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Isolation and screening of pyocyanin producing *Pseudomonas* spp. from soil

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Abstract

Pseudomonas spp. produces large quantities of water soluble blue green phenazine pigment pyocyanin. The blue green chloroform soluble phenazine pigment extracted from *Pseudomonas* spp has antimicrobial activity. In present study a total of nine bacterial isolated screened from the different environment sample. The isolates obtained were gram negative, non-spore forming and exhibited fluorescence under UV light. The isolate showing blue green pigment on king's B agar medium at a temperature of 35^{o}C after 48-72 hr. of incubation and considered as having capabilities to produce pyocyanin.

Keywords: *Pseudomonas* spp, Pyocyanin, production.

Introduction

Pseudomonas spp is a gram-negative, aerobic rod shaped bacterium, ubiquitous organism in nature and widespread in soil, water and many other environment. *Pseudomonas* spp. producing a variety of extra-cellular phenazine pigments. *P. aeruginosa* was a common environmental gram-negative *Bacillus* (Rutala et al., 1997). It was an opportunistic human pathogen as well, was known for its ability to produce pigments (Kerr et al., 1999). *P. aeruginosa* was widely distributed in the environment; it was founded in soil, water, skin flora, and most man mode environments throughout the world, and had thus colonized many natural and artificial environments (Ran et al., 2003).

Pyocyanin is a water soluble blue green phenazine nitrogen-containing heterocyclic compound. Pyocyanin is redox active secondary metabolite. It is an extracellular pigment which is produced by *Pseudomonas aeruginosa*. Nearly 90 – 95% of all isolate of *P.aerogenosa* produces pyocyanin pigment, which is referred to us" blue pus". A variety of redox active phenazine compound are produced by strain of *P.aerogenosa,* including pyocyanin, phenazine 1-carboxylic acid and phenazine 1- carboxamide (Budzikiewicz, 1993). Pyocyanin production is abundant in medium with low iron content and plays an important role in iron metabolism. The presence of pyocyanin is easy to detect due to its blue color that turns stationary phase cultures of *P.aerogenosa* into green color. It has various pharmacologic effect on prokaryotic cell and also used to control phytopathogen (Sudhakar *et al*., 2013)

The aim of this study is to produce pyocyanin pigment from different *P.aerogenosa* strains isolated from different soil and to determine the pyocyanin production under submerge condition (Saha *et al.*, 2008).

Materials and Methods

Sample collection

The soil samples are collected from different area of Vapi, Gujarat. The soil sample are collected in sterile plastic (zipper) polythene bags by digging the lake shore 5-10 cm deep from different sites around the lake and rhizospheric soil of some inhabitant plants. A total of 15 soil samples were collected at the Different sampling sites; about 50 g of soil was collected in sterile bags for each one.

Isolation of Bacterial species:

The collected soil sample were spread on nutrient agar media and incubated overnight at 30°C. After incubation blue green pigmented colonies were selected. The identification of *Pseudomonas* species was done based on morphological, cultural, biochemical and physicochemical characteristics as suggested by Schaad *et al*., (2001).

Media used

Nutrient agar, king B, king A, Citrimide agar Glutamic acid medium, Muller Hinton medium, Mineral salt medium was used for the growth of *P.aerogenosa* and detection of pyocyanin.

Production of Pyocyanin

Selected single colonies from nutrient agar, were inoculated into king's B Broth (KB) (KB: Peptone 20 g; Glycerol 10g; MgSo4 1.5 g; K2PO4 1.5 g; D.W 1000 ml) and incubated for overnight at 37°C on 120 rpm rotary shaker for 24 -48 hours and were observed for color change. The Pigment was extracted using chloroform system.

