International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.5, No.3, pp 1101-1106, April-June 2013

IPACT-2013[14th – 15th March 2013]

National Conference on Industrial Pollution And Control Technology-2013

Analysis Of Pyocyanin Compound And Its Antagonistic Activity Against Phytopathogens

T. Sudhakar[1]*, S. Karpagam[2] and Sabapathy Shiyama[3]

[1]Research Scholar, Department of Biomedical Engineering, Sathyabama University, Chennai, Tamil Nadu 600 119,India.
[2]Associate Professor, Department of Plant Biology and Biotechnology, Queen Mary's College, Chennai, Tamil Nadu 600 004, India.
[3]Jaya College of Arts & Science, Tiruninravur, Chennai, Tamil Nadu 602 024, India.

***Corres. author: tsr.182001@yahoo.co.in**
. Mob: 98404 01909,

Abstract: The characterization of a *Pseudomonas aeruginosa* is the production of soluble pigment pyocyanin which has the property of antagonism towards bacteria and fungi. This strain was isolated from the clinical sample and the pigment was optimized by using various solid and liquid media. The pigment was extracted using chloroform resulting in a bluish colored compound and upon addition of 0.2 N HCl giving red color. Further, the pigment was partially purified using chromatographic technique and the maximum absorbance peak at 278 nm, characteristic of pyocyanin. The molecular weight of the compound was analyzed using Gas Chromatography – Mass Spectrometer (GC–MS) and found to be 210.23 kDa with an Rf value 11.94. The pigment was subjected to antagonistic activity against commonly encountered phytopathogens and its inhibitory concentration was observed at 64 µg/ml. The growth suppression phenomenon of this pigment isolated from the strain against fungi. This study reveals that the strain can be economically used as biocontrol agent against common phytopathogenic fungi.
Keywords: pyocyanin, GC-MS, phytopathogens.

Introduction

Pseudomonas species a gram negative bacteria which has the characteristic feature of showing resistance to many drugs, this property may be due to the production of soluble pigment namely pyocyanin which has the property of inhibiting many bacterial colonization and fungal growth both *in vivo* and *in vitro* condition apart from inhibiting the growth of microbes and showing resistance to various chemotherapeutic agents this gram negative bacteria can be isolated from many sources which is ubiquitous in nature. Apart from parasitism it can

be even used to control environmental hazards such as biodegradation, bioremediation simultaneously can be used even to control the phytopathogens[5]. In this study the bacteria was isolated from the wound swab and a pure isolate was studied as *P. aeruginosa*. Further pigment was produced by using various media, it was extracted and used to control the growth of microbes. So this shows that the pigment has the capacity to inhibit the growth of pathogens both in clinical as well as in environmental hazards. This work indicates that this pigment even can be used to control the environmental pollution by applying pyocyanin as an indicator.

Materials And Methods

Isolation Of Bacterial Strain

A total of 50 clinical isolate of different sample was collected from Sharp's lab, Chennai and characterized as *P. aeruginosa* based upon preliminary test and biochemical analysis[3]. Out of 50 strains one strain isolated from wound swab (WS1) was very effective against fungi that strain was further studied.

Production of pyocyanin pigment by using *P. aeruginosa* WS1 strain

P. aeruginosa WS1 strain produces soluble pigment pyocyanin, the production was seen by inoculating this strain in Pseudomonas broth then they were incubated at 37^{o}C for 24 hrs and observed for color change.

Extraction Of Pigment

Pigment was separated by the addition of chloroform solvent system. Further it was extracted by adding 0.2N HCl and further subjected to purification.

Purification Of Pigment

Extracted pigment was further purified by using column chromatography on a silica gel column (45 x 3.5 cm) and eluted by using solvent of chloroform. The purity of the compound was further done by using TLC technique.

UV Absorption Range Of Pigment

Red color pigment which was obtained by adding 0.2N HCl was separated and subjected to UV-spectrophotometric analysis and the maximum absorbance of pigment was read by using T-1800 UV spec.

