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Production optimization, characterization and antimicrobial activity of pyocyanin from *Pseudomonas aeruginosa* SPC B 65

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ABSTRACT

Siderophores can act as biostatic compounds by drastically reducing the amount of ferric ions available to microbes. Pyocyanin, produced by *Pseudomonas aeruginosa* was found to show anti bacterial and anti fungal activities. Of the various parameters optimized, a temperature of 35° C, pH of 7.5 and ferric chloride concentration of 10 µM was found to be optimum for pyocyanin production. Maximum pyocyanin production was noticed in the absence of NaCl. High thermostability was also noted for the pigment.

KEYWORDS

Siderophore;
Pyocyanin;
Pseudomonas aeruginosa;
Optimization,
Characterization;
Thermostability.

INTRODUCTION

Leaf infecting phytopathogenic fungi pause a serious threat to agriculture due to its immense destructive activity on crop plants. Chemicals of wide variety are used as an effective control to these fungi but cause destruction to the already fragile ecosystem through bioaccumulation and biomagnification. Hence biological control using microorganisms are gaining momentum as an effective alternative to chemical agents. Several forms of bacteria like *Pseudomonas, Enterobacter, Aeromonas, Serratia, Bacillus and Burkholderia* are being used as antifungal agents[1-3]. Of these, *Pseudomonas*, especially fluorescent *Pseudomonas* have drawn world wide attention due to the production of secondary metabolites like siderophores, antibiotics, HCN, enzymes and phytohormones[4]. They thus not only produce antifungal compounds but also act by enhancing growth and inducing defense in the crop plant. *Pseudomonas fluorescens, P. cepacia, P. aureofaciens and P. putida* are the four major species showing antifungal activity. Anti fungal activity of *Pseudomonas* may be mediated by three distinct ways- production of chitinolytic enzymes that degrade fungal cell wall, siderophores that delimit iron and by other non chelating antibiotics.

Siderophores are secondary metabolites which act as iron chelating agents. They are produced under iron limiting conditions and functions as biostatic compounds by drastically reducing the amount of ferric ions available to certain rhizosphere microflora[5]. Some siderophores may also act as antibiotics by destroying fungal cells through lysis. The important siderophores produced by *Pseudomonas* include pyochelin, pyocyanin, pyoverdine and salicylic acid. 2, 4-diacetylphloroglucinol (PHL), pyoluteorin (PLT), pyrrolnitrin, cepabactin, ornibactin are some of the antibiotics produced by *Pseudomonas*[3, 6].

FULL PAPER

Pseudomonas aeruginosa is an opportunistic pathogen that can cause infections in respiratory tract, wounds and urinary tract of immunocompromised or immunodeficient host[7]. The virulence of the bacteria lies mainly in the blue pigment pyocyanin that is known to have deleterious effect on a number of prokaryotic organisms, possibly due to the formation of hydroxyl radical[8]. However the bacteria are not used as a biocontrol agent, due to its human pathogenicity. As a soil dwelling saprobe, it is also not possible to use the avirulent strains of the same in controlling leaf infecting fungi.

In the present study, antifungal activity of *Pseudomonas aeruginosa* is compared with two strains of *P. fluorescens*, commercially available as biocontrol agent. The process parameters for maximal production of pyocyanin are optimized. Characterization of pyocyanin and its anti microbial activity is studied.

EXPERIMENTAL

Microorganisms

Four phytopathogenic fungi were isolated in pure culture from diseased plant parts- *Pestalotia palmarum* from coconut leaves, *Oidium heveae* from rubber leaf, *Fusarium oxysporum* from infected fruits of tomato and *Curvularia lunata* from paddy leaf. *Pseudomonas fluorescens* SPC B 60 and SPC B 61, commercially available as biocontrol agents and *P. aeruginosa* SPC B 65, isolated from soil following procedure of Dubey and Maheshwari[9] were used in the study.

Bioassay

For antifungal assay, a small square (1 cm) of the fungal mycelium was placed in the center of PDA plate. After 24 hours of incubation, the bacteria was streaked as a square along the perimeter of the plates, unstreaked plates were taken as the control. The plates were incubated at 30°C for 5 days and the zone of inhibition was measured. To test the role of chitinase enzyme, the three strains of *Pseudomonas* were grown in modified Mandel and Reese medium[10] with 1% chitin as the sole carbon source.

Characterisation of Pyocyanin

The solubility of pyocyanin in various polar and non-polar solvents was studied. Rf value of pyocyanin in various running solvents were determined using TLC with silica gel G as adsorbent.