Extraction and purification of pyocyanin

King's B broth medium was used for the extraction of pigment where the organism was inoculated and incubated for 2-3 days at 35°C. The change in color of the pigment to blush green indicated the pigment production. The color change the pigment was extracted from culture supernatants and measured based on the absorbance of pyocyanin in acidic solution at 520 nm (Baron, 1981). The broth culture was centrifuged at 5000 rpm for 10 minutes. The culture supernatants were transferred into new test tubes and extracted with chloroform (1:2) and the aqueous phase was removed. The bottom layer was re-extracted with 1 ml of 0.2 N HCl until color change was observed. Following this, the absorbance of the pigment solution was measured using spectrophotometer at 520 nm.

Results and Discussion

The colony morphology and cultural characteristics of the isolated organisms was identified as *Pseudomonas aeruginosa.* Gram staining and motility showed gram negative rods with actively motile organism. Pigment production was accomplished after overnight incubation. Soluble pigments namely pyocyanin production were indicated by color change in the solid media. In case of liquid media, pyocyanin production was demonstrated in shades of green color. The change in color of the pigment to deep pink observed upon addition of chloroform and 0.2N HCl that confirmed the presence of pyocyanin. The absorbance of this solution was maximum at 520 nm. A total of 12 different of pyocyanin producing *pseudomonas* strain was isolated in pure form and characterized for their morphological characteristic (Table1.1) upon screening in liquid media maximum pyocyanin production was shown by isolate DN9 (Liang et al., 2011).

Table 1.1: Morphological and cultural characteristics of isolates:-

Sr. no	Sample	Isolate	Gram Staining	Motility	Pigment	Capsule staining & Endospore staining
1	Garden soil 1.	DN1	Gram Negative,rods	Motile	Green	Negative
2	Agricultural Soil 1.	DN2	Gram Negative, short rods	Motile	Green	Negative
3	Garbage soil 1	DN3	Gram Negative,rods	Motile	Green	Negative
4	Agricultural soil 2	DN4	Gram Negative, short rods	Motile	Blue green	Negative
5	Garden soil 2	DN5	Gram Negative, short rods	Motile	green	Negative
6	Rhizospheric soil 1	DN6	Gram Negative, short rods	Motile	Green	Negative
7	Agricultural soil 3	DN7	Gram Negative, short rods	Motile	Green	Negative
8	Agricultural soil 4	DN8	Gram Negative, short rods	Motile	Blue green	Negative
9	Garbage soil 2	DN9	Gram Negative, rods	Motile	Blue green	Negative
10	Rhizospheric soil 2	DN10	Gram Negative, short rods	Motile	Blue green	Negative
11	Slimy soil	DN11	Gram Negative, rods	Motile	Green	Negative
12	Agricultural soil 5	DN12	Gram Negative,rods	Motile	Green	Negative

