International Standard Serial Number (ISSN): 2319-8141

International Journal of Universal Pharmacy and Bio Sciences 10(3): May-June 2021

INTERNATIONAL JOURNAL OF UNIVERSAL PHARMACY AND BIO SCIENCES

IMPACT FACTOR 4.018*

ICV 6.16*

Pharmaceutical Sciences **Research Article……!!!**

STUDIES OF EFFECTS OF FUNGAL SIDEROPHORE ON MICROORGANISM

Ms. Yadav S.B.*, Mr. Jadhav S. S.[1] , Mrs. Khamkar S. P.[2]

Sadguru Gadage Maharaj College, Karad.

KEYWORDS:

Siderophore, Czaky's assay, Arnow's assay, CAS (Chrome Azurol Sulphonate), SM (Succinate media), Grim and Allen's media.

For Correspondence:
Ms. Yadav S.B.*

ADDRESS:

Sadguru Gadage Maharaj College, Karad.

ABSTRACT

Siderophore production shown by fungi isolated from soil sample collected from Kas plateu in western ghat. The potent fungal Siderophore isolated was grown on CAS (chrome Azurol Sulphonate) agar media and detection of siderophore was checked by Czaky's assay and Arnow's assay. Quantitative estimation of Siderophore was calculated with standard hydroxamate siderophore. The different 4 media prepared (SM, CAS, Nutrient Broth media, and Grim and Allen's media) for collecting more quantity of production of Siderophore. While using this media it was found that, the Siderophore production is more into the Grimm and Allen's medium and nutrient broth medium. This study will help in determining the Siderophore is a light weight iron chealating compound which helps into reduce deficiency of iron into body.

INTRODUCTION:

Iron (Fe) as the fourth most abundant element in the terrestrial environment and is greatly needed by most microorganisms in order to perform several metabolic functions. It is greatly involved in the reduction of oxygen for ATP synthesis, in the reduction of DNA ribonucleotide precursors, and in the production of heme. This element exists in two readily inter-convertible oxidation states, Fe (II) and Fe (III). Such ability to convert between the two states allows iron to play an essential role in numerous electron transfer processes. Such ability to convert between the two states allows iron to play an essential role in numerous electron transfer processes.

Siderophores are low-molecular weight (600 to 1500 Daltons) secondary metabolites with iron-chelating potential. Siderophore not only chelates iron but also it chelates some heavy metals and essential metals. These are compounds with small peptidic molecules having side chains and functional groups which have high-affinity ligand to bind ferric ions and transport them through the cell membrane. Various types of bacteria, fungi, actinomycets are produced siderophore and are majorly classified into 4 types (carboxylate, hydroxamates, catecholates, and mixed type) based on their functional groups and structural feactures. Microbial siderophores strongly chelate iron and enhance iron uptake by forming a ferric–siderophore complex even at very low concentrations.[1,2,3]

AIM AND OBJECTIVES

AIM :-

To isolate and identify the siderophore producing fungi and their effect on microbial growth.

OBJECTIVES :-

1. Screening of siderophore producing fungi from the soil.
2. Detection of the potent siderophore producing fungi.
3. Extraction and purification of siderophore.
4. Effect of siderophore on microbial growth.

MATERIAL AND METHODS :-

1. **Collection of soil samples:-**

Composite soil sampling :- The soil sample are taken from the western ghat of Kas plateu of satara district by using composite soil sample method for the Siderophore producing fungus from soil.[4,5]

2. Method used for screening :-

Serial dilution of soil sample :-

Take 1 gm of soil sample

↓

Add it into test tube having 10 ml of sterile distilled water

↓

Then take 1 ml of suspension into further tubes filled with 9ml of distilled water

↓

Repeat it for further 8 dilutions

↓

Take 0.1ml of last most 2 dilutions and spread it on CAS agar medium

↓

Incubate for 48 hrs and observe the growth of fungus.[5,6]

3. Media preparation :-

Preparation of CAS agar medium

1. Preparation of Blue Dye

2. Preparation of Basal Agar

3. Preparation of CAS Agar Plates

The basal agar medium and blue dye sterilized separately. After sterilization, add 10 ml of Blue dye into 100 ml of agar medium. Mix it properly and pour it into sterilized petriplates.[7,8]

Screening of Siderophore producing fungi :-

Procedure :-

- The soil sample collected from various areas of satara district are serially diluted.
- The dilutions are spread on CAS agar medium.
- After the incubation period of 24 Hrs, the mycelial growth of fungus takes place and after further incubation of 48 hrs the production of spores and siderophore takes place by the formation of zone orange pink coloured zone of production of siderophore.

