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PYOVERDINE PRODUCTION BY NOVEL STRAIN OF *PSEUDOMONAS* FROM MARINE SOURCE OF ALIBAGH, MAHARASHTRA

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ABSTRACT

Using Zobell agar 42 bacterial isolates were obtained from marine water samples. These isolates were screened for the production of the pigment Pyoverdine by cultivating them on Pseudomonas Isolation Agar (PIA). Eight isolates were confirmed as pyoverdine producers by U.V. studies and were identified based on their morphological and biochemical characterization. These isolates were further evaluated for the production of pyoverdine in succinate media and estimated both qualitative and quantitatively using CAS assay. Isolate RSML 05741 was found to be the most efficient producer with 87% siderophore production. pH 8 and 30^{0}C were reported as most suitable parameters for maximum siderophore production.

Key words: *Pyoverdine, Pseudomonas, U.V., CAS assay,*

INTRODUCTION:

The marine environment, with its physicochemical properties, is a unique habitat for a variety of living forms. Microbes, which adopt this diverse environment and produce a variety of

metabolites, play a significant role in fields like Agriculture, pharmaceuticals, etc. (Armstrong *et al.*, 2001). Pyoverdine (PVD) is a green-yellow diffusible florescent siderophore produced by *Pseudomonas* species in iron-limiting conditions. (Meyer &Abdallah,1978) PVD gene expression depends on intracellular iron concentration; hence, it is synthesized in an iron-limiting environment. There are about 50 different pyroverdines described (Visca *et al*., 2007, Budzikiewicz, 2004). Siderophores have great applications in plant growth promoters, bio-control, ecological sectors and medicine as a potent drug for iron-deficient diseases. Pyoverdine produced by *Pseudomonas* species is a mixed type of iron scavenger that contains both hydroxamate and catecholate functional groups (Meyer & Hornsperger, 1978, Meyer and Stintzi, 1998).

The present investigation is an effort to isolate marine *Pseudomonas* and analyze its valued potential for the production of pyroverdine.

Material and methods:

Marine water sample was collected in sterile bottles from Alibagh beach, located in District Raigad ($18^0$38' 29'' N $72^0$52'' 20' E; temperature 29.7 ^{O}C), Maharashtra (India), and brought to the lab within 24 hours. Marine bacterial isolates were obtained using the standard tenfold dilution method, and by cultivating microbes on sterile Zobell marine agar (peptone5g, sodium chloride19.45g, yeast extract1, magnesium chloride 8.8g, ferric citrate 0.1g, sodium sulfate 3.24g, calcium chloride 1.8 g, potassium chloride 0.55g,sodium bicarbonate 0.16g, potassium bromide 0.08g, strontium chloride 0.16 g, disodium phosphate 0.008g, boric acid 0.022g, sodium silicate 0.004 g, sodium fluorate 0.0016g, distilled water 1000ml, agar 15g.,pH7.6) plates after incubation at 28 °C for 72 hrs. Upon incubation, isolated colonies were purified by sub culturing and maintained on Nutrient agar slants at 4^OC. Isolated colonies were then screened for *Pseudomonas* using Pseudomonas isolation agar (PIA) at the same ambient conditions and identified according to Bergey's Manual of Systematic Bacteriology (Palleroni,1984) based on morphological and biochemical characters up to genus level. Which were then evaluated for their potential for the production of Pyoverdine. All experiments were conducted in triplicate.

Screening of Marine *Pseudomonas* for pyoverdine production:

Marine *Pseudomonas* species were screened for the production of Pyoverdine by simply growing them in flasks containing sterilized iron-deficient succinate medium (succinate 4.0 gm, KH_2PO_4, 3.0 gm, K_2HPO_4 100mgm, $(NH_4)_2SO_4$ 1.0gm, $MgS0_4.7H_2O$-20 mg, pH 7.) Growth medium was extracted with 8 hydroxyquinoline and chloroform to remove traces of iron. (Tailor and Joshi, 2012) Flasks were incubated at 120 rpm for 48 hr. on rotary shaking

incubator at 28±2^{O}C till the visible yellow florescent diffusible pigment appeared. The absorption maxima of cell-free supernatant at 404 nm and instant appearance of golden yellow color after reacting with CAS reagent confirmed production of the Pvd type of siderophore.

Pyoverdine production by *Pseudomonas* isolates:

All isolates were tested for pyoverdine production on Chrome azurol S agar medium. 24 hours old cultures were spot inoculated on Chrome azurol sulphonate agar medium plates separately and incubated at 28±2^{0}C for 72 hrs in the dark. Development of yellow - orange zones around the growth was considered as positive for pyoverdine production (Schwyn and Neilands, 1987).