Production media and optimization of Process parameters

The production media used was the King's B broth[11] containing (g/L) : peptone, 20.0; glycerol, 15.0; K_2HPO_4, 2.5 and $MgSO_4.7H_2O$, 6.0. A loop full of bacteria was inoculated in to 50 ml of medium, taken in Erlenmeyer flask and was incubated for 5 days at 30°C with shaking at 150 rev/min. Pyocyanin from this culture was extracted with chloroform in five steps, in each step, 10 ml of chloroform was used. The chloroform extract was made up to 50 ml and the absorbance was read at 684nm. The parameters studied for maximal synthesis of the pigment include temperature (20-50° C), pH (3.0-8.5), incubation period (1-10 days), $FeCl_3$ concentration (0-50 μM) and NaCl concentration (0- 0.4 M).

Antimicrobial activity of pyocyanin

The antimicrobial activity of pyocyanin was determined using disc diffusion method[12]. To obtain aqueous extract of pyocyanin, equal volumes of chloroform extract and acidified water was shaken well. The aqueous and chloroform extract were tested for antibacterial (NA plates) and antifungal (PDA plates) activity. To determine the role of nutrient availability as a mode of resistance to pyocyanin, fungi were grown in glucose minimal medium[9]. The fungi were now tested for its susceptibility to pyocyanin. Thermostability of the pigment was tested by subjecting it to temperatures 30-100° C for 1 hour, and then measuring its antibacterial activity.

RESULTS AND DISCUSSION

All the 3 strains of *Pseudomonas* exhibited antifungal activity. *P. fluorescens* has been reported to have antagonistic effects against soil borne pathogens like *Fusarium oxysporum*[13], *Macrophomia phaseolina* and *Sclerotinia sclerotiorum*[4], *Gaeumannomyces graminis*[14] and *Pythium ultimum*[15]. Saikia *et al*.,[16] had reported antifungal activity of *P. aeruginosa* against root infecting pathogen like *Rhizoctonia solani*. In the

present study, the bacterial strains not only reduced the growth of *Fusarium*, a root infecting fungi, but also were efficient against leaf infecting forms like *Curvularia*, *Oidium and Pestalotia*. In addition to restricting the vegetative growth, *P. aeruginosa* was found to completely inhibit spore formation in all the four fungal strains studied. Microorganisms possessing chitinolytic enzymes are found to be potential antifungal agents. These enzymes play a key role in bacterium-fungus and fungus-fungus antagonism[17]. However, *P.aeruginosa* failed to grow in modified Mandel and Reese medium containing chitin as the sole carbon source indicating the absence of chitinolytic enzymes. Hence the mode of antifungal activity may be through antibiotics and siderophores. The bacteria showed considerable amount of pyocyanin production, the pigment got diffused throughout the medium.

Characterization of pyocyanin

Pyocyanin produced by *P. aeruginosa* showed varying solubility in different solvents, maximum solubility was seen in chloroform (TABLE 1.) In the visible spectrum, the pyocyanin extracted with chloroform showed maximum absorbance at 684 nm in the visible spectrum. The pigment possesses value of 0.17. 0.2 and 0.16 in running solvents- diethyl ether-acetone (9:1), chloroform- acetone- petroleum ether (2:1:3) and methanol: water (1:3) respectively.

Optimization of Parameters

Maximum pyocyanin production was noticed at 35° C (Figure 1), no growth was noted beyond 45°C. The production of pyocyanin got increased in alkaline pH, reaching a peak at 7.5 (Figure 2.). This result can be correlated to the findings of Budzikiewicz[18] who stressed the importance of alkalinity in preventing siderophore destruction. van Rij *et al*.,[19] also reported a similar enhancement of siderophore production by *P. chlororaphis* in alkaline pH.

TABLE 1 Solubility of pyocyanin in various solvents

Solvent	Solubility
Diethy lether	No solubility
Petroleum benzene	No solubility
Glycerol	No solubility
Water	Low solubility
Methanol	Low solubility
Ethanol	Low solubility
Xylol	Low solubility
Tertiary Butyl Alcohol	Moderate solubility
Iso Propyl Alcohol	Moderate solubility
Acetone	Moderate solubility
Toluene	Moderate solubility
Chloro form	High solubility

