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Siderophore producing *Pseudomonas* spp. isolated from rhizospheric soil and enhancing iron content in *Arachis hypogaea* L. plant

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Abstract *Pseudomonas aeruginosa* PSA01 and *Pseudomonas fluorescences* PSF02 were isolated from rhizospheric of agriculture soil Salem district in Tamil Nadu, India, and confirmed species using biochemical test and molecular phylogeny. Both isolates proved to produce siderophore and solubilized phosphate. Pot experiment resulted to promote the growth of *Arachis hypogaea*. Inoculated PSA01 and PSF02 significantly increased in the root length, shoot length, fresh weight and dry weight, iron and oil content as compared to untreated control. Total chlorophyll and carotenoids content increased in treated plants. Dual culture technique showed a good biocontrol efficiency of PSF02 activity against *Fusarium oxysporum.* PGPR culture expressed plant growth potential and inhibited phytopathogen could be exploited as effective bioinoculant for sustainable farming of peanut.

Keywords: PGPR, Siderophore, P Solubilization, Plant Growth Promotion, Biocontrol, *Arachis hypogaea*

Introduction

Siderophore producing Plant Growth Promoting Rhizobacteria (PGPR) is required iron in plants by causing solubilization and chelation of organic and inorganic complexes in soil (Singh *et al.*, 2017). It helps to protect phytopathogens (Saha and Sarkar, 2016) due to iron starvation or iron-deficient between the plant and pathogen (Sayyed *et al*., 2019). PGPR associated directly to the roots and on the surface of root tissue or within the soil that influenced by the root system. Siderophore producing PGPR for the solubilization and transport of iron from phases to soluble Fe^{3+} complexes that can be taken up by active transport mechanism. The siderophores are non-ribosomal peptides bonds (Hu and Xu, 2011). It is a multidentate, organic, oxygen donor ligands and classified to hydroxamate, catecholate, carboxylate and to facilitate solubilization and chelation of transport of iron to cells (Sayyed *et al*., 2019).

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Pseudomonas is most widely reported as PGPR as a potential to produce plant growth promotor, phytohormones, phosphate solublization, hydrogen cyanide, siderophore, antibiotics, hydrolytic enzymes and antimicrobial compounds (Noori and Saud, 2012). *Arachis hypogaea* L. is oilseed crop in India which occupying 45 % of total oilseed production of the country. Traditional groundnut production relies on chemical fertilizers which are expensive. PGPR is a group of bacteria that enhances the plant growth and yield through growth promoting substances. Siderophore producing PGPR play an important role to provide iron nutrition to the plants and help in plant growth promotion and prevent the plant pathogen from the iron nutrition thereby restricting its growth and thus help in biological control of phytopathogens (Shaikh *et al.*, 2014; Sayyed *et al*., 2019).

Siderophore producing *Pseudomonas* is regarded as potential of PGPR and siderophore production is influenced by physicochemical factors (Sayyed *et al*., 2019). The physicochemical factor influences the siderophore production of PGPR. The best physicochemical condition supports the growth and siderophore production in PGPR. Therefore, the present study was conducted to find out the potent of *Pseudomonas* to optimize the conditions of siderophore production and to evaluate biocontrol efficacy in oilseed crops and biocontrol potential against *Fusarium oxysporum*.

Materials and Methods

Soil bacteria isolation and identification

Rhizosphere soils were collected from different crops including, *Zea mays, Arachis hypogaea* in PSA01 (11.8284 ºN, 78.0723 ºE) and PSF02 (11.8386 ºN, 78.0645 ºE), Salem district Tamil Nadu, India. Rhizospheric bacteria were isolated from serial dilution plate technique (Wilson and Knight, 1952). Biochemical characterization: the isolates were characterized on biochemical test eg. indole, catalase, urease, citrate, ammonia, nitrate producing abilities to identify bacteria to genera level (Gupta *et al*., 2000).

The 16S rRNA gene sequencing was analysed by Sambrook and Russel (2001). 16S rRNA gene of PSA01 &PSF02 was amplified by PCR using universal bacterial primers 27F 5' AGAGTTTGTCGTGGCTCAG 3' and 1492R 5' GGTTACCTTGTTACGACTT 3'(Eden *et al*., 1991). The similarity of 16S rRNA gene sequencing was aligned using BLAST programme of GenBank database (NCBI).