For determination of % CAS decolorizing siderophore units the cultures grown earlier were centrifuged at 10,000 rpm for 20 min. The supernatant was adjusted to pH 7 and was analyzed for appearance of pyoverdine by Universal Chemical assay i.e. Chrome Azurol Sulphonate (CAS) assay (Schwyn and Neilands, 1987). For this, 0.5ml culture supernatant and 0.5ml CAS reagent were mixed. and incubated for 5 minutes at room temperature, followed by spectral-based studies of the mixture at 630 nm that revealed the percentage of siderophore units. (Neilands,1987)

A standard was prepared by mixing sterile medium and CAS reagent. The siderophore units responsible for percent decolorization of blue colored CAS reagent for each isolate were determined using the following formula. (Payne, 1994) Both qualitative and quantitative studies are carried out in triplicate.

$$\% \text{ pyoverdine units} = \frac{Ar - As}{Ar} \times 100$$

Where, Ar = absorbance of reference (un-inoculated SM +CAS reagent) and

As = absorbance of sample (supernatant of production medium + CAS reagent) at 630nm.

Optimization of Siderophore Production:

Isolate with potential to produce high pyoverdine production was further evaluated by CAS assay (as mentioned earlier) under optimization with pH (4 to 9) and temperature (20 to 40

^{O}C) parameters. Each set with a standard variable was inoculated with 1% (v/v) active culture and incubated at 180 rpm for 40 h and siderophore production was measured at 630 nm & calculated (Fazary *et al*., 2016)

Results and Discussion:

Isolation and screening of pyoverdine-producing marine *Pseudomonas*:

Total 42 marine bacteria were isolated using zobell agar from which 18 isolates were obtained after screening on *Pseudomonas* isolation agar and finally eight isolates producing pyoverdine on succinate medium were obtained.(Fig1)

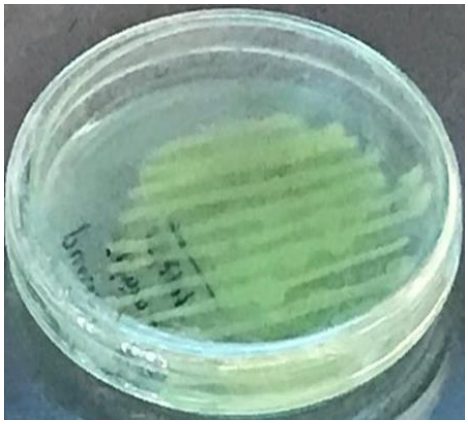

Fig1: Growth of isolate on succinate medium

These pyoverdine producing isolates were studied for morphological, cultural and biochemical characteristics and identified as *Pseudomonas.* Results of pyoverdine production in terms of % CAS decolorizing units of siderophore by all eight pseudomonas isolates are presented in Table 1.

Table1 : Isolate wise siderophore production

Sr.No.	Isolate	Ar	As	% siderophore production
1	RSML05741	0.72	0.12	83.33
2	RSML05742	0.72	0.31	56.94
3	RSML05743	0.72	0.15	79.16

4	RSML05744	0.72	0.29	59.72
5	RSML05745	0.72	0.16	77.77
6	RSML05746	0.72	0.17	76.38
7	RSML05747	0.72	0.21	70.83
8	RSML05748	0.72	0.28	61.11

Ar: Absorbance of reference As: Absorbance of sample

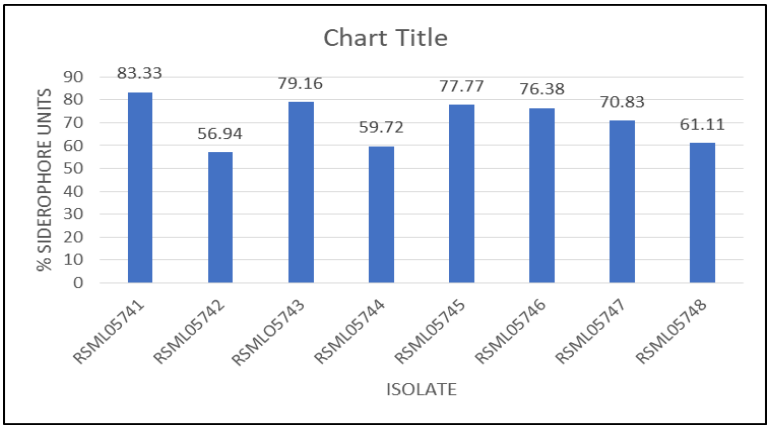

Fig 2: Isolate wise siderophore production

It is evident from the results presented in the table 1and Fig 2 that, production of % siderophore unit by eight isolates ranged from 56.94 to 83.33 % . Maximum 83.33 % siderophore units were reported by isolate RSML 05741 and lowest 56.94 % by isolate RSML 05742.Hence Isolate RSML 05741 was selected for morphological, cultural biochemical followed by production of pyoverdine and optimization studies.