GC-MS Analysis Of The Compound

The gas chromatography combined with mass spectrometry detection technique is a qualitative and quantitative analysis of the crude extracts with high sensitivity even with smaller amount of compounds. Consequently identification of the chemical moiety of crude extract of pyocyanin which showed previous antifungal activities against the selected phytopathogens *A. flavus, A. fumigatus* and *Candida* species was analyzed.

The GC-MS analysis was done with standard specification by dissolving 100mg of pyocyanin with 1ml chloroform. The liquid sample of 1ul was injected into column of GC-MS model (Joel GC-Mate II Mass spectrometer) HP5 silica column as stationary phase and helium as a carrier gas with the flow rate of 25ml / min. The temperature gradient program was adopted for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was 70°C and gradually accelerated to 250°C at a rate of 15°C / min. The sample was injected at 220°C after 10 min. The maximum peak representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds.

Demonstration Of The Antifungal Activity By MIC

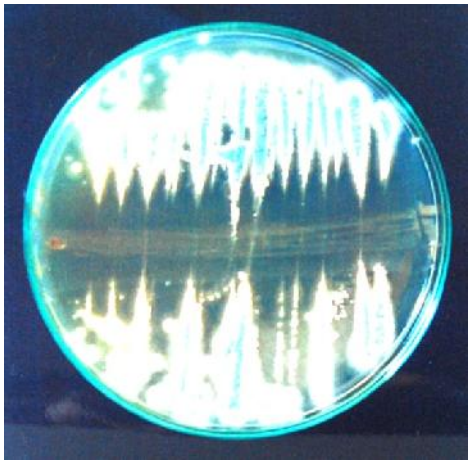
The MIC of the compound against *Candida* species, *A. flavus, A. fumigatus* was performed as per NCCLS norms[1].

Results And Discussion

Isolation Of *Pseudomonas* Sp. WS1

The colony morphology and biochemical characteristics of the isolated organism WS1 was identified as *Pseudomonas aeruginosa*. This strain was selected based upon cross streak method among fifty isolates (Fig 1).

Fig 1. Cross streak method of pyocyanin



Production And Extraction Of Pigment

Pigment production was accomplished after overnight incubation. Soluble pigment production namely pyocyanin was indicated by change in color in Pseudomonas broth with a green shade indicating the production of pyocyanin pigment, which was further extracted by the addition of chloroform. The pigment was separated as a blue color compound at the organic phase. The chemical nature of pyocyanin was confirmed with the appearance of red color upon addition of 0.2N HCl.

Purification Of The Pigment

In column chromatography technique, a single band of blue color fraction was observed and further its purity was checked by subjecting to Thin Layer chromatography using silica gel as a stationary phase and chloroform as a mobile phase. The corresponding TLC Rf value was found to be 0.71 indicating the presence of pyocyanin pigment.

UV-Spectrophotometric Analysis

The partially purified compound was subjected to UV-spectrophotometer and the absorbance of this solution was maximum at 278nm. This peak indicates the presence of pyocyanin compound (Fig. 2).