Various fungi producing siderophore were isolated on plates. Orange-pink colored zones were of these siderophore producers were measured and maximum siderophore producing fungus was selected for further study.[9,10]

Detection of siderophore :-

- Czaky's assay and Arnow's assay :-

Czaky's assay used for Hydroxamate type of siderophore and Arnow's assay mainly used for Catechol type of siderophore.

Detection of Hydroxamate Siderophores :-

Presence of hydroxamate-type siderophores was determined using the quantitative Iron Perchlorate Test and the confirmatory colorimetric Csáky Assay.

Detection of Catechol Siderophores :-

The occurrence of catechol-type siderophores were detected using the quantitative Iron Perchlorate Test and the confirmatory colorimetric Arnow's Test.

Positive control– Pure catecholate solution. **Negative control** – Uninoculated Fe- Medium.From above assays the fungus forming siederophore is of the Hydroxamate type.[11,12,13,14]

Extraction of Siderophore :-

The extraction of siderophore is carried out by the three phase extraction method i.e. chloroform-phenol-ether extraction.[15]

Purification of siderophore by Thin Layer Chromatography (TLC) :-

Thin layer chromatography by using silica gel and Solvent system based on various ratios of n-Butanol, acetic acid and distilled water (4:1:1). [16,17]

Effect of siderophore on microorganisms :-

The microorganisms like *E. coli*, *Staphylococcus aureous*, *Pseudomonas aeroginosa* are used for determination of effect of siderophore on growth of these microorganisms.[18,19,20]

Study of media :-

Different media preparations, like SM (succinate medium), CAS medium, Nutrient broth medium and Grimm-allen's medium were used.[21,22]

Results and Discussion

1. Collection of soil:-

The soil collected was from Kas plateu area of satara by using composite soil method. The soil was brownish black in colour.

2. Screening of sidereophore producing fungi:-

The orange pink coloured zone was observed on plates which is of siderophore production.

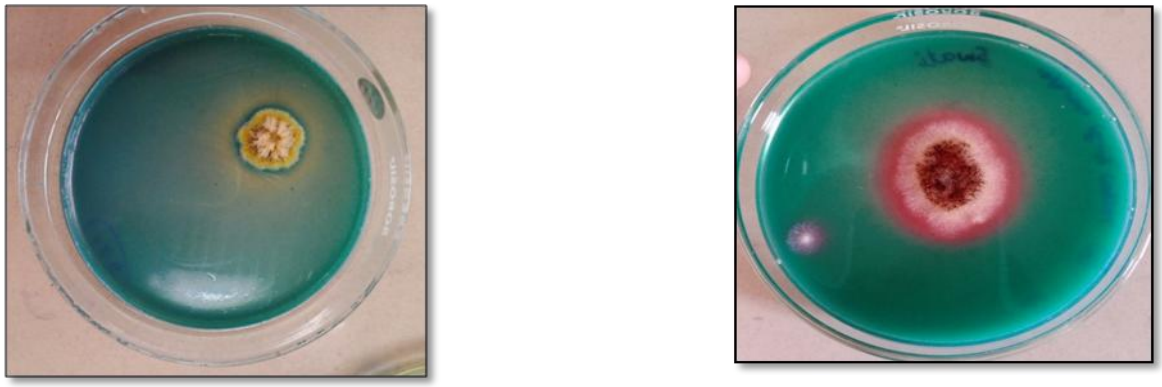


Fig - Isolated fungus

3. Detection of siderophore :-

On the basis of detection of type of siderophore, it was detected by Czaky's assay and Arow's assay.

4. Detection of Hydroxamate type of siderophore :-

Presence of hydroxamate type of siderophore was determined by quantitative iron perchlorate test and the Czacky's assay.

Czacky's assay :-

In the iron perchlorate test was indicated that the presence of an orange coloured ferric hydroxamate.