Assay for indoleacetic acid (IAA) production

IAA was performed in the concentrations of tryptophan (0, 50, 150, 300, 400, and 500 µg/ml). It was centrifuged at 3000 rpm for 35 min and the cell-free supernatant (2-3 ml) was mixed with 2-3 drops of orthophosphoric acid, allowed to react with 4 ml of Salkowski's reagent. The reaction mixture was incubated at 28 ℃ for 30 min and observed for the development of a pink colour as a positive indication of IAA production. Optical density was 530 nm. IAA concentrations was estimated by standard curve in the range of 10-100 µg ml^{-1} (Ahmad *et al*., 2005).

Screeniong for phosphate (P) solubilization

Bacteria isolates were transferred to Pikovskaya's agar plates. The plates were incubated at 28 ±1 ℃ for 3 days, observed for clear zones around the colonies due to solubilization of inorganic phosphate (Sharma *et al*., 2013).

Screening for siderophore production

The Chrome Azurol Sulfonate (CAS) assay (Schwyn & Neilands, 1987) for the qualitative assay cultures were spot inoculated onto the blue agar and incubated at 37^{o}C at 24 - 48 h. The results were observed actively growing culture on appearance of orange-halo zones formed after 72 h of incubation at 28 ℃. The sizes of yellow-orange haloes around the growth indicated total siderophores activity.

Siderophore assay

1 ml of cell free supernatant and 1 ml of CAS reagent were added in siderophore production which measured at 630 nm. The uninoculated broth with CAS reagent served as control. Amount of siderophore units (SU) was calculated by using the following formula (Payne 1994):- % SU = Ar-As/Ar × 100 where, SU = Siderophore Unit, Ar = Absorbance of reference at 630 nm and As = Absorbance of the sample at 630 nm.

Type determination of siderophore

Neilands assay ($FeCl_3$ test): 0.5ml culture filtrate was added to 0.5 ml of 2 % aqueous ferric chloride solution. Then, the reddish brown or orange colour was showed as $FeCl_3$ test that was positive for siderophore production.

Arnow's assay (Catcholate type of siderophore): 1ml culture filtrate and 1ml of 0.5N hydrochloric acid, 1ml of nitrite molybdate reagent and 1ml of 1N sodium hydroxide solution were added. The formation of red colour was presented as indication of catechol type of siderophore (Arnow, 1937).

Tetrazolium test (Hydroxamate type of siderophore): a pinch of tetrazolium salt and 2 drops of 2 N NaOH were added to 0.1 ml culture filtrate and observed for colour change. The appearance of a deep red colour was presented as hydroxamate-type of siderophore (Snow, 1954).

Optimization of siderophore production

Influence of different carbon sources: 100 ml of succinate broth was supplemented with 0.1g of different carbon sources eg xylose, Arabinose, Maltose, Cellobiose, and Galactose. Each flask was incubated at 37 ºC for 24 h in a rotary shaker 120 rpm. The growth and siderophore production was estimated (Tailor and joshi, 2012).

Different nitrogen sources: 100 ml of succinate broth containing 0.1 g of different nitrogen sources eg urea, ammonium chloride, potassium nitrate, and sodium sulphate. Each flask was incubated at 37 ℃ for 24 h in a rotary shaker 120 rpm. the growth and siderophore production was estimated (Tailor and joshi, 2012).

Different metal irons: the influence of metal irons on siderophore production was done. Each 100 ml of succinate broth was supplemented with 10 µm of iron, potassium, zinc, magnesium, cobalt, mercury. Each flask was incubated at 30 ºC for 24- 48 h. The growth and siderophore production was estimated.

Different amino acid: the effect of different amino acids, succinate broth was determined at 0.1% Trytophane, Histidine, Cystein, Tyrosin, and Glysine, and inoculated at 30 ºC for 36 h under shaking at 100 rpm. The growth and siderophore production was measured.

