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THE QUALITATIVE AND QUANTITATIVE ASSAY OF SIDEROPHORE PRODUCTION BY SOME MICROORGANISMS AND EFFECT OF DIFFERENT MEDIA ON ITS PRODUCTION

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ABSTRACT

The production of siderophore by biocontrol agents (BGA) and plant growth promoting microbes (PGPM) is one of the important mechanisms for plant growth promotion and disease suppression. Microorganisms compete for iron by releasing siderophores. In this experiment, three fungi (*Trichoderma viride*-1 and *T. harzianum*-1 *and Candida famata*-1) and three bacteria (*Bacillus subtilis*-1, *B. megatericus* 1, *Pseudomonas aeroginosa*1) are taken for their evaluation as siderophore producer by both qualitative and quantitative assay. All fungi and bacteria gave positive response to qualitative assay. In quantitative assay, among the fungi, *C. famata* gave maximum (60.00%) siderophore while among the bacteria and fungi, *P. aeroginosa* yielded highest (80.50) percentage of siderophore. Moreover, effect of different media (MEB, NB, SMB, BRB and CCAB) on the siderophore production of *P. aerogenosa* was recorded where MEB supported maximum percentage of siderophore production (80.50%) but NB did not support. In modern science, production of pure siderophore in commercial way is very necessary as application of siderophore is in increasing trends in agriculture, medical science etc. Therefore, this work may be helpful for mass production of siderophore from microbes.

Key words: Microorganism, Siderophore, Qualitative, Quantitative.

INTRODUCTION

Siderophores are extracellular, small (low molecular weight < 1000 Daltons) compounds, which selectively bind iron (Fe^{3+}). The siderophores are generally produced by microorganisms, both aerobic and facultative anaerobic and monocotyledonous plants under low-iron stress conditions[1]. The production of siderophore by biocontrol agents (BGA) and plant growth promoting microbes (PGPM) is one of the important mechanisms for plant

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growth promotion[2,3]and disease suppression[4-7]. Siderophore producing bacteria have been used as biocontrol agents to combat plant pathogens[8]. Iron plays a central role in the energy metabolism of aerobic and semi-aerobic microorganisms[9]. Its availability in soil for microorganisms and plants drops dramatically with increasing pH above 6. The first report of a siderophore production was reported from *Ustilago sphaerogena*[10]. Then gradually, it was revealed that several fungi and bacteria are able to produce siderophores. Microorganisms compete for iron by releasing siderophores[11]. Typically, microbial sideophores are classified as catecholares, hydroxamates and α-carboxylates, depending on chemical nature of their coordination sites with iron[12,13]. Some sideophores are as phenolates[14] and others as mixed (both hydroxamate and catecholate functional groups)[15]. Pseudomonads generally produce fluorescent yellow-green and water soluble siderophores with both hydroxamate and phenolate groups; these siderophores have been classified as either pyoverdins or pseudobactins (Fig. 1). Iron competition in *Pseudomonads* has been intensively studied and the role of the siderophore produced by *Pseudomonads* species were clearly demonstrated in the biological control of diseases[16,7,17,10]. *Pseudomonads* possess many traits that make them well suited as biocontrol and growth-promoting agents[18]. In addition, *pseudomonads* are responsible for the natural suppressiveness of some soils to soil borne pathogens[19-21]. Some fungi produce carboxylate while others produced hydroxamate type of siderophores. *Rhodotoula pilimanae* secreted rhodotorulic acid (Fig. 2) siderophore[22,23].