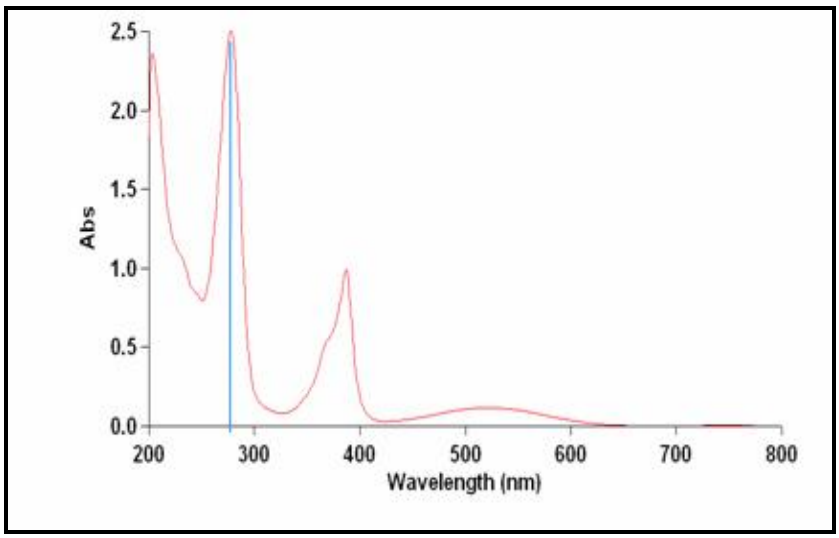


Fig 2. UV Absorption spectra of *P. aeruginosa* WS1 showing λ_{max} at 278nm

GC-MS Analysis

Pyocyanin compound which was synthesized was subjected to GC-MS which has the intense molecular ion at m/z 210.23 defining the molecular weight as 210. The mass spectrum showed intense ions at m/z 210 and other ions 140, 125, 168, 194, 181, 156, 108, 92. The spectrum was in good agreement with the early report[9] and the Pyocyanin GC was done and the retention time was found to be at11.04 mts (Fig 3 & 4).