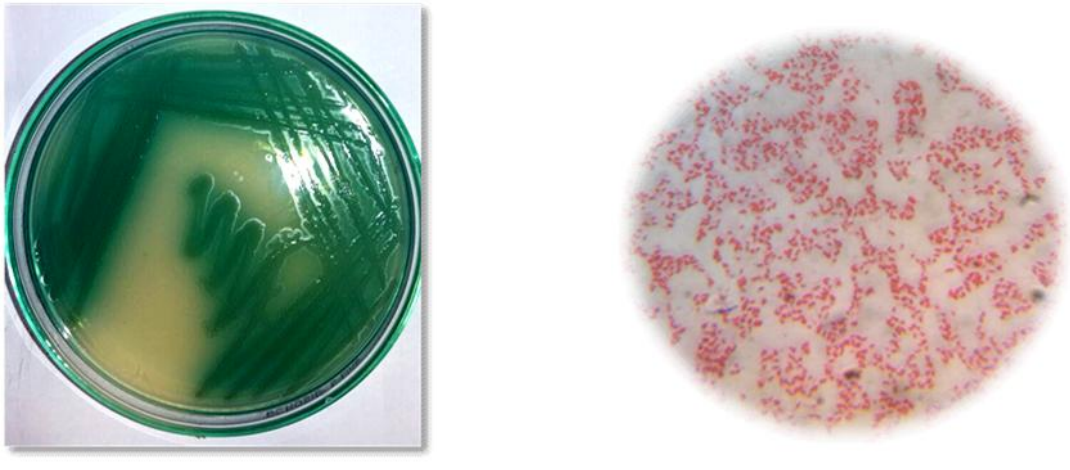

Fig.1.1 : Isolate DN9. A: Growth on king's B medium. B: Gram's staining

Table1.3 Pyocyanin production yield by isolates

Sr.No	Symbols for expected *Pseudomonas*	Pyocyanin yield (µg/ml)		
		48h	72h	96
S1	DN1	1.23	3.56	2.50
2	DN2	3.89	6.36	2.78
3	DN3	2.47	5.75	3.85
4	DN4	1.2	2.9	1.7
5	DN5	5.75	10	4.7
6	DN6	0.52	2.12	1.23
7	DN7	2.49	5.47	3.47
8	DN8	0.7	1.28	0.89
9	DN9	6.25	10.9	6.45
10	DN10	1.90	4.89	2.44
11	DN11	2.96	6.10	3.45
12	DN12	1.78	3.85	1.5

Effect of incubation period on pyocyanin production

The effect of incubation period was determined by production medium with 1% inoculum. The inoculated flasks were incubated for 144 h and pyocyanin production was estimated at an interval of 24 h. A gradual increase in pyocyanin yield was observed from 24-72 h. Maximum pyocyanin production with 11.44 µg/ml was observed at 72 h (fig.1.2) further increase in incubation time results in decreased pyocyanin production. Saha et al., (2008) reported that pyocyanin pigment showed a steady increase in concentration throughout the culture period of 72 h. Onbasli and Belma., (2008) showed that the highest pyocyanin production occurred after 72 h of incubation.