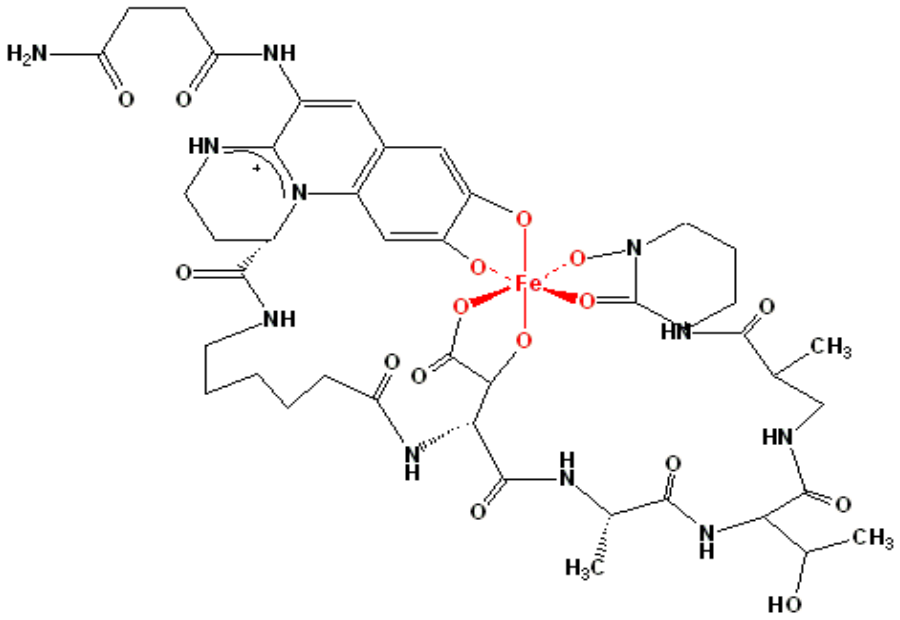

Fig. 1: Structure of Pseudobactin

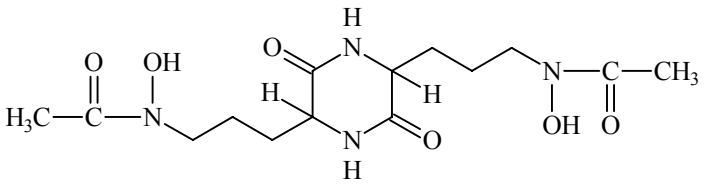

Fig. 2: Structure of rhodotorulic acid

Recently, microbioal siderophores are isolated, purified and utilized, in addition to agriculture field[2], in medical science for siderophore antibiotic preparation (Trojan horse antibiotics)[24,25], in MRI (Magnetic Resonance Imaging) technique[26] in cancer therapy[27], as antimalaria[28], antisleeping sickness[29].

The main objectives of this study were to screen some microbes for their ability of siderophore production, quantitative assay of it and effect of different media on its production.

EXPERIMENTAL

Material and methods

Detection in plate culture- In case of bacteria, universal Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neiland (1987)[22] to detect the siderophore production. ME agar medium with Chromo Azurol S (CAS) (blue agar) was inoculated in the plate with 24 hr old bacteria and kept for incubation at 30^{o}C for 72 hr. The blue colour of the medium to orange or presence of yellow to light orange halo surrounding the colony indicates the production of siderophore.

In case of fungi, the universal CAS assay was modified (CAS agar half plate[30]) to test the ability of fungal species to produce iron binding compounds of siderophore type in solid medium avoiding the growth inhibition caused by the toxicity of the CAS blue agar medium. Petri dishes (10 cm in diameter) were prepared with the MEA medium. After solidifying, the medium was cut into halves, one of which was replaced by CAS blue agar, the halves containing culture medium were inoculated with species taken from stock culture. The inoculums was placed as far as possible from the borderline between the two media, plates were incubated in the dark at 28^{o}C for 6 days.

Quantitative estimation

MEB medium was prepared and used for siderophore production. 24 hr old culture of microorganisms were used to inoculate for 24 hr at 30^{o}C with constant shaking at 120

r.p.m. Following the inoculation, fermented broth was centrifuged (10,000 r.p.m. for 15 min) and cell free supernatant was subjected to detection and estimation of siderophore. Quantitative estimation was done by CAS – Shuttle assays[31,32]. In which 0.5 mL of culture supernatant was mixed with 0.5 mL of CAS reagent and absorbance was measured at 630 nm against a reference consisting of 0.5 mL of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content in the liquor were calculated by using following formula :

$$\% \text{ Siderophore units} = \frac{Ar - As}{Ar} \times 100 \quad \ldots(1)$$

Where Ar = Absorbance of reference at 630 nm (CAS reagent)

As = Absorbance of sample at 630 nm.

