Optimization of MM9 Medium Constituents for Enhancement of Siderophoregenesis in Marine *Pseudomonas putida* Using Response Surface Methodology

R. M. Murugappan · A. Aravinth · R. Rajaroobia ·
M. Karthikeyan · M. R. Alamelu

Received: 11 November 2011 / Accepted: 16 February 2012 / Published online: 18 March 2012
© Association of Microbiologists of India 2012

Abstract *Pseudomonas putida* (CMMB2) was isolated from open ocean water of Gulf of Mannar. The isolate was identified based on 16S rRNA gene sequencing and phylogenetic analysis. Chrome azurol sulphonate assay confirms siderophore production by the isolate. Nature of siderophore produced by the isolate was found to be of mixed type. Siderophore production was found to be inversely proportional to iron concentration of the medium. Maximum siderophore production was observed with MM9 medium. Siderophore production was found to be influenced by different carbon, nitrogen and amino acid sources. Optimization of MM9 medium nutrient composition by response surface methodology (RSM) enhances siderophore production. Application of RSM is one of the strategic attempts in cost effective siderophore production process. Presence of aromatic ring in the siderophore with (C–O) and (C=C) stretching was ascertained by FTIR spectral analysis. Mass spectral analysis revealed the presence of chromophore in the pyoverdine siderophore. Cell free supernatant and purified siderophore was found to inhibit the growth of bacterial and fungal pathogens.

Keywords Siderophore · MM9 medium · RSM · Antagonism

Introduction

Iron is an important bioactive metal indispensable for the growth and metabolism of bacteria. Iron plays a key role in electron transport, oxidation–reduction reactions, detoxification of oxygen radicals, synthesis of DNA precursors and in many other biochemical processes [1]. Being a transition element, iron gets rapidly oxidized from soluble ferrous (Fe^{2+}) to insoluble ferric (Fe^{3+}) state. Dissolved iron in open ocean water is about 10^{-12} – 10^{-9} M, significantly below the concentration required for the bacterial growth (10^{-7} M) [2]. Therefore, microorganisms in the open ocean water face unique challenges to obtain iron required for their survival. In response to low iron concentration, many bacteria produce organic multidentate ligands generically known as siderophores which solubilize and transport iron into the cell [3]. Apart from iron chelation, siderophore production is considered as one of the virulence factors in bacteria. Fgaier and Eberl [4] reported that siderophore production in microorganisms possesses a competitive advantage over other species which lack the ability. Several earlier reports represent an inhibitory activity of pseudomands against several pathogenic microorganisms [5]. However, the mechanism behind their antagonistic activity remains obscure [6].

There is plethora of reports on siderophore production in terrestrial bacteria, but knowledge on siderophores produced by marine bacteria and its antagonistic activity is scarce [7]. Hence, the present study is intended to determine the siderophore producing ability of the bacterium *Pseudomonas putida* isolated from Gulf of Mannar. Influence of media, pH and nutrient sources on siderophore production was also analyzed. Nutrient constituents of MM9 medium was optimized by Central composite design (CCD) of RSM for increasing siderophore production.

R. M. Murugappan (✉) · A. Aravinth · R. Rajaroobia ·
M. Karthikeyan
Department of Zoology and Microbiology, Thiagarajar College,
Madurai, Tamil Nadu, India
e-mail: cmmbmak@gmail.com

M. R. Alamelu
Department of Mathematics, Odaiyappa College of Engineering
and Technology, Theni, Tamil Nadu, India

In vitro antagonistic activity of siderophores against bacterial and fungal pathogens was also evaluated.

Materials and Methods

Strain Isolation and Identification

Pseudomonas was isolated from the open ocean water of Gulf of Mannar (latitude 9°12'N and longitude 79°53'E) using King's B medium. The isolate was identified based on the biochemical analyses [8]. Further 16S rRNA gene sequencing was carried out for species identification. Amplification of the 16S rRNA gene was attempted by PCR using forward primer 5'-AGAGTTTGATCCTGGTC AG-3' and reverse primer 5'-TAAGGAGGTGATCCAG GC-3' [9]. The 16S rRNA gene sequence of the isolate was submitted to NCBI and compared with related gene sequences. Selected sequences were aligned in Bio-Edit. Phylogeny was examined by neighbour-joining dendrogram using MEGA software [10].