Study of characteristics and identification:

Morphological characters of isolate RSML 05741reveled as pigment producer, Gram negative, slender, motile rod shaped organism. Results of biochemical test were presented in Table 2. It is evident from the table that isolate RSML 05741 was found positive for oxidase, catalase, glucose fermentation, citrate, urease & HCN production and negative for indole, methyl red & voges proskaeur. Morphological and biochemical studies revealed that RSML

05741 is a *Pseudomonas species* as per Bergey's Manual of Systematic Bacteriology (Palleroni., 1984).

Table 2: Biochemical characteristic of potential isolate:

Sr.No	Test	Results by RSML 05741
1	Oxidase	+
2	Catalase	+++
3	Glucose	+
4	Indole	-
5	Methyl red	-
6	Voges Proskaeur	-
7	Citrate	+
8	Urease	+
9	HCN	+

+ = Positive, - = Negative, +++ = Strong Positive

The formation of the orange zone on CAS agar gives clear evidence of siderophore production (Fig. 3).

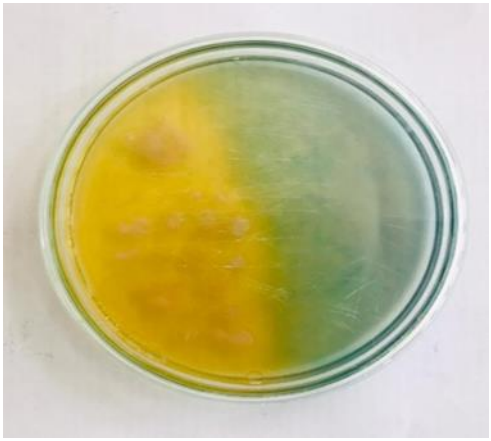

Fig.3: CAS assay; Appearance of Orange color zone indicate siderophore production.

Separated cell free supernatant from succinate broth was analyzed spectrophotometrically for CAS assay test and appearance of fluorescent color under U.V. light indicated presence of pyoverdine.(Fig 4)

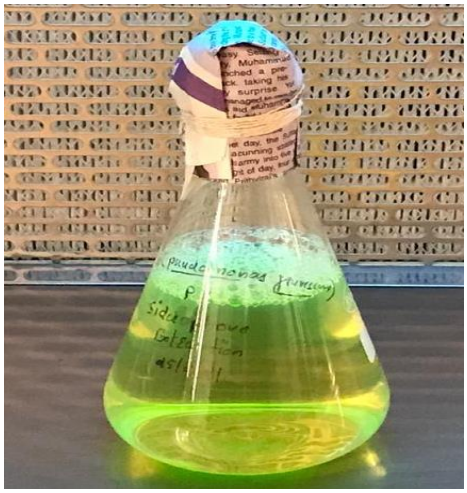

Fig4: Fluorescent colored Cell-free supernatant under U.V indicating pyoverdine

Similar results were obtained by Ghosh et al. (2015) while studying *Trichoderma* species, *Bacillus* species, and *Pseudomonas aeruginosa f*or siderophore production.

Optimization studies:

Table 3 and 4 depicts results of effect of pH and temperature on the production of pyoverdine.

Effect of pH on Pyoverdine Production:

It is evident from the Table 3 that siderophore production found increased with increase in pH from 4 to 8 and decreased at 9. Maximum 87 % siderophore units were produced at pH 8