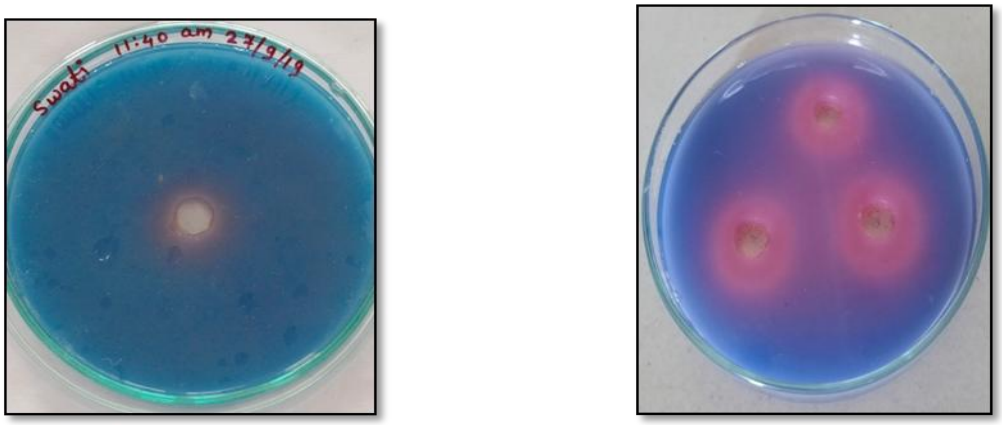
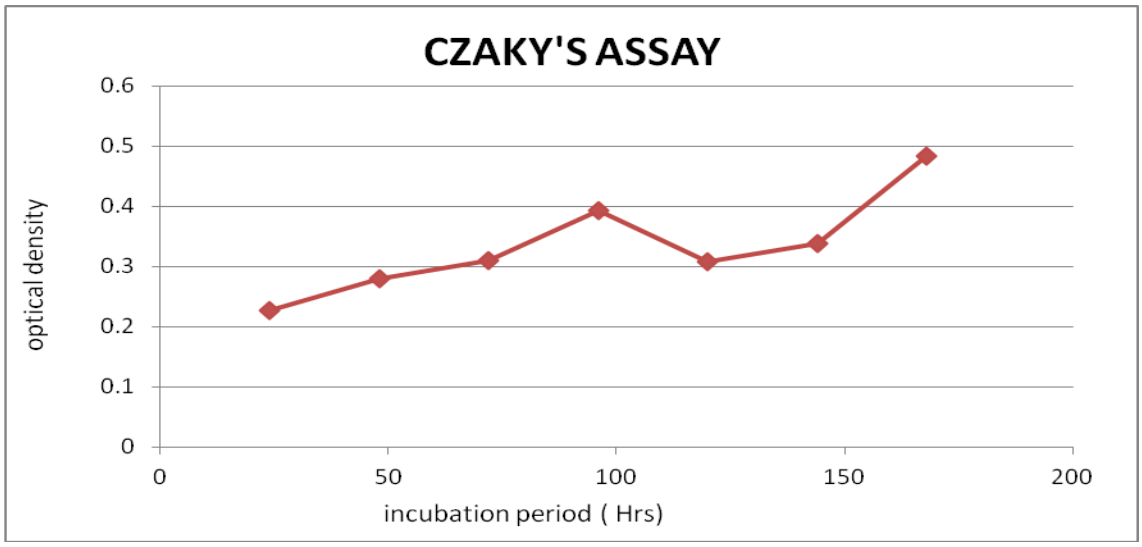


Fig – Quantitative Czaky's Assay by well method

Czaky's assay :-

Incubation period (hrs)	Optical density (O.D.)
24	0.227
48	0.279
72	0.310
96	0.392
120	0.308
144	0.337
168	0.483



5. Quantitative estimation of siderophore production :-

$$\% \text{ of siderophore} = \frac{Ar - As}{Ar} \times 100$$

Ar = Absorbance of reference (0.023)

As = absorbance of sample (0.364)