Screening for Siderophore Production

For siderophore production, one ml of the isolate (5×10^7 bacterial cells/ml) was suspended in iron depleted minimal (MM9) medium [9]. After incubation, the cell free supernatant (10,000 rpm for 15 min) was examined for siderophore production by FeCl_3 test and CAS agar plate method [11]. Nature of siderophore produced by the isolates was ascertained by Csaky, Arnow's and Vogel's assay [11]. The amount of siderophore in the culture supernatant was quantified by Chrome azurol sulphonate (CAS) shuttle assay [11]. The growth was estimated in terms of protein content [12] with bovine serum albumin (BSA) as the standard.

Influence of Media and Hours of Incubation on Siderophore Production

Influence of seven different media and hours of incubation on siderophore production was analyzed. Media were rendered iron free by treating them with 3 % 8-hydroxy-quinoline in chloroform [13]. Three replicates were maintained. Following incubation, growth and siderophore units were quantified.

Influence of Iron and pH

To determine the influence of iron and pH on siderophore synthesis, the isolate was grown in MM9 medium with different concentration of ferric ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) iron (2–20 μM) and pH (4–11) for 48 h.

Influence of Carbon, Nitrogen and Amino Acid Sources

Influence of different carbon, nitrogen and amino acid sources on growth and siderophore production of the isolate was determined separately in three different sets as described by Calvente et al. [14]. Influence of different carbon sources was ascertained by supplementing MM9 separately with 4 g l^{-1} each of glucose, fructose, sucrose, succinate, maltose and mannitol while the other constituents of the medium retained. Similarly, in another set, influence of eight different amino acids (3 g l^{-1}) namely alanine, serine, arginine, lysine, ornithine, asparagine, threonine and casamino acids were analyzed. In the third set four different nitrogen sources (10 g l^{-1}) ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$, ammonium nitrate $[\text{NH}_4(\text{NO}_3)_2]$, ammonium phosphate $[\text{NH}_4\text{PO}_4]$, ammonium chloride $[\text{NH}_4\text{Cl}]$ and sodium nitrate $[\text{NaNO}_3]$ were used for fortification studies. Each experimental set was inoculated with one ml of the isolate (5×10^7 bacterial cells/ml) and incubated in a shaker (120 rpm) at 28 °C. Following 48 h of incubation, each set was subjected for growth and siderophore quantification. Triplicates were maintained for each analysis.

Optimization for Siderophore Production in MM9 Medium by Statistical Method

Experimental Design

The interactive effect of three main constituents of MM9 medium viz., succinate, NH_4Cl and casaminoacids on siderophore production by the isolate was analyzed using response surface methodology (RSM) of CCD. Twenty sets of experiment were formulated (Design Expert software) to determine the optimum concentration of the three variables ($n = 3$) at five levels ($\alpha = 2$) with the central point consisting of 6 trials. Siderophore produced by the isolate in different experimental setup was quantified after 48 h of incubation. 3D surface graphs were generated to illustrate the interaction between the three factors and the optimum value of each factor.

Statistical Analysis

The second order polynomial equation was employed to fit the experimental data. The proposed model for the response Y_i was given in the following equation [15]:

$$Y_i = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \beta_3\chi_3 + \beta_{11}\chi_1^2 + \beta_{22}\chi_2^2 + \beta_{33}\chi_3^2 + \beta_{12}\chi_1\chi_2 + \beta_{13}\chi_1\chi_3 + \beta_{23}\chi_2\chi_3 \quad (1)$$

where Y_i = predicted response, χ_1 , χ_2 and χ_3 are independent variables, β_0 is the offset term, β_1 , β_2 and β_3 are the linear effects, β_{11} , β_{22} and β_{33} are squared effects and

β_{12} , β_{13} and β_{23} are interaction term effects. All designed experiments were conducted in triplicates. Statistical and numerical analyses were carried out by means of the analysis of variance (ANOVA).