Evaluation of bioefficacy in pot culture experiment

Seeds of *Arachis hypogaea* were soaked in water overnight and surface sterilized with 0.1% mercury chloride for 5 min and washed several times with sterilized distilled water. The garden soil and sand (2:1 w/w) was sterilized for 3 days before seeds were sown. After germination, the seedling was thinned out to be 2-3 seedlings per pot. The pots were randomly arranged in the greenhouse, the plants were irrigated with nitrogen-free sterile tap water on alternate days. Plants were measured at 60 DAI (Day after Inoculation) and separated into

leaves, shoots, roots, and plant length (cm). Three plants were taken each pot to measure the mean value for all the treated and control.

Estimation of photosynthetic pigments: chlorophyll a, b and carotenoids contents were extracted from leaves and estimated according to the method of Arnon (1949). Carotenoids content was determined according to the method of Krick and Allen (1965). Chlorophyll content was calculated using the formula of Arnon (1949).

Determination of total iron content: the plant materials (leaves, stem, seed) for total iron were measured the weighed samples (0.5g) by digesting the mixture of 30% H_2O_2 and 65% HNO_3 (3:5, v/v). The samples were transferred to 50 ml flasks and diluted with the distilled water (Poland, 2007).

Determination of total seed oil content: total seed oil content was estimated by Soxhlet asparatus. Each seed was dried and weighed before taken to the device. The chloroform was used as a solvent. The dried and powdered seed samples were taken to the soxhlet device and extracted by evaporating solvent. % Ether extract = (weight of flask + extract – tare weight of flask) / weight of sample) x100 as determination of mineral content in Indian spices by ICP-OES).

Isolation of fungal pathogen

Fusarium sp and *Cercospora* sp were isolated from naturally infected groundnut causing wilt and rot diseases using standard isolation techniques .

Antagonist activity of dual culture technique

The antagonistic properties of *Pseudomonas aeruginosa* PSA01 and *Pseudomonas fluorescences* PSF02 against *Fusarium sp* and *Cercospora* sp was done by dual culture technique. Inhibition zone and mycelial growth of tested pathogens were recorded and percent inhibition was calculated (Reshma *et al*., 2018) as $\mathbf{I\ (\%)= \frac{(C-T)\times 100}{C}}$ where I = Inhibition of Mycelial growth, C = Growth of plant pathogens in the control plate (cm), T = Growth of pathogens in dual cultures (cm).

Results

Isolation of Pseudomonas spp.

The isolates showed that Gram- negative, rod shaped and motile. The biochemical characterization of the bacterial isolates PSA01 & PSF02 are given in Table 1. The isolates were various phenotypic characters that compared with

standard strains of *Pseudomonas aeruginosa* PSA01 and *Pseudomonas fluorescences* PSF02.

Table1. Biochemical characterization of bacterial isolation

Test	PSA01	PSF02
Gram staining	-	-
Indole production	+	+
Methyl Red	+	+
Voges-proskaur	_	+
Citrate utilization	+	+
Catalase activity	+	+
Oxidaseproduction	+	+
Urea's activity	+	+

(+) Positive, (-) Negative

16S r RNA gene sequencing of *P. aeruginosa* NCBI is deposited in Genbank accession number MH128359. It showed the maximum sequence similarity 99 %. *P. fluorescenes* is deposited as accession number MK478897 which the maximum sequence similarity was 96 % (Figure 1). These isolates were confirmed to *Pseudomonas aeruginosa*, *Pseudomonas fluorescences* named as from PSA01, PSF02 (Figs. 1 and 2).

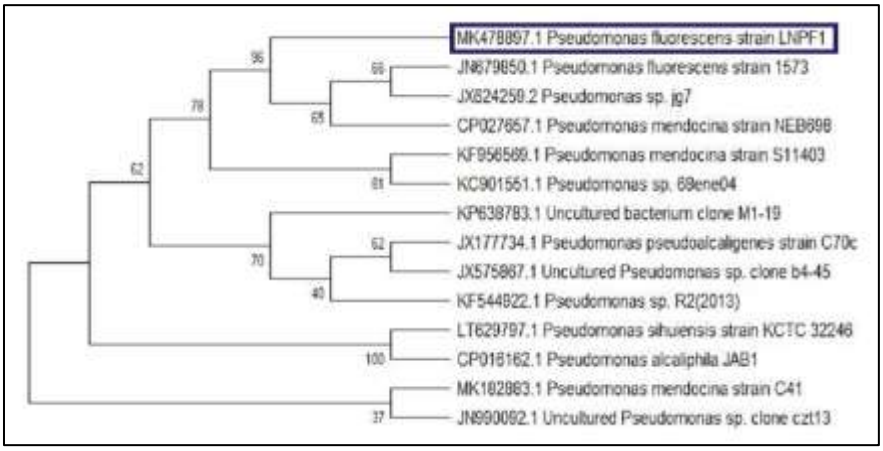

Figure 1. Phylogenetic tree on *Pseudomonas fluorescenes* 16S r RNA gene sequences were submitted Genebank under Accession number MK478897. It showed the maximum sequence similarity 96 %

