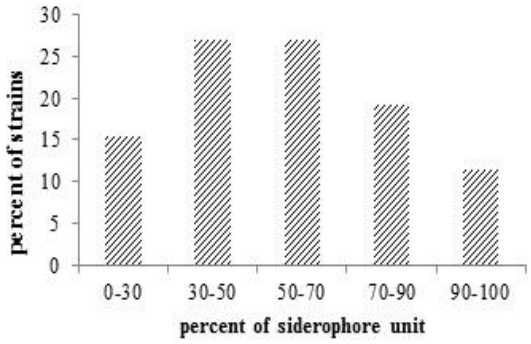

Fig. 1- Different capabilities of siderophore producing rhizobacteria based on their siderophore unit.

Table 3- Determination of siderophore types by using O-CAS method.

No	Strains	color	Siderophore type		Strains	color	Siderophore type
1	Wah1	Orange-purple	Catechol and hydroxamate	9	Hke8	Orange-purple	Catechol and hydroxamate
2	Wkh	Purple	catechol	10	Ake7	Purple	catechol
3	Ver4	Purple	catechol	11	Hke4	Orange	hydroxamate
4	Hke2	Purple	catechol	12	Ake9	Purple	catechol
5	Hke3	Orange	hydroxamate	13	Hke7	Orange	hydroxamate
6	Aes2	Purple	catechol	14	Wah2	Purple	catechol
7	Wta2	Purple	catechol	15	Ver9	Purple	catechol
8	Lka4	Purple	catechol	16	Ver10	Orange	hydroxamate

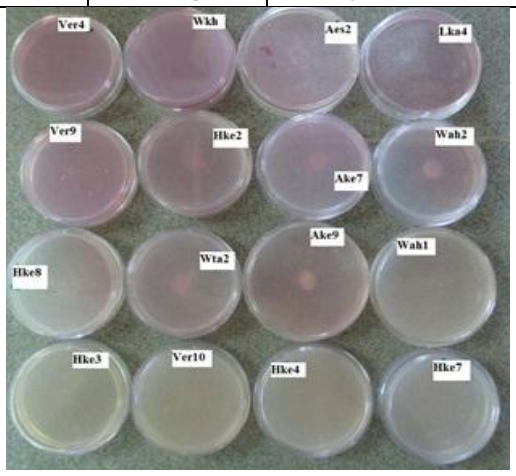

Fig. 2- O-CAS assay carried out with the best siderophore producing Bacteria.

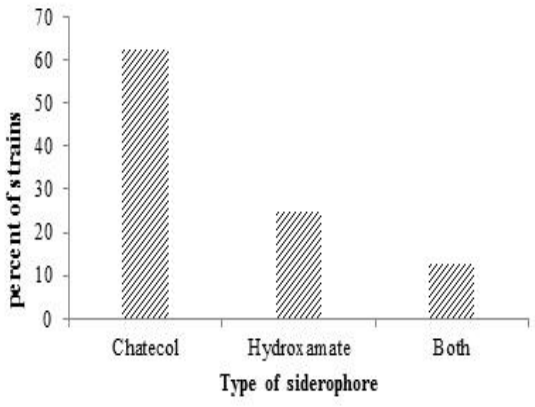

Fig. 3- Distribution of different siderophore types

between siderophore producing rhizobacteria.

Phylogenetic Analysis: The phylogenetic affiliation of the best siderophore-producing strains (*Bacillus cereus* strain Wah1 and *Enterobacter cloacae* strain Wkh) was obtained by 16S rDNA gene sequence analysis. The blast search showed that the strains Wah1 and Wkh were more similar to *Bacillus cereus* and *Enterobacter cloacae*, respectively. The 16S rDNA sequence of Wah1 and Wkh reported in this paper had been deposited in the GenBank database under accession numbers: KU744631 and KU744630, respectively.

In order to find the most similar available sequences, a BLAST search was conducted in NCBI database. 16S rDNA sequence data of the most closely related species of *Bacillus and Enterobacter* were extracted and used in a tree construction to demonstrate the taxonomy of these strains (Figures 4 and 5). The phylogenetic relationships derived from the neighbor-joining analysis of the16S rDNA gene sequence of the Wah1 and Wkh with the most validly described species of the genus *Bacillus and Enterobacter* were shown in Figures 4 and 5.