Purification of Siderophore

Purification of siderophore produced by the isolate was carried out using Amberlite XAD 400 resin and Sephadex LH-20 column as described by Sayyed and Chincholkar [16]. Purified siderophore sample was lyophilized and stored at -20°C for further structural analyses.

FTIR and Mass Spectroscopy

Lyophilized siderophore sample was pelleted with potassium bromide (KBr) and subjected to FTIR spectroscopy. Spectra were recorded in $4,000\text{--}400\text{ cm}^{-1}$ range. For mass spectrum purified siderophore was dissolved in water/methanol/trifluoroacetic acid 50:50:0.1 (v/v/v). One μl was injected and the ion of interest was selected then collided with helium gas to obtain fragments and analyzed based on m/z .

In Vitro Antagonistic Activity

Antibiosis of purified siderophore and cell free culture supernatant of *P. putida* was tested by well diffusion method. Wells of 8 mm were made on Muller Hinton agar plates swabbed with the target bacterial cultures. To elucidate the antifungal activity of the siderophores, spore ($6 \times 10^6\text{ ml}^{-1}$) suspension of the test fungi were mixed with molten potato dextrose agar and poured into the sterile petri plates. 20 μl of cell free supernatant filtrate and purified siderophore were loaded onto the wells. Antibacterial and antifungal potential of the siderophore was determined by measuring the zone of inhibition after 48 h of incubation.

Result and Discussion

Strain Identification and Phylogenetic Analysis

The bacterial isolate was identified as *Pseudomonas* based on the morphological and biochemical characteristics. Identification of the isolate was further confirmed by 16S rRNA gene sequencing (GenBank no. GQ404505). Phylogenetic tree generated by the neighbor-joining method for the isolate is shown in Fig. 1. Based on the sequence analysis and phylogenetic tree generated the isolate was confirmed as *P. putida*. The dendrogram shows reliability of branching order based on bootstrap analysis. The fluorescent *Pseudomonas* from the open ocean was chosen for the following reasons: firstly *Pseudomonas* represent one of the important Gram-negative aerobic bacteria of γ -proteobacteria known for their capacity to colonize various ecological niches; secondly, controversy exist on siderophore contribution to the bio-control capacity of the fluorescent *Pseudomonas*; thirdly, little is known about the influence of nutrient sources on siderophore production.

Siderophore Characterization

Development of orange halo in CAS agar is an indicative of siderophore production by the isolate. Cell free supernatant exhibits an absorbance maximum at 404 nm, which confirms pyoverdinin type of siderophore. Further positive results with Csaky's and Arnow's assays (absorbance maxima at 525 and 510 nm, respectively) revealed the presence of mixed (hydroxamate and catecholate) type of siderophore.

Influence of Different Physico-Chemical Parameters on Siderophore Production

Of the seven different media analyzed, maximum siderophore production was observed with MM9 salts medium

Fig. 1 Phylogenetic analysis of 16S rRNA gene sequence of *Pseudomonas putida*

Table 1 Influence of different media and nutrients on siderophoregenesis of marine *P. putida*

Media	SU (%)	Sugars	SU (%)	Nitrogen sources	SU (%)	Amino acids	SU (%)
ZMB	60	Mannitol	57	(NH ₄) ₂ SO ₄	66	Arginine	62
MM9	71	Glucose	71	NH ₄ Cl	71	Serine	64
KBM	66	Fructose	47	NH ₄ PO ₄	59	Lysine	66
SWNB	52	Sucrose	54	NH ₄ (NO ₃) ₂	51	Ornithine	68
IDSMB	34	Maltose	40	NaNO ₃	53	Threonine	62
ASW	45	Succinate	73			Asparagine	68
LB	42					Alanine	57
						Casamino acids	71

MM9 minimal medium, SWNB sea water nutrient broth medium, ZMB Zobell marine broth, LB Luria–Bertani broth, KBM King's B medium, ASW artificial sea water medium, IDSMB iron deficient low nutrient artificial sea water medium, SU siderophore units