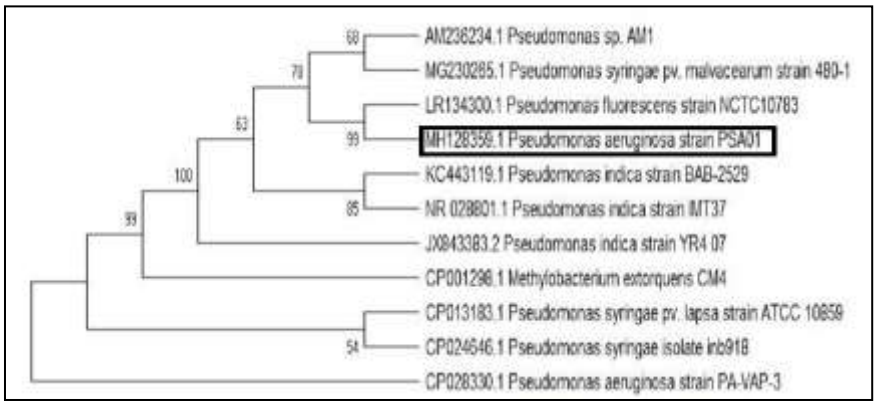

Figure 2. Phylogenetic tree on *Pseudomonas aeruginosa* 16S r RNA gene sequences were submitted Genebank under Accession number MH128359. It showed the maximum sequence similarity 99 %

Assay for indoleacetic acid (IAA) production

Quantitative of IAA production was reported with different concentration of tryptophan. Result showed that the production of IAA was not observed with no addition of trytophane. The addition of tryptophan from 50 to 500 µg/ mL leading to increase the production of IAA. The production of IAA was highest in isolates of PSF02 and followed by PSA01 (Table 2).

Qualitative and quantitative estimation of phosphate solubilization

Phosphate solubilizing ability in pikovskaya agar, PSA01 & PSF02 was formed clear zone around the colony. The highest phosphate solubilizing activity was shown by the isolates PSF02 with an averaged clear zone of 0.5 mm.

Qualitative estimation of siderophore production

In CAS assay PSA01 & PSF02 showed positive reaction (orange colour zone) for siderophore production. The strain PSF02 produced maximum of 9.4±0.55mm orange colour zone in CAS plate. CAS plates were blue in colour because chrome azurol S dye is reacted with ferric iron. When siderophore is presented the following reaction occurs which releasing the free dye of orange in colour.

Quantitative estimation of siderophore production

Quantitative of chrome Azurol sulphonate (CAS) assay described by Schwyn and Neilands, and the OD value was measured at 630 nm. of siderophore production produced in PSA01 and PSF02 were 60.51±0.95% and 62.48±0.88%, respectively (Table 3).

Table 2**.** Quantitative analysis of IAA production (µg/ mL± SD) concentrations µg/mL

	0	50	150	300	400	500
PSA01	ND	2.13±0.15	4.67±0.15	7.67 ±0.15	10.43±0.47	15.00±0.26
PSF02	ND	2.56±0.20	5.90±0.20	11.30±0.36	15.17±0.25	18.70±0.25

ND-not detectable

Table 3**.** CAS assay based on the color change around the microbial colonies

Pseudomonas spp	**CAS Agar Plate Zone diameter in (mm)**	**Quantitative Estimation of % Siderophore**
PSA01	**6.53±0.56**	60.51±**0.95**
PSF02	**9.4±0.55**	62.48±**0.88**

Determination of siderophore

Result showed that PSA01 & PSF02 ferric chloride test was a positive result. The formation of a red colored solution was considered as an indication of catechol type of siderophore in PSA01 and PSF02 isolates were not catecholate type. The appearance of a deep red color was an indication of hydroxamate-type of siderophore.