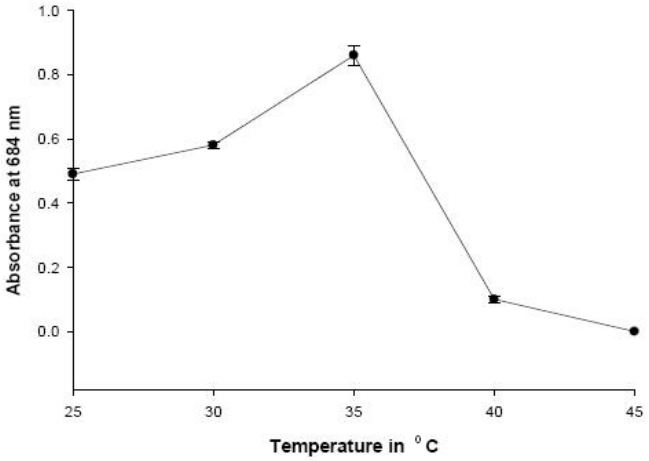

Figure 1 : Effect of temperature on pyocyanin production

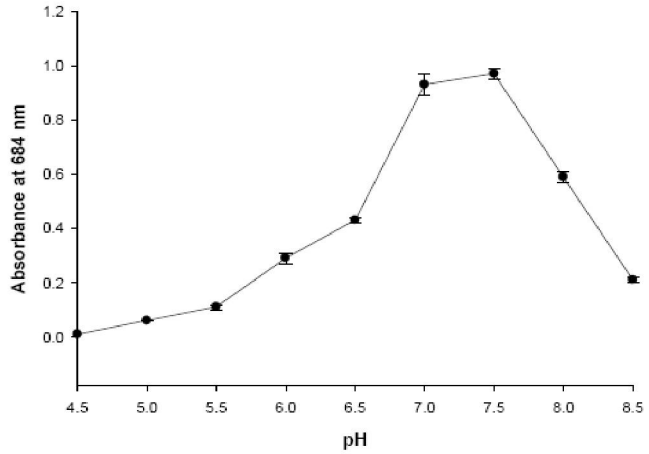

Figure 2 : Effect of pH on pyocyanin production

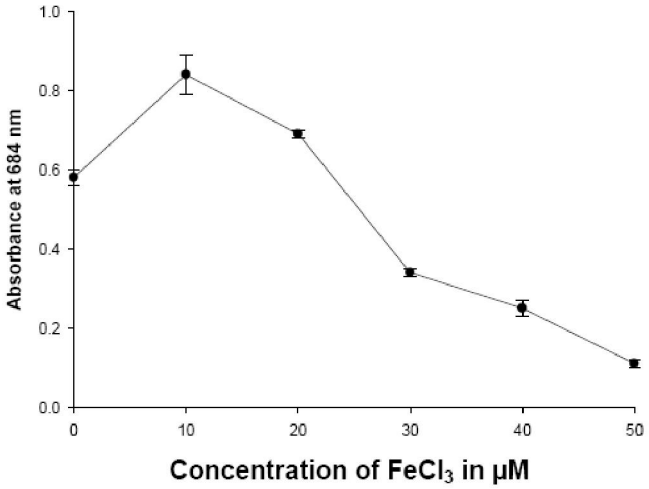

Figure 3 : Effect of pH on pyocyanin production

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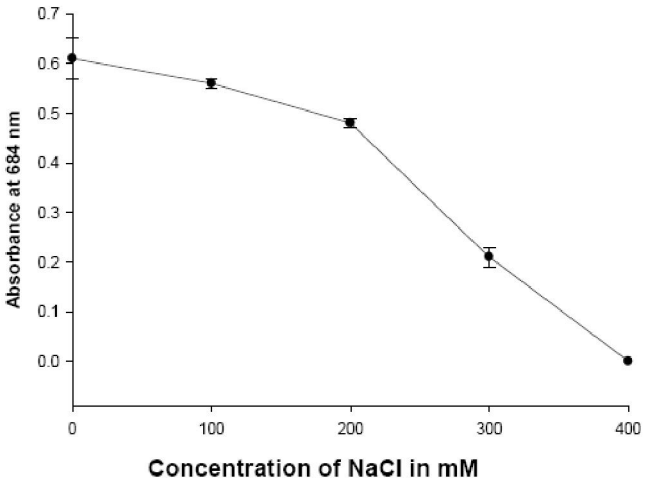