Table 2 Influence of different pH, iron and hours of incubation on growth and siderophoregenesis of marine *P. putida*

Hours	SU (%)	Growth (μg/ml)	Iron (μM)	SU (%)	Growth (μg/ml)	pH	SU (%)	Growth (μg/ml)
6	0	1	2	70	46	6	0	1
12	0	6	4	70	48	6.5	0	1
18	0	14	6	62	39	7	48	27
24	12	29	8	58	52	7.5	60	45
36	22	39	10	49	53	8	71	53
48	46	48	12	23	55	8.5	65	37
54	62	55	14	12	58	9	0	9
60	71	58	16	4	56	9.5	0	0
66	70	58	18	0	49			
72	68	56	20	0	50			

SU siderophore units

(Table 1) followed by KBM, ZMB, SWNB, ASW, LB and IDSMB (71, 66, 60, 52, 45, 42 and 34 % units, respectively at 48 h of incubation). This variation clearly indicates that siderophore production in microorganisms is substrate dependent. Resemblance of minimal media to the marine environment in its composition may be the reason for maximum siderophore production. Gram [17] stressed that the media which resemble the ambient environment enhance the biological potential of the organism. In the MM9 medium, siderophore production was observed only after 24 h of incubation (Table 2) and it was found to be directly correlated with bacterial growth (in terms of μg protein per ml). The isolate exhibit 12 h long lag phase followed by 36 h log phase (up to 48 h). Maximum siderophore production by the isolate during late log phase reveals a critical demand for iron during that phase of bacterial growth.

Increase in iron concentration of the medium had a negative effect on siderophore production whereas it does not influence the growth of the isolate. Similar biphasic relationship was observed by Barghouthi et al. [18] in

A. hydrophila 495A2. Dave and Dube [19] reported 27 μM of iron as threshold level at which fluorescent pseudomonads ceased siderophore production. Siderophore production by *Pseudomonas putida* at 2 and 16 μM was found to be 78 and 4 % units, respectively. At 18 and >18 μM iron concentrations, complete retardation of siderophore production was observed (Table 2). Suzuki et al. [20] reported that the transcription of iron regulated gene is under the negative control of fur protein (repressor) with Fe²⁺ as an essential corepressor during iron overload.

Besides iron concentration, environmental factors and growth conditions are also found to influence siderophore production. It is evident from Table 2 that there is no growth of the isolate below pH 7 and above pH 9. Maximum growth and siderophore production by the isolate was observed at pH 8 this may due to the halophilic nature of the isolate. These results are in accordance with that of Payne [11] who reported breakdown of siderophores at higher pH.

In the present study, manipulation of culture medium (MM9) composition found to influence siderophore synthesis. Of the different carbon sources analyzed maximum

Table 3 Central composite design (CCD) matrix with experimental and predicted values of siderophore production

Std	Succinate (g l ⁻¹)	NH ₄ Cl (g l ⁻¹)	Casamino acids (g l ⁻¹)	Siderophore units (%)	
				Experimental	Predicted
15	4.00	10.00	3.00	71	71.13
6	5.00	7.50	4.00	79	76.45
14	4.00	10.00	4.68	81	84.56
2	5.00	7.50	2.00	58	60.46
13	4.00	10.00	1.32	45	36.64
9	2.32	10.00	3.00	57	50.91
12	4.00	14.20	3.00	70	67.17
10	5.68	10.00	3.00	73	74.29
11	4.00	5.80	3.00	66	64.03
3	3.00	12.50	2.00	30	35.93
1	3.00	7.50	2.00	28	33.56
8	5.00	12.50	4.00	80	77.81
4	5.00	12.50	2.00	60	62.82
7	3.00	12.50	4.00	76	76.92
5	3.00	7.50	4.00	75	75.55

Table 4 Analysis of variance for the regression model of siderophore production obtained from experimental results