$$\% \text{ of siderophore} = \frac{0.023 - 0.071}{0.023} \times 100$$

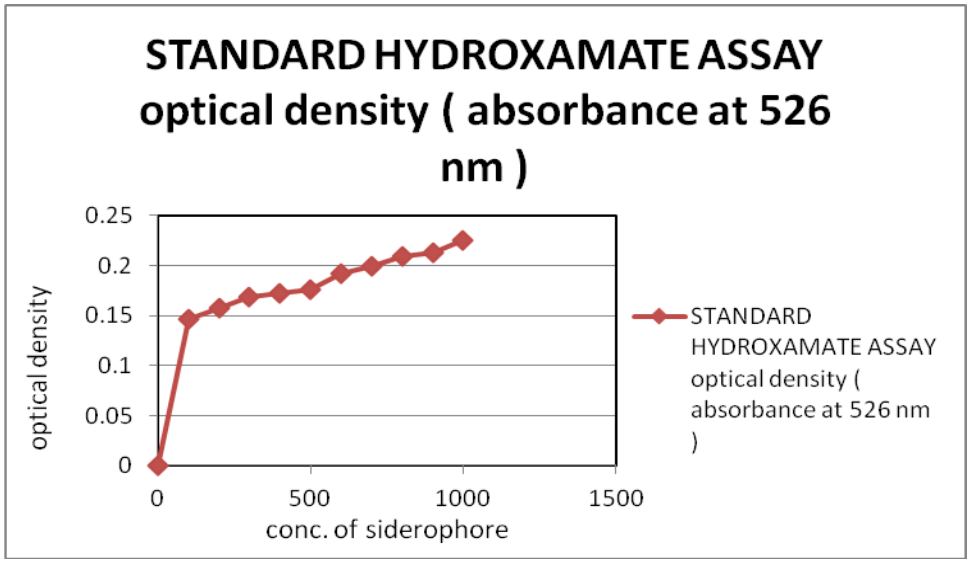
$$= \frac{0.048}{0.023} \times 100$$

$$= 208$$

It is concluded that the percentage of siderophore is more than **100 %** so the fungus is definitely produces siderophore .

6. **Standard hydraxamate siderophoer assay :-**

Conc. of siderophore (µg/ml)	Optical density (526 nm)
000	0.000
100	0.147
200	0.158
300	0.169
400	0.172
500	0.176
600	0.192
700	0.199
800	0.209
900	0.213
1000	0.225



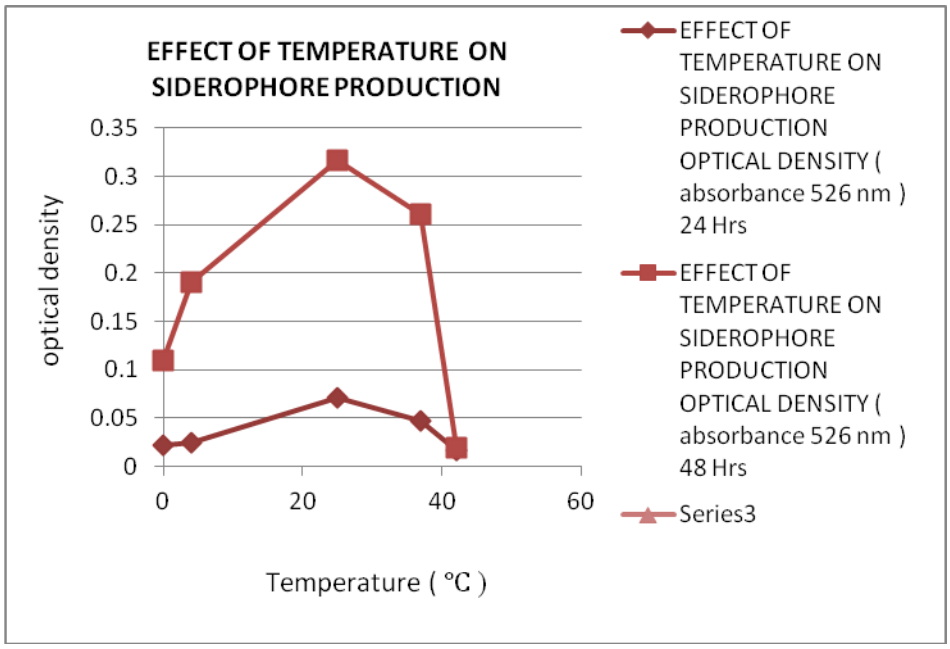
For quantitative estimation of siderophore standard assay was carried out using known concentration of pure hydroxamate. From the graph the concentrating of unknown was found to be 800 µg/ml at optical density of absorbance 526 nm is **209.**

7. Effect of different parameters on production of Siderophore :- The effect of temperature and ph were studied.

8. **Effect of temperature on siderophore production:-**

The siderophore producing fungus were inoculated in CAS medium and incubated at different temperatures (0ºC, 4 ºC, room temp, 37 ºC, 42 ºC) for 24 hrs and 48 hrs and siderophore production was analysed.