Optimization of physiochemical parameter for siderophore production Influence of carbon sources and Nitrogen sources

The optimization was carried out with the different source of nitrogen such as urea,ammonium chloride, potassium nitrate, sodium sulphate, ammonium sulphate both the bacterial isolates showed maximum production of siderophore as NH_4SO_4 (76.14 %). The carbon source and its availability play a role in regulating siderophore production. The various carbon sources tested the optimum siderophore such as Xylose, Arabinose, Maltose, Cellobiose, Galactose yielded xylose in PSF02. In maximum yield of siderophore production was in PSF02 xylose (88.9 %) (Table 4).

Table 4**.** Optimization of carbon source and Nitrogen source

Influence on carbon source	% of SU		Influence on Nitrogen source	% of SU	
	PSAO1	**PSFO2**		**PSAO1**	**PSFO2**
Xylose	70.05	88.9	**NH_4 SO_4**	76.14	64.05
Arabinose	85	87.52	**NH_4 Cl_2**	82.52	77
Maltose	67.9	75.03	**Urea**	70.19	57.32
Cellobiose	65	67	**KNO_3**	67	58.62
Galactose	77	68.5	**Na_2 SO_4**	87	83

Influence of metal irons and amino acids

The siderophore production was carried out in SM medium with different metal ions such as, zinc, magnesium, cobalt, copper and mercury. In both the bacterial isolates showed a maximum siderophore production was in PSF02 cu^{2+} (87.63%). Both bacterial isolates in succinate broth showed the maximum of siderophore production in Trytophane (87.02) (Table 5).

Table 5**.** Optimization of Metal irons and Amino acids

Influence on Metal irons	% of SU		Influence on Amino acids	% of SU	
	PSAO1	**PSFO2**		**PSAO1**	**PSFO2**
K2+	56.17	59.02	**Tryptophan**	61	87.02
Zn2+	50.25	59	**Histidine**	78.92	48
Mg2+	54.01	61	**Cystein**	49	59.93
Cu2+	73.67	87.63	**Tyrosine**	36	39
Co2+	55	53	**Glycine**	56.52	44

Evaluation of bioefficacy of isolates (Pot culture studies)

The siderophore producing isolates were inoculated to *Arachis hypogaea.* After 60 days of inoculation, it was significantly enhanced in PSF02 that root and shoot length, fresh and dry weight as compared to the inoculated PSAO1+PSF02, inoculated PSA01 and uninoculated plants are shown in Table 6 and Figure 3.

Table 6. Effect on growth parameters of *Arachis hypogaea* (60 DAI =Days after Inoculation)

Treatment	Leaf length (cm)	Shoot length (cm)	Root length (cm)	Fresh Weight (g)	Dry Weight (g)	Number of nuts
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Control	2.66 ±2.51	31 ±0.816	15 ± 4.08	6.8 ± 0.20	3.6 ± 0.5	5
PSF02	6.66 ±1.52	39.6 ±1.63	33.1 ± 1.24	12.9 ± 0.68	7.3 ± 0.60	12
PSA01	3.66 ±1.15	36.6 ±1.24	30 ± 1.63	10.1 ± 0.36	4.5 ±0.2	9
PSA01+PSF02	4 ±1.73	38.9 ±1.24	31.2 ± 1.24	10.6 ± 0.25	5.8 ± 0.21	10

Figure 3. Pot culture studies on *Arachis hypogaea*

Estimation of total iron content and oil content

Iron content in *Arachis hypogaea* which treated with PSF02 significantly increased in leaves (551.52 ±6.00 µg/g), shoots (581.17 ±7.08), seeds (672.25 ±6.06 µg/g) when compared to inoculated PSA01+PSF02, inoculated PSA01, uninoculated plants. Result increased in oil content in PSF02 50.3 ±1.2% and followed by inoculated PSA01+PSF02 (47.5 ±1.08%) and inoculated PSA01 (42.53 ±0.54 %) and uninoculated control (34.36 ±1.36 %) as seen in Table 7.

Arachis hypogaea showed significantly increased total chlorophyll and carotenoids content in inoculated PSF02 as compares to inoculated PSA01+PSF02, PSA01 and uninoculated plants (Table 8).