Source	SS	df	MS	F value	Prob (p) > F
Siderophore production: ($R^2 = 0.9479$, adj $R^2 = 0.9011$, pred $R^2 = 0.6052$, CV = 7.29 %)					
Model	4109.55	9	456.62	20.24	<0.0001
A-Succinate	659.57	1	659.57	29.23	0.0003
B-NH ₄ Cl	11.861	1	11.86	0.53	0.4851
C-Casamino acids	2771.32	1	2771.32	122.82	<0.0001
A × B	0.000	1	0.000	0.000	1.0000
A × C	338.00	1	338.00	14.98	0.0031
B × C	0.50	1	0.50	0.022	0.8846
A ²	131.09	1	131.09	5.81	0.0367
B ²	55.10	1	55.10	2.44	0.1492
C ²	199.76	1	199.76	8.85	0.0139
Residual	225.65	10	22.56		
Lack of fit	22.65	5	45.13		
Pure error	0.000	5	0.000		
Total	4335.20	19			

SS sum of squares, df degrees of freedom, MS mean squares

siderophore production (in % units) was observed on supplementation of MM9 medium with succinate followed by glucose, mannitol, sucrose, fructose and maltose (73, 71, 57, 54, 47 and 40 % units, respectively). Role of succinate in siderophore production can be substantiated by the

Fig. 2 Three-dimensional response surface curve for siderophore production

Fig. 3 FT-IR spectra of purified siderophore

structure of pyoverdins in which the three amino moiety of the chromophore is substituted with various acyl groups derived from succinate, malate, α -ketoglutarate [21].

Of the different amino acids tested maximum siderophore production was observed when the growth medium was supplemented with casamino acids, followed by ornithine, asparagine, lysine, rine, arginine, threonine and alanine (Table 1). Of the various nitrogen sources tested, maximum siderophore yield (71 % units) was obtained on supplementation of MM9 medium with ammonium chloride (Table 1).

Response surface methodology

From response surface analysis, the optimal concentration of succinate, NH_4Cl and casamino acids were found to be 4.00, 10.00 and 3.00 g l^{-1} , respectively (Table 3). The final response equation for siderophore production is given below

$$\begin{aligned} \text{Siderophore} = & +71.14 + 6.95\chi_1 + 0.93\chi_2 + 14.25\chi_3 \\ & - 3.02\chi_1^2 - 1.96\chi_2^2 - 3.72\chi_3^2 \\ & + 0.000\chi_1\chi_2 - 6.50\chi_1\chi_3 - 0.25\chi_2\chi_3 \end{aligned}$$

The model equation for siderophore production showed significant positive linear effects for succinate and casamino acids. Statistical significance of respective model equation was checked using analysis of variance and the results are summarized in Table 4. The response of the different variables revealed that χ_1 , χ_3 , $\chi_1 \times \chi_3$, χ_1^2 and χ_3^2 are significant model terms for (p values < 0.05) siderophore production. The interactive effect of succinate and NH_4Cl was found to be non significant ($p > 0.05$). In the present study, the R^2 value was 0.9749. The closer the R^2 value to 1.0 the stronger the model and the better in

predicted response. An adequate precision of 15.183 for siderophore production was recorded. The predicted R^2 of 0.6052 was in reasonable agreement with the adjusted R^2 of 0.9011. A relatively lower value of the coefficient of variation ($\text{CV} = 7.29\%$) indicated a good precision and reliability of the experiment (Table 4).

Response surface curves were generated by plotting the response (siderophore production) on the Z-axis against two of the three selected independent variables while keeping the other independent variable at its 'O' level (Fig. 2 a–c). It is obvious from the response surface curves that succinate and casamino acids have significant effect on siderophore production. A linear increase in siderophore production was observed with succinate and casamino acid concentration. Siderophore production was found to increase with increasing concentration of NH_4Cl up to 10 g l^{-1} . Predicted and experimental responses reflected the accuracy and applicability of RSM for optimal production of siderophore. Similarly Su et al. [22] enhanced phenazine-1-carboxylic acid production in wild and mutant strains of *Pseudomonas* using RSM.

