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ISOLATION OF *PSEUDOMONAS SPP*. AND USE OF STATISTICAL DESIGN FOR OPTIMIZATION OF PROCESS PARAMETERS FOR SIDEROPHORE PRODUCTION

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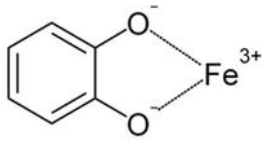
ABSTRACT
In this study, two bacterial strains were isolated from waste water samples and they were further tested for siderophore producing ability. These two isolates were labelled as *R.Y.1* and *R.Y.2*. Among these two isolates, *R.Y.1* strain was found to produce more siderophore, and therefore was selected for further study. Molecular identification of *R.Y.1* was done by 16s rRNA analysis and identified the *R.Y.1* strain as *Pseudomonas aeruginosa*. For exact media formulation the Placket-Burman design (PBD) was used. This has showed to be efficient and effective approach to systematic investigation on the target important factor. PBD is an effective screening design which considerably diminishes the number of experiments and gives information for the number of the evaluation of the target factors as much as possible. Only the most effective factors with positive significance are selected for further optimization. The less Significant or high negative effect on response value has been omitted in further experiments. In this study PBD for seven variables with eight experimental runs were designed with low and high value of each media components. Significant process parameters (pH, Time, N_2 source and temperature.) for production of Siderophore were determined by using Placket-Burman statistical design technique. Further optimization of individual selected parameter was carried out for maximum production of siderophore.

KEY WORDS: PBD, Siderophore Optimization, *Pseudomonas spp.*

INTRODUCTION

Iron is essential for almost all life forms for processes such as respiration and DNA synthesis. Despite being one of the most abundant elements in the Earth's crust, the bioavailability of iron in many environments such as the soil or sea is limited by the very low solubility of the Fe^{3+} ion. This is the predominant state of iron in aqueous, non-acidic and oxygenated environments. It accumulates in common mineral phases such as iron oxides and hydroxides (the minerals that are responsible for red and yellow soil colours) hence cannot be readily utilized by organisms. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+}complexes that can be taken up by active transport mechanisms. Many siderophores are nonribosomal peptides, although several are biosynthesized independently. Siderophores are amongst the strongest binders to Fe^{3+} 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 desferrioxamine B gaining widespread use in treatments for iron poisoning and thalassemia. Besides siderophores, some pathogenic bacteria produce hemophores (heme binding scavenging proteins) or have receptors that bind directly to iron/heme proteins. In eukaryotes, other strategies to enhance iron solubility and uptake are the acidification of the surroundings (*e.g.* used by plant roots) or the extracellular reduction of Fe^{3+} into the more soluble Fe^{2+} ions.



Structure of Siderophore-Iron Complex

Siderophore have applications in medicine for iron and aluminum overload therapy and antibiotics for improved targeting. Siderophores are useful as drugs in facilitating iron mobilization in humans, especially in the treatment of iron diseases, due to their high affinity for iron. One potentially powerful application is to use the iron transport abilities of siderophores to carry drugs into cells by preparation of conjugates between siderophores and antimicrobial agents. Because microbes recognize and utilize only certain siderophores, such conjugates are anticipated to have selective antimicrobial activity. Microbial iron transport (Siderophore)-mediated drug delivery makes use of the recognition of siderophores as iron delivery agents in order to have the microbe assimilate siderophore conjugates with attached drugs. Fluorescent pseudomonads have been recognized as biocontrol agents against certain soil-borne plant pathogens. They produce yellow-green pigments (pyoverdines) which fluoresce under UV light and function as siderophores. They deprive pathogens of the iron required

for their growth and pathogenesis. To Increase the production of Siderophore by *pseudomonas* was investigated while varying cultural conditions including Temperature, pH, NaCl concentration *etc*. A practical experiment for optimization of a single factor while maintaining the all other factor at constant level does not represent the combine effect of all the factors involved. In addition it requires large number of experiments and optimum value obtained from such experiments are unreliable. So Plackett-Burman design is a well established and widely used statistical design technique for the screening of medium component which eliminates the limitation of a single factor optimization process. (Kim S.J *et al* 2008). Plackett–Burman designs are experimental designs presented in 1946 by Robin L. Plackett and J. P. Burman while working in the British Ministry of Supply. Their goal was to find experimental designs for investigating the dependence of some measured quantity on a number of independent variables (factors), each taking *L* levels, in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments. Interactions between the factors were considered negligible. (Robin L. Plackett and J. P. Burman 1946)

MATERIALS & METHODS

Sample collection

For the isolation of Siderophore producing microorganisms, four different sewage samples were collected in sterile bottle, and were stored in a refrigerator.