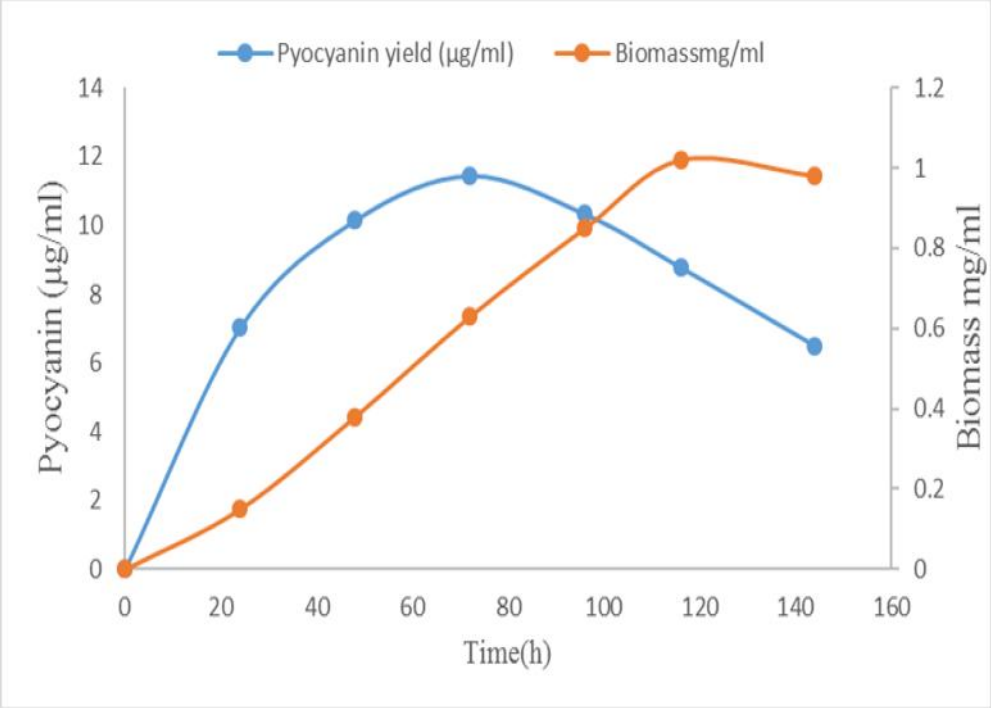

Fig.1.2: Effect of incubation period on pyocyanin production

Effect of different growth media on pyocyanin production

Various production medium were evaluated for their effect of pyocyanin production. The medium used in this study are as follows; Glutamic acid, King's B, King's A, Nutrient broth, Cetrimide broth, Mineral salt medium, Muller Hinton broth. As indicated from the (Fig.1.3), King's B medium give maximum yield of pyocyanin production (11.509 µg/ml), when compared to Glutamic acid (9.971 µg/ml), King's A medium (7.753 µg/ml). Barakat et al., (2015) reported that marine *Pseudomonas aeruginosa* produces highest yield of blue green pigment of 26 µg/ml concentration on king's B medium.

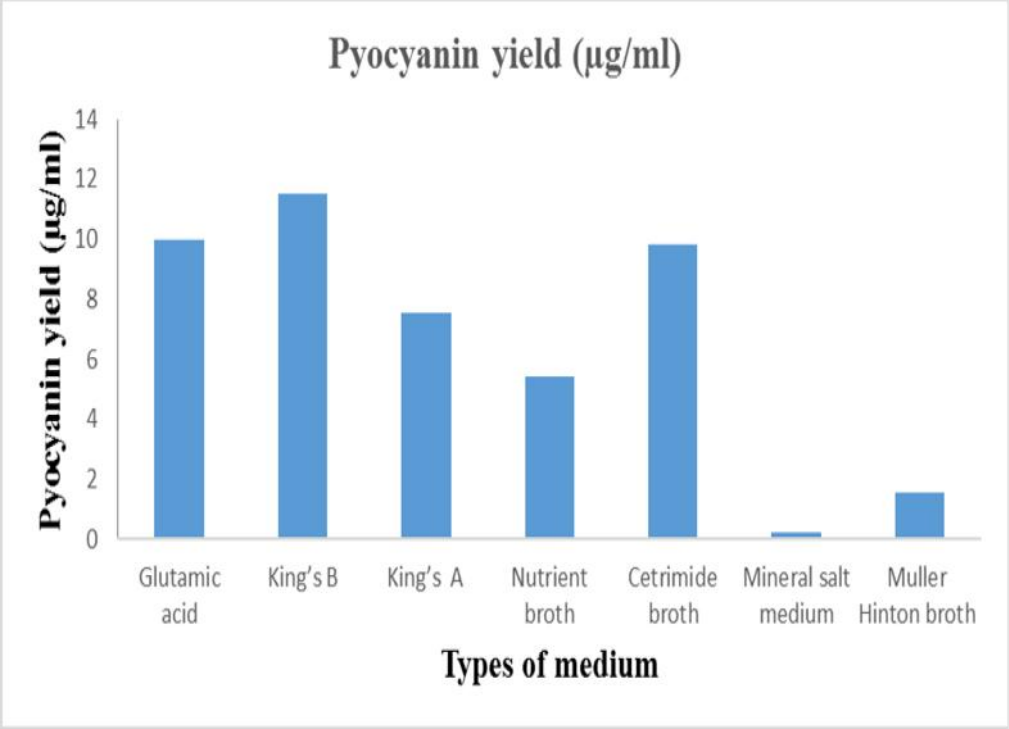

Fig 1.3 Effect of different growth media on pyocyanin production

Summary and Conclusion

In the present study, pyocyanin pigment was produced by *Pseudomonas* DN9 on various liquid media. The pigment was extracted using chloroform which produced blue color and turned to red on addition of 0.2N HCl. The extracted pigment showed a maximum absorbance at 520 nm confirmed the presence of pyocyanin. The higher pyocyanin pigment producer isolate DN9 isolated was selected for medium composition optimization.

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