Isolation, Purification, and Chemical Characterization of the Dihydroxamate-Type Siderophore from Penicillium chrysogenum NCIM 707

R. Sangeetha Kumari[1], R. Aravind[1] A.Ganesh[2], and V.Kaviyarasan[1]

[1] CAS in Botany, University of Madras, Guindy campus, Chennai –600025.
[2] CAS in Zoology, University of Madras, Guindy campus, Chennai –600025.
Email: manikavi53@gmail.com, sangkumari@gmail.com, Ph: +919444255225.

Abstract— **Iron is essential to the majority of microorganisms; it is an important cofactor in many cellular processes and enzymes. However in an aerobic environment and at biological pH, iron is primarily found as insoluble oxyhydroxides and is unavailable to microorganisms. Many fungi have the ability to produce siderophores, low molecular weight compounds that have a high affinity for Fe3+. During a screening programme, a strain of *Pencillium chyrsogenum* (NCIM 707) produces a large number of extra cellular siderophores in an iron deficient medium. The test fungus was further grown in different production media with changes in iron source. The extra cellular siderophore has extracted through XAD 2 polymer resin and characterized hydroxymate siderophore using iron percholate assay. The extracted compound was analyzed by HPLC and GC MS for its purity of the siderophore and elucidated the structure of the compound. Further the siderophore was screened with various wound enhancing pathogens and work was extended to find the possible action on wound healing.**

Keywords- **Chrome Azurol Sulphonate (CAS); Iron Perchlorate, hydroxymate; DNA Cleavage.**

I. Introduction

Microorganisms expressed a variety of low molecular weight, high affinity chelating agents that solubilize ferric iron in the environment and transport it into the cell (7). These compounds are known generically as Siderophores (5). Due to their conformation, chelation and acquisition properties, they have attracted for therapeutic purpose. Siderophores are commonly produced by aerobic and facultative anaerobic bacteria and fungi under iron limiting condition. A variety of fungi are known to produce and excrete desferrisiderophore complex under iron limitation. A new hydroxymate type siderophore was synthesized and characterized in terms of its acid-base behavior; they form a stable ferric complex and were investigated by spectrophotometric methods (10). Almost all fungal siderophores are exclusively hydroxymate type. In an ecological study was used to detect mobilization of iron by testing for siderophores, Schwyn and Neilands (11) were developed CAS medium with specific compounds for siderophore screening studies. Since CAS assay is high in sensitivity it is able to be used on agar plates. The Fe (III) gives the agar a rich blue colour and concentration of siderophores excreted by iron starved organisms results in a colour change to orange. Many siderophores are non ribosomal peptides, although several are biosynthesized independently. Siderophores are amongst the strongest binders to Fe3+ known, with enterobactin being one of the strongest of these. Because of this property, they have attracted interest from medical science in metal chelation therapy, with the siderophore deferroxamine B gaining widespread use in treatments for iron poisoning and thalassemia. In general, siderophores are classified as hydroxamates or catecholates and more recently with new group polycarboxylates. Many strains have been shown to simultaneously synthesize more than one type of hydroxymate siderophore (9). The coprogens are fungal siderophores composed trans fusarinine subunits. These linear siderophores take either dihydroxamate or trihydroxamate forms. Rhodotorulic acid and dimerum acid are the two known dihydroxamate siderophore (9). No evidence was found for catecholate siderophore. Most of the fungal cultures were shown to secrete siderophores in liquid culture (3). To understand the production of siderophores, knowledge of the organization and regulation of siderophore synthesis is essential. Five separate gene clusters are involved in siderophore biosynthesis. Although the total numbers of genes are involved in the biosynthetic pathway is not known. The completation pattern suggest that minimum of twelve genes are needed (8). The present investigation describes diabetic wound helation involving fungal dihydroxamate siderophores.