Antagonist activity in dual culture technique

Result showed that *Pseudomonas* sp. effectively inhibited of the tested fungi in dual culture at high radial growth in PSF02 (6.5 cm) against *Fusarium* sp and the inhibition over 16.66 % and inhibited *Cercospora* sp which showed

a radial growth in PSF02 of 5.2 cm which inhibition over 32.05 %.b Antagonist activity in dual culture showed highly inhibition growth in PSF02 compared to PSA01 (Table 9).

Table 7. Fe content and total seed oil content of *Arachis hypogaea* plants

Treatment	Leaf (µg/g)	Shoot (µg/g)	Seed (µg/g)	Total seed oil content %
Control	310.34 ± 4.66	453.10 ± 7.18	357.91 ±11.6	34.36 ±1.36
PSF02	551.52 ± 6.00	581.17 ± 7.08	672.25 ±6.06	50.3 ± 1.2
PSA01	509.35 ± 3.26	524.09 ± 4.28	518.10 ± 7.60	42.53 ± 0.54
PSF02+PSA01	525.69 ±6.03	556.16 ± 5.79	585.21 ± 8.73	47.5 ± 1.08

Table 8. Estimation of total chlorophyll & carotenoids content

Treatment	*Arachishypogaea*	
	Chlorophyll mg g^{-1}	Carotenoids mg g^{-1}
Control	0.023 ±0.03	0.021 ±0.02
PSF02	1.150 ±0.28	0.059 ±0.10
PSAO1	0.113 ±0.26	0.035 ±0.05
PSF02+PSA01	0.125 ±0.16	0.046 ±0.04

Table 9. Antagonist activity of dual culture technique

Plant pathogens	Dual culture technique			
	PSA01		PSF02	
	Radial growth cm	Inhibition over Control %	Radial growth cm	Inhibition over Control %
Fusariurm sp.	5.9	24.35	6.5	16.66
Cercospora sp.	5.2	33.33	5.2	32.05

Discussion

Pseudomonas was gram negative bacteria and confirmed that belongs to *Pseudomonas*. The morphology of *Pseudomonas* spp. showed a Gram-negative, pink colored, appearance with rod-shaped bacteria. This was agreed with the

findings by Tripathi *et al*. (2011). The qualitative estimation of siderophores production showed that the isolates *P. fluorescens* and *P. aeruginosa* are powerful producers with limited iron. The siderophore was produced by *P. fluorescens* and *P. aeruginosa* indicated as a biocontrol against soil-borne pathogens. Similarly, earlier reports stated that *P. fluorescens* was given the high yield of siderophores under iron stress condition (Sayyed *et al.*, 2005). It had been observed in the CAS reagent from blue to orange in CAS agar that the siderophore production was positive by the change of colour (Pahari *et al* . 2016). Three bacterial colonies were able to produce yellow green fluorescent pigment on Kings B agar medium (MehriInes *et al*., 2012)The optimum condition in different pH levels resulted that *Pseudomonas aeurginosa* was optimum to produce siderophore at pH 7.0. The production of siderophore depended pH of medium. It was very important to maintain pH during fermentation broth between 7.0-7.5 which is the optimum for siderophore production in *P. fluorescences.* The siderophore produced by *Azotobacter* sp. is an iron chelating substance with the ability to bind different metal ions to increase in molybdenum and cadmium (Neilands, 1974). Carbon sources play a secondary metabolism and important role to regulate siderophore production. Siderophore production varied with the type of carbon source in *Pseudomonas* sp. (Sayyed *et al.*, 2005). The effect of minerals towards siderophore the production was carried out in mannitol medium amended with different mineral ions. The metal ions cu^{2+} in the medium enhanced the siderophore production. Bhattacharjee and Dey (2014) stated that microorganisms used for the biofertilizer are *Bacillus, Pseudomonas, Lactobacillus*, photosynthetic bacteria, nitrogen-fixing bacteria but the present study was used *Pseudomonas* tested in pot experiment showed a good the effect of growth promoting activity and yield of *Arachis hypogaea*. This realization is particularly concerned to be an important to understand on siderophore production by *P. aeruginosa* as it was stated by Harrison *et al*. (2006).

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