Figure 4 : Effect of NaCl on pyocyanin production

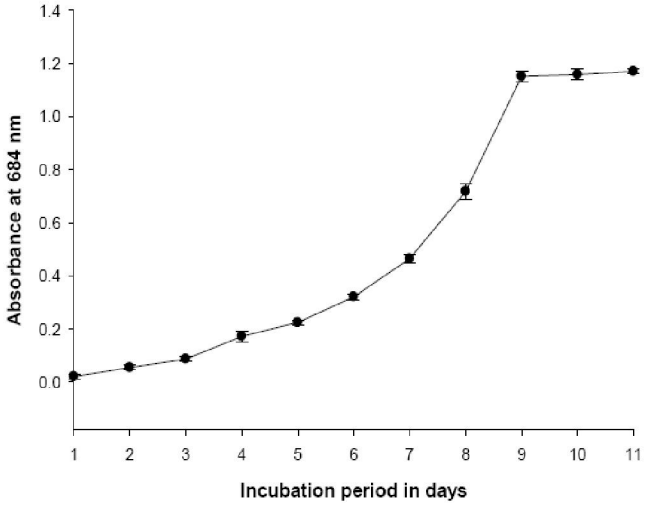

Figure 5 : Effect of incubation days on pyocyanin production

TABLE 2 : Anti Bacterial activity of pyocyanin

	Zoneofinhibition(mm)	
Bacteria	**Chloroformextract**	**Aqueousextract**
Bacillus thuringiensis	4.0	5.0
Bacillus coagulans	12.0	13.0
Bacillus subtilis	3.0	3.0
Staphylococcus aureus	2.0	3.0
Escherichia coli	-	-
Pseudomonas aeruginosa B25	-	-
Pseudomonas fluorescence B20	-	-
Pseudomonas fluorescence B21	6.0	7.0

Ferric chloride concentration of 10 μM was found to be optimal for pyocyanin production (Figure 3.). Neilands[20] had reported that 10 μM concentration of $FeCl_3$ is essential for good growth of *Pseudomonas* but resulted in only modest yield of siderophores. Van Rij *et al*.,[19] noticed an increase in siderophore production at 78 μM concentration of $FeCl_3$. Rachid and Ahmed[21], reported that even very low concentrations of $FeCl_3$ (50g/l) could adversely affect siderophore production by *P. fluorescens* in King's B medium.

Sodium chloride stress in the medium was found to affect pyocyanin production (Figure 4.). At a concentration of 0.4 M, there was complete loss of siderophore production. In *P. chlororaphis*, complete inhibition of siderophore production was noticed at 0.1 M NaCl concentration[19] *P. aeruginosa* showed increased production of pyocyanin with increase in incubation period, reaching the maximum at 9 days of incubation, production thereafter remains more or less constant (Figure 5.)

Antibacterial activity

Pyocyanin was found to show varied antibacterial activity against test bacteria (Figure 6, TABLE 2.). No antimicrobial activity was noted against *E. coli*, *P. aeruginosa* SPC B 65 and *P. fluorescens* SPC B 60. Waksman and Woodruff[22], had reported that the antibiotic action of pyocyanin was dependent up on the growth medium. With the studies of Hassan and Fridovich[23], it is now clear that the anti microbial activity of pyocyanin is determined by several factors like nutrient availability, molecular oxygen and possession of specific enzymes that could make an organism resistant to its attack. They noticed that *E. coli* grown in glucose-minimal medium was susceptible to pyocyanin while increase of nutrients with in the medium resulted in high production of enzymes like superoxide dismutase and catalase that helps the bacteria to resist pyocyanin toxicity. Thus many bacteria, apparently resistant to pyocyanin in culture media will become susceptible in minimal nutrient conditions such as those in rhizoplane and phylloplane. However *P. aeruginosa* showed a better survival capacity even in high pyocyanin concentrations by exhibiting low permeability, active extrusion and high catalase production[23].

Thermostability of the pigment

The siderophore showed high thermal stability. Even after exposing to 100° C for an hour, no decrease in

antibacterial activity was seen. The pigment also showed high shelf life and can be stored at room temperature for long periods without much loss of activity.

Anti fungal activity

Pyocyanin showed no antagonistic effect to fungi grown in PDA plates. However, the pigment showed anti fungal activity to all the four test fungi grown in glucose minimal medium (TABLE 3.). Hence pyocyanin can be used s an effective anti microbial agent either alone or in combination with other anti microbial agents against leaf infecting microbes; its antagonistic action can be increased with increasing concentration of the pigment.