Table 5 Determination of functional groups in the siderophore by FT-IR spectra

Peak ranges (cm^{-1})	Functional groups
3135.60	Primary alcoholic OH
1615.09	$\text{C}=\text{O}_{\text{Str}}$
1,402	C–H
1036.55	$\text{Ar}-(\text{C}-\text{O})_{\text{Str}}$
2360.44	$(\text{C}=\text{C})_{\text{Ar}}$

Ar aromatic group, Str stretching

Fig. 4 Mass spectrum of the purified siderophore

Infra Red Spectroscopy

The IR spectrum of the purified siderophore showed a broad peak at 3135.60 cm^{-1} characteristic of a primary alcoholic group (--O--H stretching). A strong peak observed at 1615.09 cm^{-1} indicates C=O stretching. The peak at $1,402\text{ cm}^{-1}$ may be due to C--H bending vibration. Infrared spectrum provides evidence for the possible presence of aromatic ring in the siderophore (Fig. 3; Table 5). A strong peak at 1036.55 cm^{-1} indicates Ar--C--O) stretching of phenolic OH group. A peak at 2360.44 cm^{-1} represents C=C stretching in the aromatic ring.

Mass Spectra

Mass spectrometry provides structural evidence on the presence of pyoverdin chromophore. Figure 4 represents a molecular ion peak at 428.64 m/z and fragmented ions peak at 428.37 , 339.30 , 297.27 , 277.25 , 217.24 , 204.23 and 88.06 m/z . Molecular ion peak at 428.37 m/z represent the chromophore. A peak at 217.24 and 204.23 m/z represents the most characteristic pyoverdin and its ring cleavage product.

Antagonistic Activity

Antagonistic activity of purified siderophore and cell free culture supernatant of *P.putida* was presented in Table 6 and Fig. 5. Fungi tested were found to be more susceptible than bacteria. Cell free supernatant of *P. putida* had a

greater antagonistic activity towards fungi than purified siderophores. This may due to the cumulative effect of secondary metabolites and siderophores present in the cell free supernatant. These findings are in the line with Yu et al. [23]. Purified siderophores had a greater antagonistic activity towards the bacteria tested than the cell free supernatant. Of the five different bacterial pathogens tested, *Bacillus subtilis* (NCIM 2063) was found to be resistant to both the cell free supernatant and purified siderophores (Table 6). Based on the above results it is evident that siderophore production impart antagonistic trait in *P. putida* against several pathogens. Therefore,

Table 6 Antimicrobial activity of cell free supernatant and purified siderophore of marine *Pseudomonas putida*

Isolates	Zone of inhibition (mm)	
	Cell free supernatant	Purified siderophore
<i>Staphylococcus aureus</i> (NCIM 2079)	9	12
<i>Bacillus subtilis</i> (NCIM 2063)	–	–
<i>Aeromonas hydrophila</i> (MTCC 646)	13	16
<i>Vibrio parahaemolyticus</i> (MTCC 451)	11	15
<i>Vibrio harveyi</i> (MTCC 3438)	10	14
<i>Asperillus niger</i> (NCIM 586)	15	11
<i>Microsporium gypseum</i> (MTCC 2830)	13	12
<i>Aspergillus flavus</i> (MTCC 7133)	18	14
<i>Penicilium oxalicum</i> (MTCC 4931)	13	9
<i>Fusarium oxysporum</i> (MTCC 4894)	14	10

Fig. 5 In vitro antibiosis of cell free supernatant and purified siderophore. *Asperillus niger* NCIM 586 (**a**, **d**); *Penicilium oxalicum* MTCC 4931 (**b**, **e**); *Aspergillus flavus* MTCC 7133 (**c**, **f**);

Staphylococcus aureus NCIM 2079 (**g**, **j**); *Aeromonas hydrophila* MTCC 646 (**h**, **k**); *Vibrio parahaemolyticus* MTCC 451 (**i**, **l**)

siderophore producing *P. putida* can be used as antagonistic biocontrol agents to displace and inhibit the proliferation of pathogens by the iron competition process.

Acknowledgments University Grants Commission (UGC), Government of India, is gratefully acknowledged for the financial support. Authors thank the Management of Thiagarajar College, Madurai and Odaiyappa College of Engineering and Technology, Theni for their support.