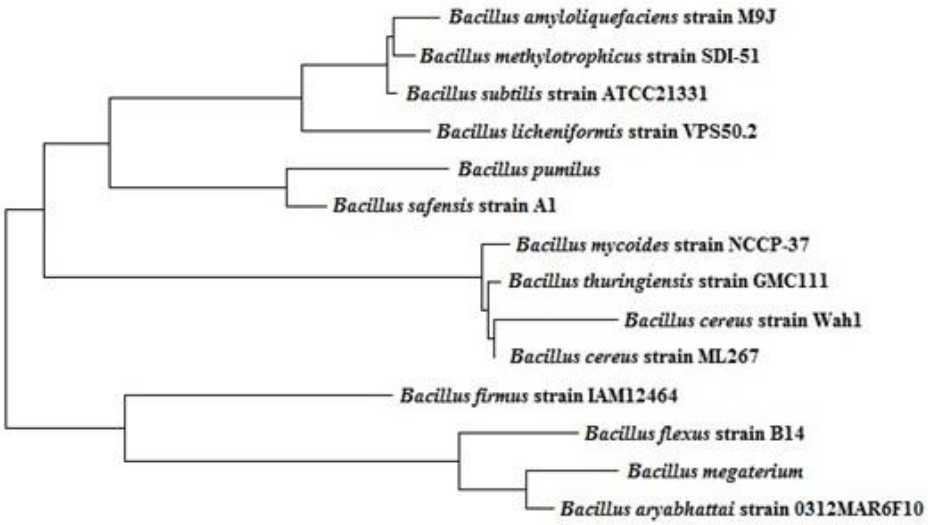

Fig. 4- Neighbor-joining tree based on *16S rRNA* gene sequences, showing relationships of *Bacillus cereus* strain Wah1 with closely related members of the genus *Bacillus*.

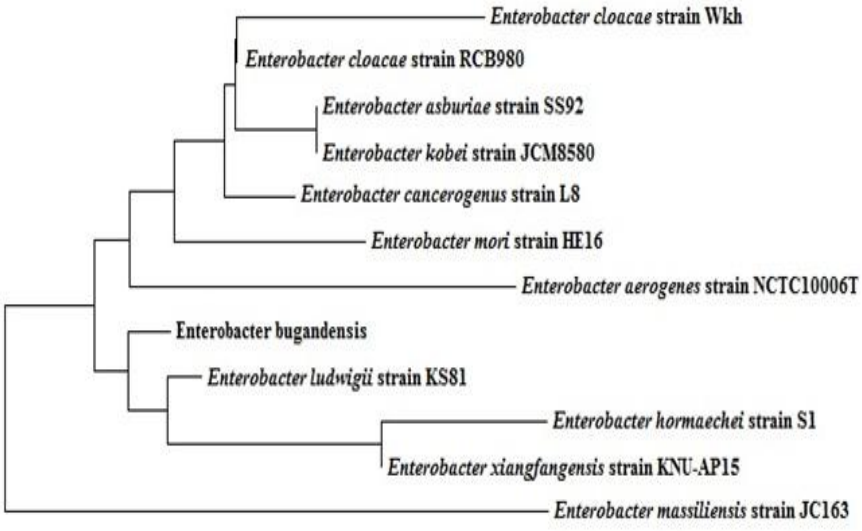

Fig. 5- Neighbor-joining tree based on 16S rRNA gene sequences, showing relationships of *Enterobacter cloacae* strain Wkh with closely related members of the genus *Enterobacter*.

Discussion and conclusion

The ability of siderophore production or absorption is one of the traits that cause the superiority of microorganisms in different environments (33) and facilitate the relationship between plant and bacteria and create a community around the roots, stems and leaves (34). This mechanism involves producing siderophore with high affinity to Fe^{3+} and absorption of siderophore loaded with iron (35).

Heretofore, several studies have been conducted to isolate the siderophore-producing rhizobacteria, all of whom confirmed that many strains can produce siderophores (36-39). Tian et al. could isolate 229 siderophore-producing bacteria (20). Their results showed that seventy five percent of siderophore-producing strains could produce siderophore unit between 20 and 40 percent and less than 25 percent between 40 and 60 percent of siderophore unit. Sayyed et al., optimized best conditions for the production of siderophores by two *pseudomonas* strains (40). These strains could produce approximately 85 percent of siderophore unit. Tailor and Joshi assessed 63 strains of *pseudomonas* for siderophore production (19). Their results showed that only 12 strains were able to produce siderophore and among these 12 strains, 7 strains were accounted for more than 85 percent of the siderophore unit. The best strain in the optimal conditions was accounted for 96% of the siderophore unit. Zhu and Yang investigated the ability of different strains for the production of siderophores (22). Their results showed that 19 strains could produce siderophores and strain MB8 produced a relatively high level of siderophore (70.38 % siderophore unit). By studying different researches on the capability of different bacteria for producing bacteria, it seems that the best strains in our study could produce more siderophores. In our study, results showed that more than 21 percent of siderophore-producing strains could produce siderophore unit more than 70 percent and the best one was accounted for more than 97 percent of siderophore unit.

Pérez-Miranda et al. introduced O-CAS assay method (30). Their results showed that color changes from blue to purple correspond to catechol-type siderophores and changes from blue to orange are consistent with hydroxamate-type siderophores. Sullivan et al. screened siderophore-producing bacteria from African dust using O-CAS method (18). Their results showed that 53 percent of bacteria could produce siderophores. Among siderophore-producing bacteria, two strains produced catechol siderophores and 10 strains produced hydroxamate siderophores, but most of them (approximately 90 percent) produced carboxylate siderophores (18). In our study, we used O-CAS assay to determine the type of siderophores in the best strains (having more than 70 percent siderophore unit). Most of the strains could produce catechol siderophores.