Effect of different media

In case of effect of different media for production of siderophore, MEB (2% Malt Extract, pH 5.6)[30], CAAB (containing g L^{-1} Cas-amino acid, 5.0; K_2HPO_4, 1.18; and $MgSO_4$ 7 H_2O, 0.25 pH 5.6) [15], BRB (containing g L^{-1} K_2HPO_4, 0.1; KH_2PO_4, 3.0; $MgSO_4$ $7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0 and Succinic acid, 4.0, pH 5.6) (Barbhaiya and Rao 1985), SMB (consisting of g L^{-1} K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4$ $7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; and Succinic acid, 4.0; pH 5.6)[15] and Nutrient Broth (containing g L^{-1} peptone, 5.0; beef extract, 3.0; NaCl, 5.0; distilled water, 1L; pH 5.6) media were prepared and used for siderophore production. 24 Hr. old culture of *Psedomonas aeruginosa*-1 was used to inoculate for 24 hr at 30°C with constant shaking at 120 r.p.m . Remaining procedure is same as earlier.

RESULTS AND DISCUSSION

The results presented in the Table 1 indicated that all bacteria and fungi tested gave positive response to siderophore production. Out of them *A. aeruginosa-1* indicated that it forms more yellow zone, then *B. subtilis*-1 and *C. famata*-1. On the other hand, fungal antagonist *T. viride*-1 and *T. harzianum*-I gave brown zone surrounding growth colony. It indicated that both fungi produced less siderophore in comparison to bacteria. In our experiment, *Trichoderma viride* and *T harzianum* indicated their positive response of siderophore production. It is at par the report of other workers[33]. The yeast *Candida famata* showed the ability of siderophore production. The secreted siderophores by this yeast were phenolate and hydroxymate type. Among the other yeast *Saccharomyces* sp and *Rhodotorula* sp gave 74.53% and 87.37% of siderophore, respectively[33]. Earlier, Schwn and Neiland[22] reported *Rhodotoula pilimanae* secreted rhodotorulic acid siderophore. Dave and

Dube (2000)[23] studied the siderophores of twenty fungi belonging to Zygomycotina (5 Mucorales), Ascomycotina (7 aspergilli, 6 penicillia, *Neurospora crassa*) and Deuteromycotina (*Fusarium dimerum*). They concluded that Mucorales produced carboxylate while others produced hydroxamate type of siderophores.

Table 1: Detection of siderophore in CAS-MEA and modified CAS-MEA medium in plate culture

S. No.	Antagonist	Colour of zone
1.	*T. viride*-1	+
2.	*T.harzianum*-1	+
3.	*P. aeruginosa*-1	++
4.	*B. megatericus*-1	+
5.	*B. subtilis*-1	+
6.	*C. famata*-1	++

+ = brown, ++ = yellow

Among the bacteria tested in this study, all gave siderophore positive but *P. aeruginosa* gave more yellow zone than other bacteria and yeasts and fungi (Table 1). Siderophores are synthesized by many bacteria *Psedomonas* sp, *Azobacter*, *Bacillus*, *Enterobacter*, *Serratia*, *Staphylococcus* sp, *Azospirillum* and *Rhizobium*[2,34,35,].

The results presented in Table 2 showed that *P. aeroginosa* -1 produced maximum percentage of siderophore (80.50) followed by *B. subtilis-1* (65.00), *C. famata*-1 (60.00), *B megatericus*-1 (50.00) and *T. harzianum*-1 (40.00) and *T. viride*-1 (30.00). Different organisms produced different percentage of siderophores in their culture as reported by many authors[29,33,3]. Hussein and Joo (2012)[33] reported that *T. harzianum* produced 92.33% of siderophore in MEB medium but in our study this *T. harzianum*-1 produced less amount and it was 40.00%. It may be due to different isolate of *T. harzianum*. Moreover, siderophore production depends on other factors such as iron content in medium, other minerals also influence its production. Zn^{2+} and Cu^{2+} increase florescent siderophore production[36]. Cu^{2+} and Ni^{2+} also promote the production of yellow pigment or siderophore in *P. florescence –putida*[37]. Hussein and Joo[33] also observed that *Aspergillus niger* produced 87.99% *Metarhizium anisopliae* 85.92% and *Penicillium digitatum* 84.26% of siderophore in quantitative estimation.