References

- Hider R, Kong X (2010) Chemistry and biology of siderophores. *Nat Prod Rep* 27:637–657
- Vraspir JM, Butler A (2009) Chemistry of marine ligands and siderophores. *Annu Rev Mar Sci* 1:43–63
- Boukhalfa H, Reilly SD, Michalczyk R, Iyer S, Neu MP (2006) Iron (III) coordination properties of a pyoverdine siderophore produced by *Pseudomonas putida* ATCC 33015. *Inorg Chem* 45:5607–5616
- Fgaier H, Eberl HJ (2011) Antagonistic control of microbial pathogens under iron limitations by siderophore producing bacteria in a chemostat setup. *J Theor Biol* 273:103–114
- Viswanathan R, Samiyappan R (2007) Siderophores and iron nutrition on the *Pseudomonas* mediated antagonism against *Colletotrichum falcatum* in sugarcane. *Sugar Tech* 9:57–60
- Hofte M, Altier N (2010) *Fluorescence pseudomonads* as biocontrol agents for sustainable agricultural systems. *Res Microbiol* 161:464–471
- Butler A (2005) Marine siderophores and microbial iron mobilization. *Biometals* 18:369–374
- Brenner DJ, Krieg NR, Staley JT (2005) The proteobacteria, vol 2, Part B, the gammaproteobacteria. In: George GM (ed) *Bergey's manual of systematic bacteriology*. Springer, East Lansing, pp 491–545
- Murugappan RM, Aravinth A, Karthikeyan M (2011) Chemical and structural characterization of hydroxamate siderophore produced by marine *Vibrio harveyi*. *J Ind Microbiol Biotechnol* 38:265–273
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5:150–163
- Payne SM (1994) Detection, isolation and characterization of siderophores. *Method Enzymol* 235:329–344
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254
- Raaska L, Viikari L, Mattila-Sandholm T (1993) Detection of siderophores in growing cultures of *Pseudomonas* spp. *J Ind Microbiol* 11:181–186
- Calvente V, de Orellano MES, Benuzzi D, de Tosetti MIS (2001) Effects of nitrogen source and pH on siderophore production by *Rhodotorula* strains and their application to control of phytopathogenic moulds. *J Ind Microbiol Biotechnol* 26:226–229
- Sunitha K, Lee JK, Tae-Kwang O (1999) Optimization of medium components for phytase production by *E. coli* using response surface methodology. *Bioprocess Eng* 21:477–481
- Sayeed RZ, Chincholkar SB (2006) Purification of siderophores of *Alkaligenes faecalis* on Amberlite XAD. *Biores Technol* 97:1026–1029
- Gram L (1996) The influence of substrates on siderophore production by fish spoilage bacteria. *J Microbiol Methods* 25:199–205
- Barghouthi S, Young R, Olson MO, Arceneaux JE, Clem LW, Byers BR (1989) Amonabactin, a novel tryptophan- or phenylalanine containing phenolate siderophore in *Aeromonas hydrophila*. *J Bacteriol* 171:1811–1816
- Dave BP, Dube HC (2000) Regulation of siderophore production by iron Fe(III) in certain fungi and fluorescent Pseudomonads. *Indian J Exp Biol* 38:297–299
- Suzuki K, Tanabe T, Moon Y, Funahashi T, Nakao H, Narimatsu S, Yamamoto S (2006) Identification and transcriptional organization of aerobactin transport and biosynthesis cluster genes of *Vibrio hollisae*. *Res Microbiol* 157:730–740
- Villegas MED (2007) Biotechnological production of siderophores. In: Varma A, Chincholkar SB (eds) *Microbial siderophores*. Springerlink, Heidelberg
- Su JJ, Zhou Q, Zhang HY, Li YQ, Huang XQ, Xu YQ (2010) Medium optimization for phenazine-1-carboxylic acid production by a *gacA qscR* double mutant of *Pseudomonas* sp. M18 using response surface methodology. *Biores Technol* 101:4089–4095
- Yu X, Ai C, Xin L, Zhou G (2011) The siderophore-producing bacterium *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium* wilt and promotes the growth of pepper. *Eur J Soil Biol* 47:138–145