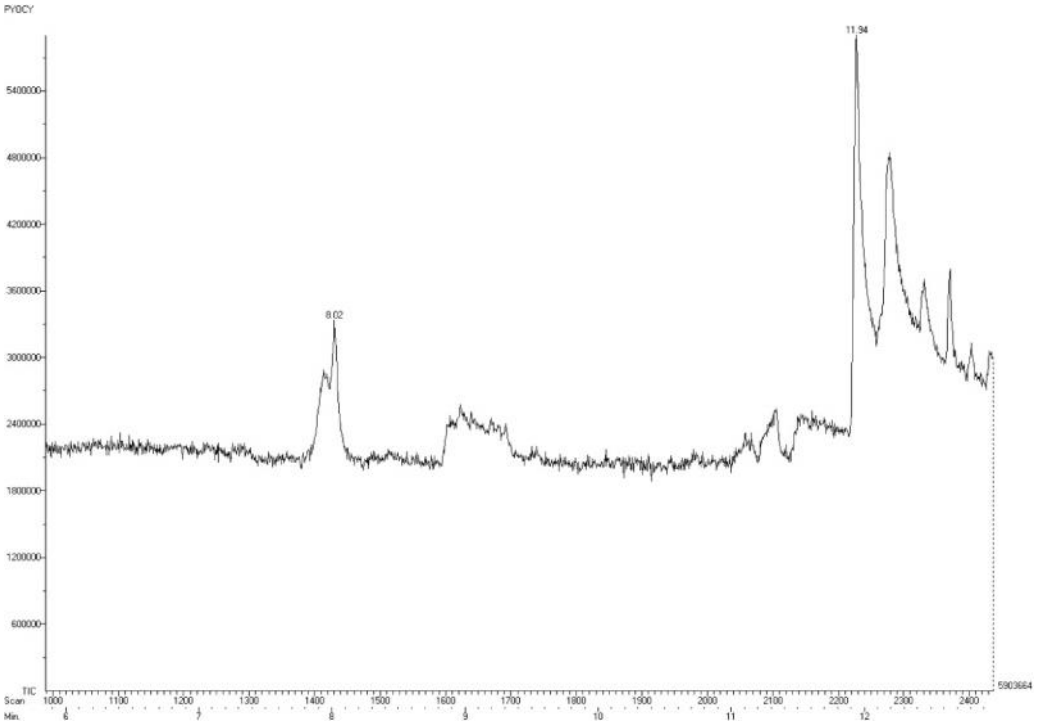


Fig 3. Gas Chromatogram pattern of pyocyanin pigment

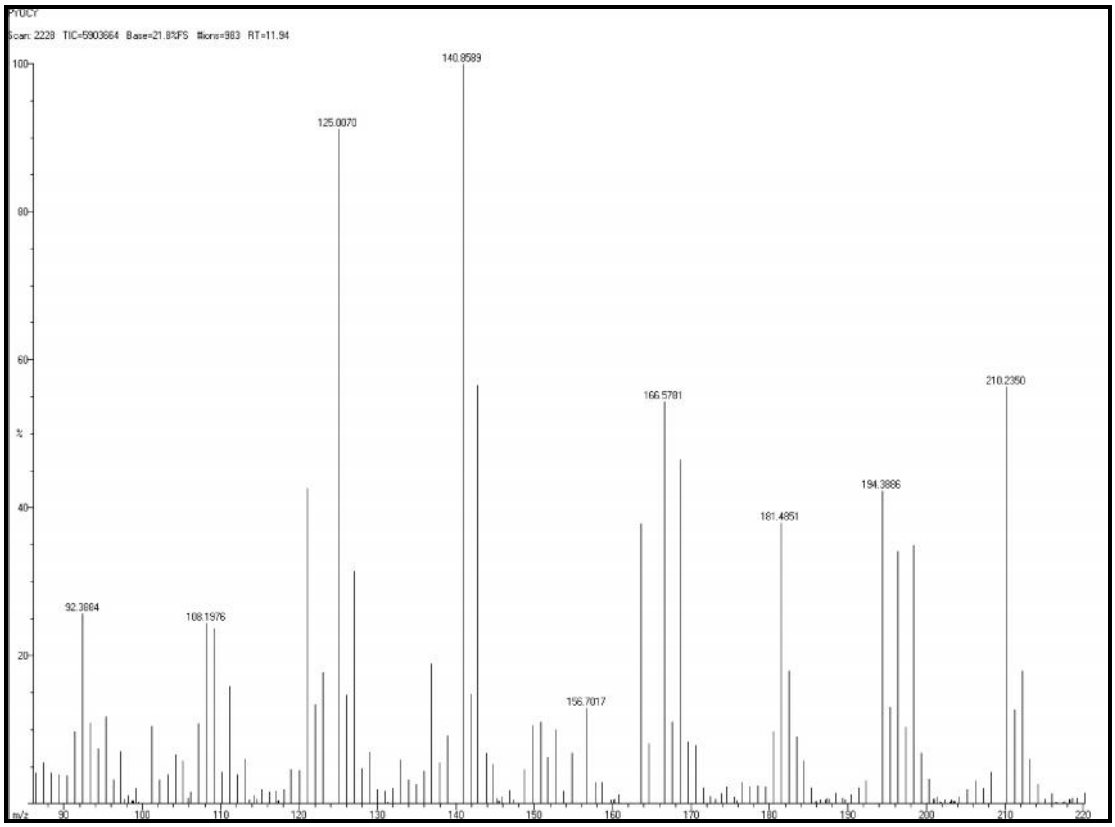


Fig 4. Mass spectrometry analysis of Pyocyanin pigment

Table1 showing MIC pattern of pyocyanin pigment

Strain	Phytopathogens	MIC range
P. aeruginosa WS1	*A. fumigatus*	64ug / ml
	A. flavus	64ug / ml
	Candida sp.	128ug / ml

Antimicrobial Activity By MIC Method

The pigment produced by *P. aeruginosa WS1* was subjected antifungal activity against *Candida sp.*, *A. flavus* and *A. fumigatus*. On the whole it showed a good antifungal activity (Table 1).

Discussion

Pyocyanin a water soluble bio-active compound produced by *P. aeruginosa*, has the capacity to arrest the electron transport chain of the fungi and exhibit the antifungal activity[7]. The organism isolated from wound swab was designated as WS1 strain and confirmed as *P. aeruginosa* based upon the preliminary examination and biochemical test[3]. This strain was selected and used for the study by checking the potency of the strain WS1 by determining the antifungal activity using cross streak method against fungi (*Candida, Aspergillus* species) from which among all the strain WS1 was every effective and further used for other studies[7].