Table 2: Quantitative estimation of siderophore produced by microorganisms

S. No.	Antagonist	Siderophore (%)
1	*T. viride*-1	30.50e
2	*T. harzianum*-1	40.25d
3	*P. aeruginosa*-1	80.50a
4	*B. megatericus*-1	50.27c
5	*B. subtilis*-1	65.00b
6	*C. famata* -1	60.00b
	SEm ±	2.003
	CD ($p \leq 0.05$)	9.5678

Note: Same letter shows data are statistically same while different letter indicates they are statistically different as per Duncan analysis

The culture filtrate of *P. aeruginosa*-1 grown in five different media showing changes of colors (brown-yellow) were subjected to quantitative estimation of siderophore production. Quantitative estimation of siderophore production of *P. aeruginosa*-1 in different media (Table 3) indicated that at pH 5.6 and at temperature 30^{o}C, MEB medium exhibited maximum percentage of siderophore production unit (80.50%) followed by SMB (50.00%), BRB (40%) and CCAB (12%).

Table 3: Quantitative estimation of Siderophore production of *P. aeruginosa* in different media

S. No.	Media	Siderophore (%)
1	SMB	50.00b*
2	NB	00.00
3	CCAB	12.00d
4	BRB	40.00c
5	MEB	80.50a
	SEm ±	3.782
	CD ($p \leq 0.05$)	9.7823

*Average of five replicas: Note: Same letter shows data are statistically same while different letter indicates they are statistically different as per Duncan analysis

Moreover, in NB medium, siderophore production by the *P. aeruginosa*-I was nil i.e. this bacterium cannot produce siderophore in NB medium. This result was at per with other workers[3]. They observed that in NB medium, *fluorescent Pseudomonas* could not secret siderophore, while SM medium supported best siderophore production but in our experiment, MEB supported maximum siderophore secretion. It may be due to different isolate of *P. aeroginosa* 1.

CONCLUSION

In conclusion, the tested fungi (*Trichoderma viride, T. harzianum and Candida famata*) all produce siderophore in qualitative test half CAS-ME agar medium and quantitative test CAS-ME broth media. These three fungi produce 30- 60% of siderophore in CAS-ME medium. All tested bacteria (P aeroginosa-1, B. subtilis 1, B megatericus 1) produce siderophore in qualitative CAS-ME media and qualitative CAS-ME broth. Moreover, they produce siderophore from 50-80.50% while *P aeroginosa*-1 is the best producer. Different media (MB, CCAB, BRB, MEB) supported siderophore production of *P. aeroginosa* 1 except NB. Therefore, this study indicated the siderophore production ability by these microbes is in good amount, which are universally recognized biocontrol agents and plant growth promoting agents. Modern application of siderophore in agriculture, medical science and environment science are increasing. This study may help for more production of siderophore in commercial way and more application of it in modern science.

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REFERENCES

1. C. Ratledge and L. G. Dover, Annu. Rev. Microbiol., **54**, 881-941 (2000).
2. J. W. Kloepper, J. Leong, M. Teinize and M. N. Schroth, Nature, **286**, 885-886 (1980).
3. R. Z. Sayyed, M. D. Badgujar, H. M. Sonawane, M. M. Mhaske and S. B. Chincholkar, Indian J. Biotechnol., **4**, 484-490 (2005).
4. E. Husen, Indo. J. Agric. Sci., **4(1)**, 27-31 (2003).
5. C. Seuk, T. Paulita and R. Baker, J. Plant Pathol., **4(3)**, 218-225 (1988).