Enrichment of bacterial culture

For the selective enrichment of microorganisms, one ml of each of four samples was inoculated in nutrient broth and incubated at room temperature on rotary shaker for four days.

Isolation of microorganisms

After incubation, the enriched culture was serially diluted in sterile saline up to 10^{-7} dilution. 0.1ml culture from 10^{-4}dilution was spread on sterile nutrient agar plates and incubated at room temperature for 48 h.

After incubation the plates were observed for the presence of pigmented bluish green colored colonies. Selected colonies were retreated on the sterile nutrient agar media to get pure culture. The obtained isolates were labeled as *R.Y.1* and *R.Y.2*. Morphological characteristics of both the isolates were recorded.

Biochemical characterization

The morphological and biochemical characterization of isolates*R.Y.1* and *R.Y.2* were performed by using following tests. The Gram's nature of the isolated organisms was determined by a standard Gram staining procedure. The isolates were checked for their ability to convert citrate into oxaloacetate by standard method of citrate utilization test. For this, isolates were inoculated in Simon's citrate media and incubated at room temperature for 24 h. Change in colouration from green to bright blue indicates positive test. The isolates were also checked for their ability to utilize various carbon sources by growing them in minimal medium containing 0.1% carbon different sources at 37^0c for 24 h.

Filter paper strip dipped in NNN'N'-tetramethyl paraphenyl enediaminedi hydrochloride (TMPD or DMPD) reagent was smeared with single colony and observed for color change. Formation of blue color within 10 seconds indicates positive oxidase test. Casein hydrolysis test was conducted on milk agar, which is a complex media containing casein, peptone and beef extract. The isolates were streaked on milk agar plates, incubated at 37^0c for 24 h and observed for zone of casein hydrolysis. To perform catalase test, 1ml of hydrogen peroxide (H_2O_2) solution was taken in a test tube and the isolated colony was immersed in to it and observed for effervescence.

Siderophore detection

Detection of siderophore produced by selected isolate (*R.Y.1*) was done by following methods

Siderophore production by the isolated strains of *Pseudomonas* was determined by chrome azurol S assay. The strains were spread over cetrimide agar and incubated for 48 h at 30 ^{0}C. After incubation, a thin layer of CAS reagent in 0.7% agar was overlayed on the bacterial growth and plates were again incubated for 24 h at 30 ^{0}C.

Quantification of siderophore

The quantitative estimation of siderophore produced by *pseudomonas* was done by CAS-shuttle assay. In this assay, the strains were grown on succinate medium containing gm/L of K_2HPO_4 (6.0), KH_2PO_4 (3.0), $MgSO_4$(0.2), $(NH_4)_2SO_4$(1.0) and succinic acid (4.0), pH 7.0 and incubated for 24-30h at 28^0C at 120 rpm on a rotatory shaking incubator .After incubation the fermented broth was centrifuged at 10,000 rpm at 4^0C for 10 minutes. The cell free supernatant was mixed with 0.5 ml of CAS solution and incubated for 20 min. The intensity of the color produced was determined by measuring the absorbance at 630 nm using the spectrophotometer. The sample without cell culture supernatant was used as control. The percentage of siderophore units produced was estimated as the measure of proportion of CAS color shifted using the formula [(Ar-As)/Ar] x100, where Ar is the 630_{nm} of reference (CAS assay solution + uninoculated medium) and As is the 630_{nm} of the sample (CAS assay solution + cell supernatant).

Plackett-burman experiment

In this experiment according to Plackett and Burman design 8 experimental Runs for 7 variables (A to G) at high and low levels were selected. In which one factor F(Water type) was designated as dummy variables which is used for obtaining experimental error. Each horizontal row in the given table represents a trial and each vertical column represents the H (high) and L (low) values of one variable in all the trials. The frequency of high and low values of each variable is maintained according to the rules of Plackett and Burman design. This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in more detail study to determine the optimum values used to formulate the medium.