Pyocyanin compound was produced and extracted by using Pseudomonas broth and a green shade color of the solution was obtained, extracted by adding chloroform which separated a blue color compound. It was then confirmed by adding 0.2N HCl and a pinkish red color compound was obtained which indicated the presence of pyocyanin pigment[10]. Extracted biologically compound was further partially purified by using silica gel in a column bed and a single fraction of light blue color was obtained by eluting with chloroform solvent later the purity was checked by using TLC chromatogram in which a Rf value of 0.71 was obtained[2].

A separated red color compound was subjected to UV-spectrophotometric analysis and a maximum absorption was seen for this strain WS1 at 278nm which was in accordance with the results[4] and confirms presence of pyocyanin. Later GC-MS analysis was done to see the presence of the pyocyanin compound the results of this technique reveals the molecular weight of about 210 kDa[9] and the retention time was found using GC, the eluted peak had a Rf of about 11.04 min. This peak was the major peak and a minor peak was also seen this was not taken in our work since the peak was found in negligible amount. The molecular weight of the pyocyanin compound was about 210.23 kDa[6]. This compound was further analyzed for MIC activity as per NCCLS norms and concentration of about 64ug/ml against *Aspergillus flavus* and *A. fumigatus* and 128ug/ml against *Candida* species was determined. The results were correlated with the work[4] and all these strains are used against this compound are phytopathogens in which this compound exhibited antifungal activity by arresting the electron transport chain of fungi[8]. This research work showed that a clinical isolate WS1 from which a biologically compound was obtained had the capacity to inhibit the growth of phytopathogens. This WS1 strain can be economically used to control phytopathogens as a bio-control agent.

Acknowledgement

I acknowledge Dr. R Murugesan, S.OI, SAIF, IIT Madras for his valuable assistance to carryout GCMS analysis.

References

1. Annette W Fothergill., Antifungal susceptibility testing., Clinical Laboratory and Standards Institute (CLSI) Methods, Springer-Science, 65-74.
2. Baron S.S. and Rowe J.J., Antibiotic action of Pyocyanin, Antimicrob. Ag, Chemother., 1981,20 (6), 814-820 .
3. Holt et al., Bergey's Manual of Determination bacteriology, William & Wilkins, Baltimore (1994).
4. Kerr J R, Taylor G W, Rutman A, Hoiby N, Cole P J, Wilson R., Pseudomonas Aeruginosa Pyocyanin and 1-hydroxyphenazine inhibit fungal growth, Clin Pathol., 1999,52,385-387.
5. Jayaswal R.K, Fernandez M.A and Schroeder R.G., Isolation and characterization of Pseudomonas strain that restricts growth of various phytopathogenic fungi, Appl Env Microbiol., 1990, 56 (4), 1053-1058 .

6. Jos M. Raaijmakers, Robert F. Bonsall, and David M. Weller. Effect of Population Density of Pseudomonas fluorescens on production of 2,4-Diacetylphlorglucinol in the Rhizosphere of wheat, Phytopathol., 1999, 89(6) 470-475.
7. Kerr J.R.., Suppression of fungal growth exhibited by *Pseudomonas aeruginosa,* J. Clin. Microbiol., 1994, 32, 525-527.
8. Pal R.B. and Revathi R., Susceptibility of yeast to *P. aeruginos,* Ind J Med Microbiol., 1998, 16, 72-74.
9. Robert Wilson, Tyrone Pitt, Graham Taylor, David Watson, John MacDermot, David Sykes, David Roberts and Peter Cole. Pyocyanin and 1-Hydroxyphenazine Produced by Pseudomonas aeruginosa Inhibit the Beating of Human Respiratory Cillia *in vitro,* J Clin inv.,1987, 79,221-229.
10. Wa'ad Mahmood Ra'oof and Ibraheem Abudl Rahman Latif., *In vitro* study of the swarming phenomena and antimicrobial acvitiy of pyocyanin produced by *Pseudomonas aeruginosa* isolated from different human infections, Eur J Sci Res., 2010, 47 (3), 405-421.

*****