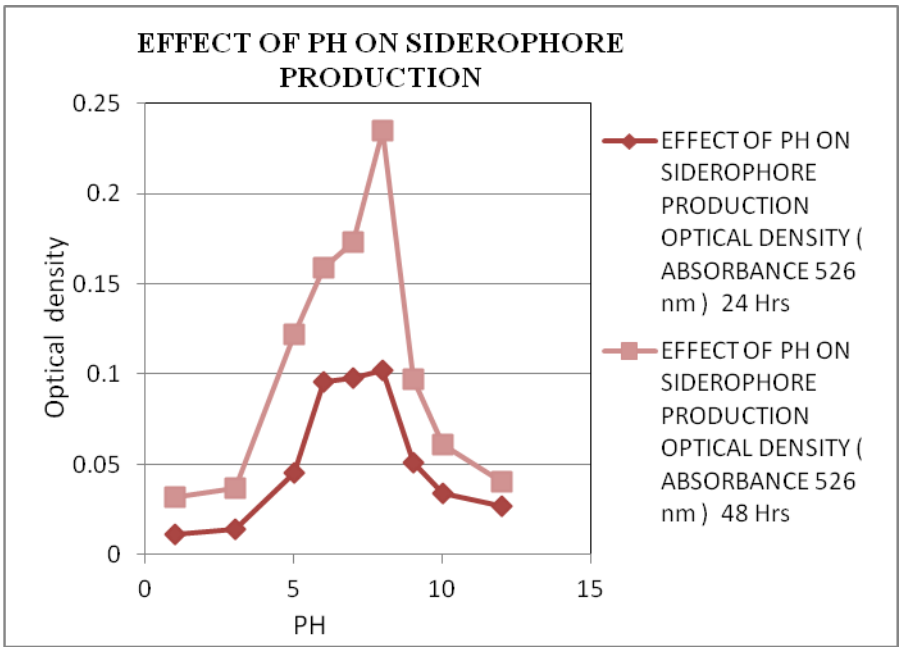
Temperature (ºC)	Optical density (526 nm)	
	24 Hrs	48 Hrs
0	0.022	0.11
4	0.024	0.19
26	0.071	0.32
37	0.047	0.27
42	0.019	0.19



9. Effect of pH on siderophore production :-

The siderophore producing fungus were inoculated in CAS medium set a different pH (3,5,7,9,12) using 1N HCl and 1N NaOH. The growth experiment was carried out for 24 hrs and 48 hrs at room temperature. In all above experiments, negative controls (plain media without any culture or uninoculated broth) were also incubated for the above all temperatures and pH.

PH	Optical density (526 nm)	
	24 Hrs	**48 Hrs**
1	0.011	0.032
3	0.014	0.037
5	0.045	0.122
6	0.096	0.159
7	0.098	0.173
8	0.102	0.235
9	0.051	0.0971
10	0.034	0.0608
12	0.027	0.0405



10. Extraction of Siderophore :- The extraction of siderophore is carried out by the three phase extraction method i.e. chloroform-phenol-ether extraction.

Fig – extraction of aqueous siderophore

11.Confirmation of Extracted Siderophore :-

The siderophore obtained from extraction was then centrifuged at 15000 rpm for 20 min and supernatant was spectrophotometrically examined at 380-450 nm.

12. Purification of Siderophore by Paper Chromatography :-

The fortmation of blue coloured spot on the paper sheet by spreading the FeCl3.HCl solution indicates that the type of siderophore is Hydroximate. The hydroximate type of siderophore gets separated from the extracted aqueous solution by using the paper chromatographic method. The determined hydroxamate siderophore value was **0.82**

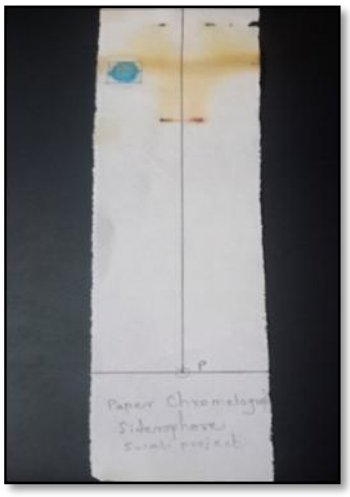


Fig :- paper Chromatography

13. Effect of Siderophore on Microorganisms :-

For the determination of effect of siderophore on microorganisms that means whether it was showing stimulatory effect or inhibitory effect on the growth of microorganisms we used the seed cultre method. That seed culture method shows that the siderophore extracted from our fungus showed the stimulatory (promotion) effect on the growth of microorganisms. This indicates that the siderophore is used as the growth stimulating compound for the microorganisms. E.coli, Pseudomonas aeroginosa and Staphylococcus aureous were examined for the detection of effect of siderophore.