II. Materials and Methods

A. Fungal Strain and Screening Assay

Penicillium chrysogenum was obtained from the National collection of Industrial Microorganisms (NCIM 707). The culture was kept on potato dextrose agar (PDA) slants and the Chrome azurol medium was prepared with 60.5mg of Chrome azurol sulphonate dye dissolved in 50ml of water, it mixed with 10ml of 1mM Fecl3. 6H2O in 10mM Hcl and 72.9mg of HDTMA in 40ml of water then autoclaved the solution and the PIPES buffer (4.032g/100ml at pH 6.8) was prepared. During Inoculation the stock solutions mixed with PDA medium,

now the medium has turned to bright blue colour. Similarly broth was also prepared for liquid assay for preliminary screening. The culture plates were incubated at 30°C for 2-3 days. The siderophore was excreted by iron starved micro organisms. This results lead to formation of orange colored halos around the fungal growth, indicated siderophore production. The reference CAS medium showed greatest absorbance (blue colour) was denoted as (Ar), samples were taken after orange zone formation was indicating siderophore removed the iron from the dye complex denoted as (As). The Percentage of the siderophore was determined by:

$$\frac{Ar - As \times 100}{Ar}$$

For liquid assay the change in colour from blue to orange after 5min, on addition of 1ml of CAS solution to 1ml of culture supernatant. The siderophore rich samples were carried out for further specific test for detection of hydroxymate or catecholate derivatives. For catechol type structure were detected by the colorimetric Arnow assay (1) in which 1ml of culture supernatant mixed with 1ml of 0.5M HCL, 1ml nitrite molybdate reagent and 1ml of NaOH. The mixture was allowed to incubate for 5mins. The catechol gives a yellow colour when reacted with nitrous acid and changes to an intense orange red when made strong basic. In which 2, 3 Dihydroxybenzoic acid (2,3DHBA) was used as a standard. The presence of hydroxymate type strain was detected by the Iron Percholate assay (2). This assay consisted in adding 2.5ml of Iron percholate solution (5mM Fe(ClO4)3 in 0.1M HClO4) to the 0.5ml of culture supernatant and allowed to incubate for 5min to developed orange red colour solution and the absorbance was measured at 480nm. Desferal used as standard

B. Media for Siderophore Production

For isolating extracellular siderophore, the fungal strain was grown on slants of chemically defined Grimm-Allen iron limited medium (6) with the following composition: K2SO4, 1.0 g; K2HPO4, 3.0 g; NH4CH3COO, 3.0 g; citric acid, 1.0 g; thiamine, 2.0 mg; sucrose, 20.0 g; CuSO4 * 5H20, 0.005 mg; ZnSO4 * 7H20, 2.0 mg; MnSO4 * H20, 0.035 mg; MgSO4 * 7H20, 80 mg; and double glass-distilled water to 1liter (pH 6.8).

C. Hydroxamate Siderophore Production:

The culture was maintained on GA medium for 3 weeks to allow incubation at 25^0C in rotary shaker for 70rpm, the mycelia were filtered off and the culture filtrate was ferrated by adding $FeCl_3$(0.1mM). The brown hydroxamate containing solution was then passed through an Amberlite XAD 2 column. After rinsing the column with distilled water, hydroxamates were eluted with methanol, the elute was evaporated to dryness and the residue dissolved in distilled water. This solution was taken for spot assay with CAS liquid solution and stored at -20^0C.

D. HPLC Analysis:

HPLC analysis was performed with concentrated siderophore using a Biorad Biologic Duoflow HPLC system with a water 7.8mmX300mm Novopak HR C_{18} hydrophobic column as the stationary phase and deaerated, filtered ddH_2O and filtered 90% acetonitrile as mobile phase. The UV detector was set at 220nm to monitor the ferric hydroxamate complexes.

E. Molecular cleavage Study:

The molecular cleavage of bacterial pathogens was performed by using purified siderophore compound. The bacterial DNA was isolated from *Staphylococcus aureus* and *Pseudomonas mirabilis,* the DNA was treated with the different concentrations of hydroxamate siderophore complexes (20µM, 40 µM, 60 µM, 80 µM and 100 µM) fortified with 5mM H_2O_2 and 100mM Tris-HCl (pH 8). All the samples were incubated for 2hrs at RT in both light and dark conditions. The samples were loaded on 1% agarose gel using 1µg/ml ethidium bromide and 6X gel loading buffer. The electrophoresis was carriedout for 1.5 hrs at 100V in TBE buffer. Bands were visualized by UV light and Photographed using Geldoc100 (Biorad) systems.