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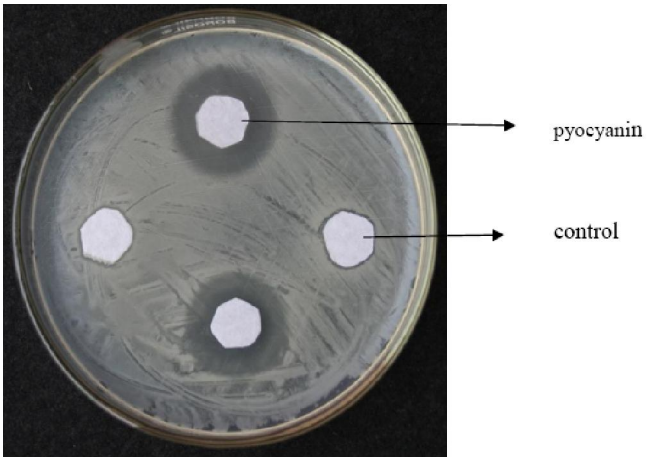

Figure 6 : Anti bacterial activity of aqueous extract of pyocyanin against *Bacillus coagulans*

TABLE 3 : Anti fungal activity of Pseudomonas

	Zone of inhibition (cm)		
	P.fluorescence **B20**	***P.fluorescence*** **B21**	***P.aeruginosa*** **B25**
Curvularia lunata	1.35 ± 0.22	1.42 ± 0.30	1.15 ± 0.15
Fusarium oxysporum	0.75 ± 0.26	0.80 ± 0.29	1.20 ± 0.12
Pestalotia palmarum	1.70 ± 0.52	1.85 ± 0.27	1.60 ± 0.80
Oidium heveae	1.60 ± 0.25	2.30 ± 0.36	1.95 ± 0.64

REFERENCES

[1] P.Lemanceau, P.A.H.M.Bakker, W.J.D.Kogel, C.Alabouvette, B.Schippers; Appl.Env.Microbiol., **59**, 74-82 **(1993)**.
[2] L.Chernin, Z.Ismailov, S.Haran, I.Chet; Appl.Env.Microbiol., **61**, 1720-1726 **(1995)**.
[3] P.Darling, M.Chan, A.D.Cox, P.A.Sokol; Infect.Immun., **66**, 874-877 **(1998)**.
[4] C.P.Gupta, R.C.Dubey, S.C.Kang, D.K.Maheshwari; Curr.Sci., **81**, 91-94 **(2001)**.
[5] R.K.Jaiswal, M.A.Fernandez, R.G.Schroeder; Appl.Env.Microbiol., **56**, 1053-1058 **(1990)**.
[6] B.K.Duffy, G.Defago; Appl.Env.Microbiol., **65**, 2429-2438 **(1999)**.
[7] R.H.Rubin; Eur.J.Clin.Microbiol Infect.Dis., **12**, 42-48 **(1993)**.
[8] R.A.Miller, G.T.Rasmussen, C.D.Cox, B.E.Britigan; Infect.Immun., **64**, 182–188 **(1996)**.
[9] R.C.Dubey, D.K.Mahehwari; Practical Microbiology. 1st Edition, S.Chand and Company Ltd; New Delhi, **(2002)**.
[10] M.Mandel, E.T.Reese; J.Bact., **79**, 816 **(1960)**.
[11] E.O.King, M.K.Ward, D.E.Raney; J.Lab.Clin.Med., **44**, 401-307 **(1995)**.
[12] L.E.Casida; Industrial microbiology, 1st Edition, New Age International (p) Ltd; New Delhi, **(1968)**.
[13] R.P.Larkin, D.R.Fravel; Plant Disease, **82**, 1022-1028 **(1998)**.
[14] L.S.Thomashow, D.M.Weller**;** J.Bact., **170**, 3499-3508 **(1998)**.
[15] H.Huang, C.Chou; Plant Pathology Bulletin, **14**, 1-12 **(2005)**.
[16] R.Saikia, R.Kumar, D.K.Arora, D.K.Gogoi, P.Azad; Folia Microbiologica **51**, 375-380 **(2006)**.
[17] S.T.Lam, T.D.Gaffney; Biotechnology in plant disease control, Wiley-Liss, Inc; New York, **(1993)**.
[18] H.Budzikiewicz; FEMS Microbiology Reviews, **104**, 209-228 **(1993)**.
[19] E.T.van Rij, M.Wesselink, T.F.C.Chin-A-Woeng, G.V.Bloemberg, B.J.J.Lugtenberg; Molecular Plant Microbe Interactions, **17**, 557–566 **(2004)**.
[20] J.B.Neilands; Structure and Bonding, **58**, 1-4 **(1984)**.
[21] D.Rachid, B.Ahmed; African J.Biotechnol., **4**, 697-702 **(2005)**.
[22] S.A.Waksman, H.B.Woodruff; J.Bact., **44**, 373-384 **(1942)**.
[23] H.M.Hassan, I.Fridovich; J.Bact., **141**, 156-163 **(1980)**.