The standard siderophore aqueous solution was used as the standard for the verification of efficiency of the siderophore obtained from our fungus.the zone of Stimulation of 48 hrs is more than the zone of stimulation at 24 hrs was observed.

Microorganisms	Zone of standard siderophore (diameter in cm)		Zone of fungal siderophore (diameter in cm)	
	24 Hrs	48 Hrs	24 Hrs	48 Hrs
E.coli	2.0	3.2	1.6	2.7
Staph aureous	2.2	2.9	1.5	2.4
Pseudomonas	2.5	3.1	1.7	2.6

Fig :- effect of siderophore on E.coli

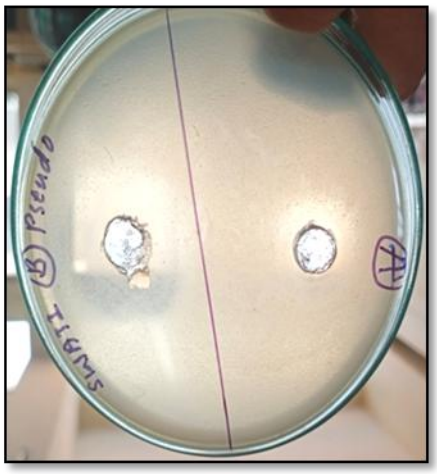


Fig:- effect of siderophore on Pseudomonas spp.

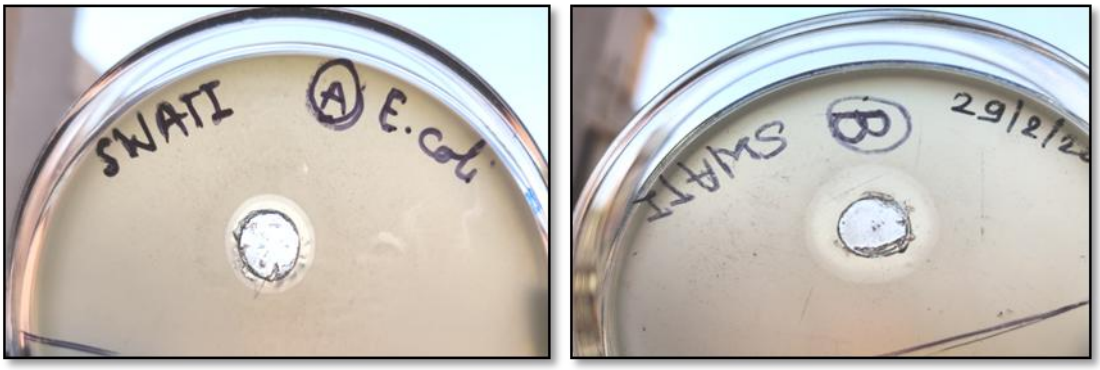


Fig :- zone of stimulation of E.coli by seed culture method

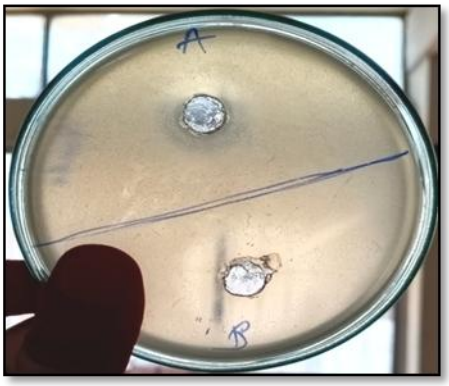


Fig:- Zone of stimulation of Staph. by seed culture method

14. **Study of effect of media on siderophore production :-**

The study of media for siderophore production we used Succinate medium (SM), Chrome Azurol Sulphonate medium (CAS)medium, Grimm-allen's medium (GM) medium, Nutrient Broth (NB) medium were used. All media used for production were in the form of liquid medium form because the detection of production and siderophore formation is more convenient than the solid medium.

Fig :- Production of siderophore in various media (CAS media, Nutrient Broth, Grimm-allen's media and Succinate media)

While studying influence of medium preparation, it was found that the growth of siderophore producing fungi was faster in Nutrient broth and Grimm-allen's medium than CAS medium and Succinate medium.