F. Antimicrobial screening on wound pathogens:

The test bacterial strains *Escherichia coli*, *Pseudomonas mirabilis*, *Streptococcus fecalis*, *Enterococcus fecalis*, and *Staphylococcus aureus* were cultured at 37°C and maintained on nutrient agar media. The bacterial inoculum was uniformly spread using sterile glass rod on a sterile Petri dish containing Nutrient Agar. Five concentrations of 200, 400, 600, 800 and 1000 ppm of siderophore substances were prepared in distilled water. The test substances of 50 µL were added to each of the 5 wells (7 mm diameter holes cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24 h at 36ºC ± 1ºC, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. Tests were performed in duplicate. Ampicillin was used as antibacterial standard drugs.

III. RESULTS AND DISSCUSSION

A. Detection of Siderophore production by Penicillium chrysogenum:

To determine if *P.chrysogenum* NCIM707 (Fig 1 and 2) was producing a siderophore under iron limiting conditions, the CAS was used. Formation of an orange halo around a disc of mycelium on CAS plate indicates that siderophore is present (Fig 3). This assay can also indicate the relative amount of siderophore is present in the supernatant of culture. The iron perchlorate assay and Arnow's assay could be used to determine the chemical type of the siderophore. In the iron perchlorate assay, the formation of an orange red color indicative of a hydroxamate type siderophore (Fig 4) the absorbance was measured at 480nm (Table1). Desferal used as standard. Arnow's assay was repeatedly negative for a catechol type siderophore.

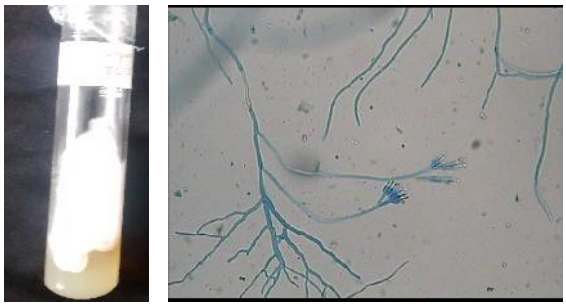

Fig. 1&2: *Penicillium chrysogenum*NCIM707

Fig 3: CAS agar Plate assay

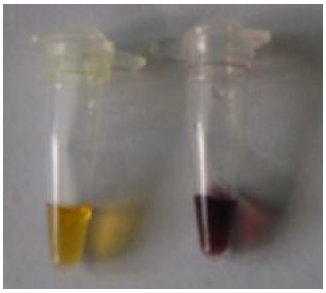

Fig 3a: CAS liquid assay

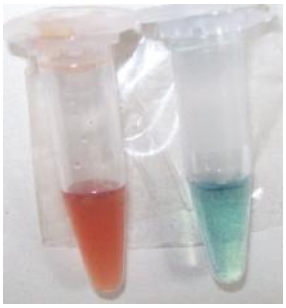

Fig 4: Ironperchlorate assay

TABLE 1: O.D VALUE FOR HYDROXAMATE SIDEROPHORE USING IRON PERCHLORATE ASSAY (ATKIN 1970)

S. NO	Samples	O.D at 480nm
1	Control	0.000
2	Standard	0.095
3	P.chrysogenum	0.076

B. Purification of Siderophore:

Once the growth conditions had been optimized, it was possible to produce large amounts of siderophore by growing Pencillium chrysogenum in batch cultures, the acidified supernatant was first purified through XAD-2 column chromatography. The yellow colour fractions were tested for their siderophore content using iron perchlorate assay (2). In addition to testing each fraction, the ddH_2O wash was also tested to ensure that most siderophore had bound the column and was not in the wash (4). The all the fractions combined, dried and dissolved in ddH_2O. This solution was taken for spot assay (Fig 6) with Iron perchlorate liquid solution and stored at -20^{0}C. The HPLC chromatogram (Fig 5) of *P.chrysogenum* showed a long single peak (2.453) of Dimerum acid (Dihydroxamate) at 220nm.

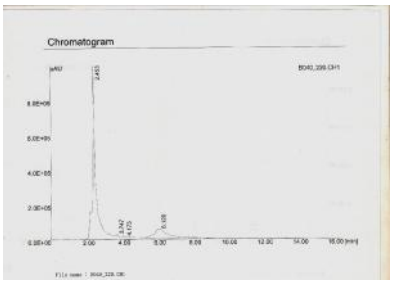

Fig 5: HPLC separation of siderophores isolated from an iron limited culture filtrate of Pencillium chrysogenum, Siderophores were separated on a C_{18} reversed phase column and monitored at 220nm.