6. S. Buysens, K. Heungens, J. Poppe and M. Hofte, Appl. Environ. Microbiol., **62(3)**, 865-871 (1996).

7. Y. Eland and R. Baker, Ecol. Epidemiol., **75**, 1047-1052 (1985).

8. L. J. Gram, Microbiol. Meth., **25(3)**, 199-205 (1996).

9. B. Schippers, Phil. Trans. R. Soc. Lond. B, **318**, 283-293 (1988).

10. J. B. Neilands, J. Am. Chem. Soc., **74**, 4846 (1952).

11. J. Leong, A. Rev. Phytopath., **24**, 187-209 (1986).

12. G. Winkelmann, Biotechnol. Adv., **8**, 207-231 (1991).

13. G. Winkelmann, Biochem. Soc. Trans., **30**, 691-695 (2002).

14. H. Haag, K. Hankte, H. Drechsel, I. Stojiljkovic, G. Jung and H. Zahner, J. Gen. Microbiol., **139**, 2159-2165 (1993).

15. J. M. Meyer and M. A. Abdallah, J. Gen. Microbiol, **107**, 319 (1978).

16. J. C. Beeker and R. Cook, J. Phytopathol., **74**, 806 (1984).

17. B. Sneh, M. Dupler, Y. Elad and R. Baker, J. Phytopathol., **74**, 1115-1124 (1984).

18. D. M. Weller, Annu. Rev. Phytopathol., **26**, 379-407 (1988).

19. D. M. Weller, J. M. Raaijmakers, B. B. McSpadden Gardener and L. S. Thomashow, Annual Rev. Phytopathol., **40**, 309-348 (2002).

20. P. Banasco, Fuente L. De La, G. Gaultieri, F. Noya and A. Arias, Soil Biol. Biochem., **10** (**10-11)**, 1317-1323 (1998).

21. J. Kraus and J. Loper, Appl. Environ. Microbiol., **61(3)**, 849-854 (1995).

22. B. Schwyn and J. B. Neilands, Anal. Biochem., **160**, 47-56 (1987).

23. B. P. Dave and H. C. Dube, Indian J. Exp. Biol., **38**, 56-62 (2000).

24. L. Vertesy, W. Aretz, H. W. Fehlhaber and H. Kogler, Helv. Chim. Acta., **78**, 46-60 (1995).

25. G. Benz, T. Schroder, J. Kurz, C. Wunsche, W. Karl, G. Steffens, J. Pfitzner and D. Schmidt, Chem., **94**, 552-553 (1982). Suppl. 1322-1335.

26. D. M. J. Doble, M. Melchior, B. Osullivan, C. Siering, J. Xu, V. C. Pierre and K. N. Raymond, Inorg. Chem., **42**, 4930-4937 (2003).

27. M. Miethke and M. A. Marahiel, Microbiol. Mol. Biol. Rev., **71(3)**, 413-461 (2007).

28. J. Gysin, Y. Crenn, Pereira da Silva and B. Luiz, Catherine, US Patent., **5**, 192-807 (1991).

29. T. Breidbach, S. Scory, R. L. Krauth-Siegel and D. Steverding, Int. J. Parasitol., **32(4)**, 473-479 (2002).

30. A. M. Milagres, A. Machuca and D. Napoleao, J. Microbiol. Methods, **37**, 1-6 (1999).

31. S. M. Payne, Method Enzymol., **235**, 329-344 (1994).

32. S. M. Pyne, Trends Microbiol., **1**, 66-69 (1993).

33. K. A. Hussein and J. H. Joo, Korean J. Soil. Sci. Fert., **45(5)**, 798-804 (2012).

34. B. R. Glick, C. L. Patten, G. Holguin and D. M. Penrose, Imperial College Press, London (1999).

35. J. E. Loper and M. D. Henkels, Appl. Environ. Microbiol., **65**, 5357-5363 (1999).

36. C. O. Dimpka, D. Merten, A. Svatos, G. Buchel and E. Kothe, Soil. Biol. Biochem., **41(1)**, 154-162 (2009).

37. A. M. Chakrabarty and S. C. Roy, Int. Biochem. J., **92**, 105-112 (1964).

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