REFERENCES :-

1. Adjimani JP, Emery T. Stereochemical aspects of iron transport in *Mycelia sterilia* EP-76. *J Bacteriol.* 1988;170:1377–1379. [PMC free article] [PubMed] [Google Scholar]
2. Aguado-Santacruz GAA, Moreno-Gómez BA, Jiménez-Francisco BB, García-Moya EB, Preciado-Ortiz RE. Impact of the microbial siderophores and phytosiderophores on the iron assimilation by plants: a synthesis. *Rev Fitotec Mex.* 2012;35:9–21. [Google Scholar]
3. Albrecht-Gary AM, Crumbliss AL. Coordination chemistry of siderophores: thermodynamics and kinetics of iron chelation and release. *Met Ions Biol Syst.* 1998;35:239–327. [PubMed] [Google Scholar]
4. http://phytopath.ca/wp-content/uploads/2014/09/Isolation-of-Soil-Microorganisms.pdf
5. George D.C. and Florence E.C. (1994) Aromatic hydrocarbons, Patty's industrial hygiene and toxicology, Vol. II, Part B, 4th edn., John Wiley and Sons, Inc., New York, 1301-1350.
6. Smith, H.W., Finkelstein, N., Aliminosa, L., Crawford, B. and Graber, M (1945) The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. Journal of Clinical Investigation, 24(3): p.388.
7. Ames-Gottfred, N. P., B. R. Christie, and D. C. Jordan. 1989. Use of the Chrome Azurol S agar plate technique to differentiate strains and field isolates of Rhizobium leguminosarum biovar trifolii. Appl. Environ. Microbiol. 55:707-710.
8. Arnold LD, Viswanatha T. The use of bis(mercaptoacetato-S,O)hydroxoiron(III) complex for the determination of hydroxamates. *J Biochem Biophys Methods.* 1983;8:307–320. doi: 10.1016/0165-022X(83)90005-2. [PubMed] [CrossRef] [Google Scholar]

9. Adhikari A, Sarker K, De Roy M, Bhattacharya I et al. 2013 – Siderophore mediated antagonism of fluorescent Pseudomonads against soil borne plant pathogenic fungi in West Bengal, India. African Journal of Microbiology Research, 7(39), 4689–4700.
10. https://doi.org/10.5943/sif/4/1/1
11. https://pubmed.ncbi.nlm.nih.gov/11233086/
12. https://www.imedpub.com/articles/studies-on-siderophore-production-by-microbial-isolates-obtained-from-aquatic-environment.pdf
13. B. Schwyn *et al.*Universal chemical assay for the detection and determination of siderophores. Anal. Biochem(1987).
14. http://www.scielo.br/pdf/pab/v43n4/a11v43n4.pdf
15. https://books.google.co.in/books?hl=en&lr=&id=NkoPEAAAQBAJ&oi=fnd&pg=PA235&dq=extraction+of+fungal+siderophore&ots=Nnlxl055DE&sig=ooVdSX7Xvik1lvRVzUhMwFovpnI
16. https://people.chem.umass.edu/samal/269/tlc.pdf
17. ftp://ftp.mn-net.com/english/Flyer_Catalogs/ Chromatography/Catalog-en/ KATEN200001-4TLC-www.pdf MACHEREY-NAGEL: Basic Principles of TLC and product catalog. http://courses.chem.psu.edu/chem36/Experiments/ PDF's_for_techniques/TLC.pdf TLC student experiments and the principles of separation.
18. Amaro, C., Aznar, R., Alcaide, E. and Lemos, M.L. (1990) Ironbinding compounds and related outer membrane proteins in Vibrio cholerae non-O1 strains from aquatic environments. Applied and Environmental Microbiology 56, 2410–2416.
19. https://sfamjournals.onlinelibrary.wiley.com/doi/abs/10.1046/j.1472-765X.2003.01290.x
20. https://www.researchgate.net/profile/Dhara_Gamit6/publication/303119418_Effect_of_siderophore_producing_microorganism_on_plant_growth_of_Cajanus_cajan_Pigeon_pea/links/5b3f08a5aca27207851c4ae4/Effect-of-siderophore-producing-microorganism-on-plant-growth-of-Cajanus-cajan-Pigeon-pea.pdf
21. https://www.imedpub.com/articles/studies-on-siderophore-production-by-microbial-isolates-obtained-from-aquatic-environment.pdf
22. https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-137-5-1185?crawler=true