Fig 6: Spot assay for XAD 2 column eluted sample of siderophore was studied using iron perchlorate solution for identification of hydroxamate siderophore

C. Antimicrobial activity on wound pathogens:

Antimicrobial activity of dihydroxamate siderophore against five tested microorganisms *Escherichia coli*, *Pseudomonas mirabilis*, *Streptococcus fecalis*, *Enterococcus fecalis*, and *Staphylococcus aureus* have been studied. As per the recorded data the compounds showed positive effects on antimicrobial activity against all the organisms.

	25µM	50 µM	75 µM	100 µM	125µM
E.coli	0.00 ± 0.00	0.00 ± 0.00	0.60 ± 0.10	0.60 ± 0.05	0.60 ± 0.05
P.mirabilis	0.00 ± 0.00	0.00 ± 0.00	0.60 ± 0.10	0.60 ± 0.10	0.60 ± 0.05
C.albicans	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00. ± 0.00	0.60 ± 0.05
S.fecalis	0.00 ± 0.00	0.00 ± 0.00	0.60 ± 0.10	0.60 ± 0.10	0.60 ± 0.05
S.aureus	0.64 ± 0.11	0.70 ± 0.11	0.71 ± 0.115	0.71 ± 0.10	0.71 ± 0.11

D. Molecular Cleavage activity on Bacterial DNA:

There was an increased concentration the cleavage efficiency was increased. A notable similarity of expression was obtained for both treated DNA with formation of Single stranded bands (SSB).

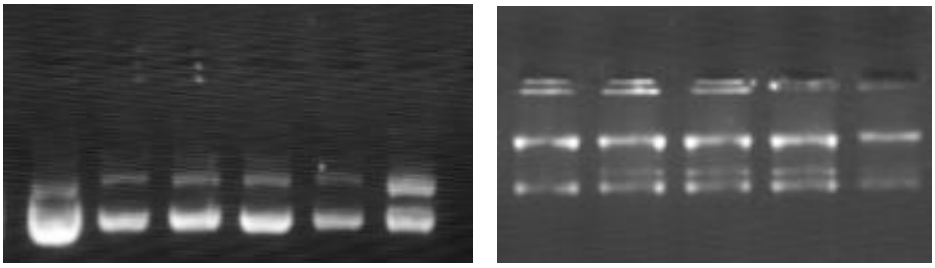

Fig 7: Molecular cleavage study on pBR 322 vector DNA, the DNA was treated with different concentration of purified hydroxamate siderophore under light and dark conditions. Lane 1: Control DNA, Lane 2, 3, 4, 5 and 6: 100µM, 200 µM, 300 µM, 400 µM and 500 µM.

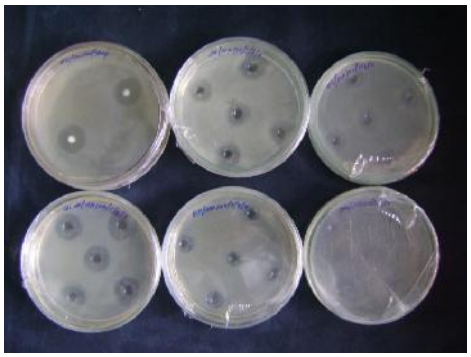

Fig 8: Antimicrobial study on wound pathogens *Escherichia coli*, *Pseudomonas mirabilis*, *Streptococcus faecalis*, *Enterococcus fecalis*, and *Staphylococcus aureus* with different concentration of hydroxamate siderophore.

IV. DISCUSSION

A large number of extracellular siderophores were isolated from the iron-starved culture medium of *P. chrysogenum*. Growth conditions were then optimized in order to achieve maximum siderophore production. Conditions that were optimized were media components, iron concentration in the media, incubation time and temperature. In general, prolonged cultivation resulted in large amounts of ferrichrome type siderophores, such as dihydroxamate. Some byproducts have been obtained which may represent condensation products as described by Moore & Emery 1976 (6). According to the application study reported that the binding activity of Siderophore compound with DNA was efficient and gave the highest rate of expression

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