Table 3: Effect of pH on siderophore production

Sr. No	pH	% siderophore units
1	4	40
2	5	50
3	6	68
4	7	83
5	8	87
6	9	69

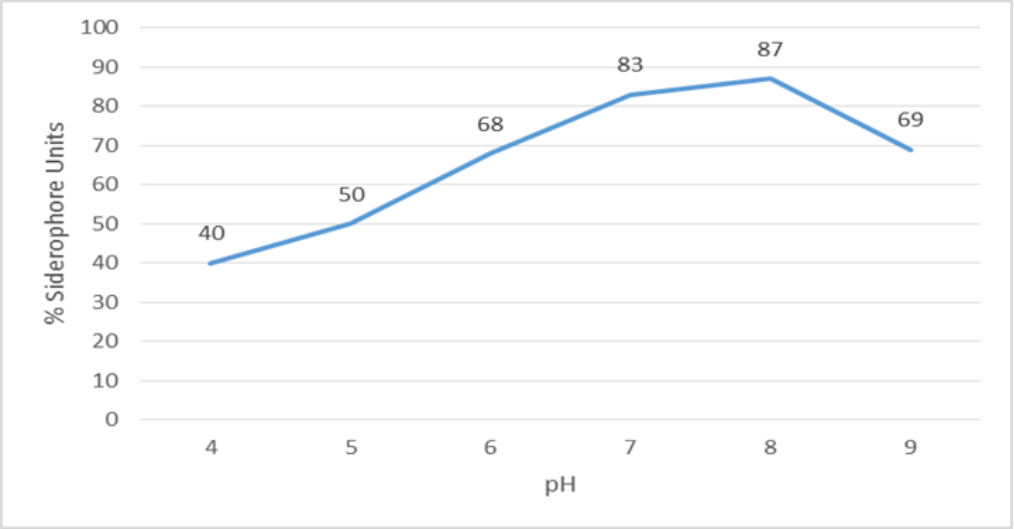

Fig **5 :** Effect of pH on Pyoverdine Production

pH plays important role in the solubility of iron and there by its availability to the growing organisms in the medium. At neutral pH (7.0), maximum siderophore yield were obtained which may be because of bacteria grow better and iron is present in insoluble form at neutral pH and therefore is not available to the bacteria. This stress of iron induces siderophore production.Previous work carried out by Sayyed *et al.*, (2005) supports the present investigation's fact that increase in pH stimulates siderophore production. On the other hand Manwar et al.,(2004) reported higher siderophore production at pH 6.

Table 4 : Effect of temperature on siderophore production

Sr.No	Temperature ^{O}C	% siderophore units
1	20	45
2	25	69
3	30	86
4	37	70
5	40	40

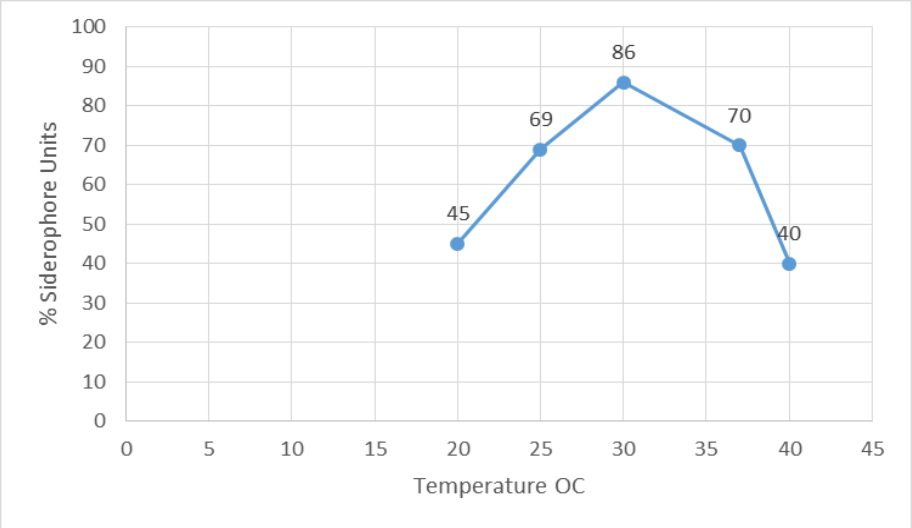

Fig 6. **:** Effect of temperature on Pyoverdine Production

It is noticed from the values of % siderophore production mentioned in Table 4 that increase in siderophore production occurred with increase in temperature up to 30 ^{O}C and decreased thereafter. Maximum 86% siderophore units was reported at 30^{O}C

Prakash and Karthikeyan (2013) obtained pseudomonas isolates from the rhizosphere soil of medicinal plant, *Acorus calamus* and were screened for siderophore production activity. They pointed out that production of siderophore was detected less frequently than other PGP characteristics.

In addition to pH, temperature also influences microbial growth and in turn, siderophore production. Previous studies of siderophore production by the plackett Burman method by *Pseudomonas* revealed elevated siderophore concentrations at 27 ^{O}C. Present isolates viz RSML 05741was found to produce optimum siderophore (86%) at 30^{O}C, but higher temperatures decreases yield.

CONCLUSION:

Isolate RSML 05741 is identified as *Pseudomonas* and found to produce 87% siderophore units. pH 8 and 30^0C were reported as optimal parameters for maximum siderophore production.

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