In our study, two best siderophore-producing strains belonged to the *Bacillus* and *Enterobacter* genus. These results indicated the importance of these genera for the production of siderophores. Different studies have shown the capability of both genera of *Enterobacter* and *Bacillus* to produce siderophores (17, 21, 41, 42, 43). For example, Van Tiel-Menkveld et al., extracted two types of siderophores from the iron-starved cultures of *Enterobacter cloacae* (43). Ramesh et al. showed that *Enterobacter cloacae* subsp. MDSR9 can produce the hydroxamate type of siderophore (17). Yaish et al. isolated PGPRs from the rhizospher of the date

palm tree. Most isolated bacteria belonged to the *Bacillus* and *Enterobacter* genera. Some of these strains were able to chelate the ferric iron and solubilize phosphorus, zinc and potassium (21). In another work, Kumar et al. (42) and Kaki et al. (41) demonstrated different capabilities of *Bacillus* strains such as production of siderophores and IAA.

Iron in the soil is in the form of insoluble Fe^{3+} under aerobic conditions that it is not available for plants or microorganisms. Iron is essential for the main physiological processes such as nitrogen fixation, photosynthesis, and respiration (44). Microorganisms and plants have developed special mechanisms to chelate insoluble iron through the release of siderophores and absorption of iron-siderophore complexes through receptor proteins from the outer membrane to meet their needs for iron (45-48). Here, we isolated strains with high capabilities to produce siderophores. The best strains, *Bacillus cereus* strain Wah1 and *Enterobacter cloacae* strain Wkh, produce siderophores more than the most identified strains in other researches until now. They could be potential biologic fertilizers and biological enemies of soil born pathogens.

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سویه‌های جدید *Bacillus cereus* Wah1 و *Enterobacter cloacae* Wkh با پتانسیل بالا برای تولید سیدروفورها

محمود ملکی [*]: استادیار اصلاح نباتات، دانشگاه تحصیلات تکمیلی صنعتی و فناوری پیشرفته، کرمان، ایران، maleki.li@gmail.com
صدیقه نوروزپور: استادیار علوم دامی، دانشگاه تحصیلات تکمیلی صنعتی و فناوری پیشرفته، کرمان، ایران، rezvannejad2002@yahoo.com
الهام رضوان نژاد: دانشیار بیوتکنولوژی، دانشگاه اصفهان، ایران، m.keyhanfar@ast.ui.ac.ir
شهریار شاکری: استادیار اصلاح نباتات، دانشگاه تحصیلات تکمیلی صنعتی و فناوری پیشرفته، کرمان، ایران، shahryar.shakeri@yahoo.com

چکیده

مقدمه: سیدروفورها مولکول‌هایی با وزن مولکولی نسبتا پایین (۵۰۰ تا ۱۰۰۰ دالتون) هستند که به عنوان لیگاندهای کلاته کننده آهن در شرایط کمبود آهن توسط بسیاری از میکروارگانیزم‌ها تولید می شوند. گیاهان می‌توانند ترکیب آهن-سیدروفور را به منظور رفع نیازشان به آهن جذب کنند. هدف از پژوهش حاضر جداسازی و شناسایی باکتری‌های تولید کننده سیدروفور است.

مواد و روش‌ها: در این پژوهش از روش سنجش مایع CAS، برای جداسازی باکتری‌های محرک رشد گیاهی (PGPR) تولید کننده سیدروفوراستفاده شد. سپس، انواع مختلف سیدروفورهای تولید شده توسط بهترین سویه‌ها، با استفاده از روش سنجش O-CAS تعیین شدند. سرانجام دو سویه برتر تولید کننده سیدروفور با استفاده از آنالیز توالی ژن *16S rDNA* شناسایی شدند.

نتایج: نتایج نشان داد که ۶۹/۳ درصد از باکتری‌های جداسازی شده توانایی تولید سیدروفور را دارند. پایین‌ترین و بالاترین میزان سیدروفورها به ترتیب به سویه های Cke1 (۱۷/۵۸ درصد واحد سیدروفوری) و Wah1 (۹۷/۷۶ درصد واحد سیدروفوری) تعلق داشتند. بر پایه روش سنجش O-CAS این سویه‌ها سیدروفورهایی از نوع کاتکول و هیدروکسامات را تولید کردند. دو سویه Wah1 و Wkh با استفاده از آنالیز توالی‌یابی ژن *16S rDNA* به ترتیب به عنوان *Bacillus cereus* و *Enterobacter cloacae* شناسایی شدند.

بحث و نتیجه‌گیری: Wah1 و Wkh سیدروفورهای بیشتری (به صورت واحد سیدروفوری) نسبت به سایر سویه‌هایی که تا کنون با روش مشابهی مطالعه شده بودند، تولید کردند. بنابراین، آن‌ها سویه‌های با ارزشی برای مطالعه پتانسیل‌شان به عنوان کودهای زیستی و حفاظت گیاه در برابر پاتوژن‌های خاکزاد خواهند بود.

واژه‌های کلیدی: باکتری، سنجش مایع CAS، سنجش O-CAS، سیدروفور

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