	A	B	C	D	E	F(D)	G
	Time	Temp	pH	Amino acid	Succinic acid	Water Type	N_2 Source
	H-12	H-40	H-07	H-01	H-04	H-DI	H-01
	L-03	L-28	L-05	L-0.1	L-01	L-I	L-0.1
Experiments							
1	H	H	H	L	H	L	H
2	L	H	H	H	L	H	L
3	L	L	H	H	H	L	H
4	H	L	L	H	H	H	L
5	L	H	L	L	H	H	H
6	H	L	H	L	L	H	H
7	H	H	L	H	L	L	L
8	L	L	L	L	L	L	L

FIGURE 1: Placket –Burman Design for seven variables

	A	B	C	D	E	F(D)	G
	Time	Temp	pH	Amino acid	Succinic acid	Water Type	N_2 Source
	H-12	H-40	H-07	H-01	H-04	H-DI	H-01
	L-03	L-28	L-05	L-0.1	L-01	L-I	L-0.1
Experiments							
1	12	40	07	0.1	04	I	01
2	03	40	07	01	01	DI	0.1
3	03	28	07	01	04	I	01
4	12	28	05	01	04	DI	0.1
5	03	40	05	0.1	04	DI	01
6	12	28	07	0.1	01	DI	01
7	12	40	05	01	01	I	0.1
8	03	28	05	0.1	01	I	0.1

FIGURE 2: H-High and L-Low values selected for each variable

Optimization of siderophore production

To optimize the production of Siderophore, the effect of significant variables selected according to Plackett-Burman design were checked. Out of 7, the variables which were found to play important role in production process were individually optimized. These were checked by incubating the bacteria in production medium. The percentage of Siderophore produced was determined by the proportion of CAS color shifted using the formula mentioned above.

RESULTS & DISCUSSION

Isolation of microorganisms

The growth was observed on all four plates of nutrient agar. However only the greenish yellow colored colonies, which are peculiar characteristics of *Pseudomonas spp*. were selected as potential isolates. Two greenish yellow colored isolates were obtained from two different sewage samples. These isolates were labeled as *R.Y.1*and *R.Y.2*.

TABLE1: Morphological and Biochemical characterization of isolate *R.Y.1* and isolate *R.Y.2*

Characters	Observations	
	R.Y.1	*R.Y.2*
Morphological		
Greenish yellow pigment	+	+
Cell Shape	Coco bacilli	short rod
Gram staining	Gram negative	Gram negative
Motility	Actively Motile	Motile
Biochemical		
Casein hydrolysis	positive	positive
Catalase	positive	positive
Oxidase	positive	positive
Sugar utilization(Glu)	positive	positive
Citrate test	positive	positive

Morphological and biochemical characteristics ofisolates

After growth on nutrient agar both isolates *R.Y.1*and *R.Y.2*formed greenish yellow colored colonies by producing a diffusible pigment. The biochemical characters were performed by using standard methods described in Bergey's Manual of Determinative Bacteriology. According to King EO.*et al.,* (1954) *Pseudomonas aeruginosa* colonies appear green to bluish-green due to production of pyocyanin pigments. The results (Table 1) obtained with morphological and biochemical characteristics for *R.Y.1*and *R.Y.2*were

compared with the characters of reference *Pseudomonas aeruginosa* (Bergey's Manual of Determinative Bacteriology) and it was found that *R.Y.1*exhibits more similarity with the *Pseudomonasaeruginosa.* The 16s rDNA gene sequence of *R.Y.1* and *R.Y.2* isolates was used to BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4

The sequencing and phylogentic analysis identified the*R.Y.1*as *Pseudomonas aeruginosa* (Genbank Accession Number JX661716.2).

Siderophore detection

Siderophore production by isolated strains of *Pseudomonas aeruginosa* (*R.Y.1*) was tested by a Chromeazurol S assay as described in the method. Yellow orange zones, an indicator of Siderophore production (Schwyn B and Neilands JB 1987) were observed around the colonies. Similarly Liquid assay was also done to confirm the result.

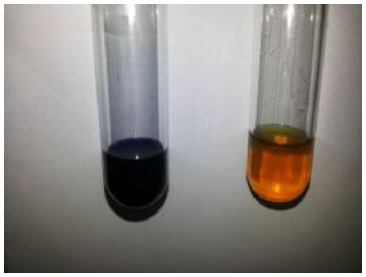


FIGURE 1: Chromazurol S assay in Liquid Medium

Siderophore characterization

Characterization of Siderophore production was performed by Tetrazolium and Neilands Spectrophotometric Assay (for Hydroxamate type of siderophore) and Arnow's and Spectrophotometric Assay (for Catecholate type of siderophore). It was found that the isolate could produce both hydroxamate and catecholate type of siderophores

Siderophore quantification

The quantitative estimation of Siderophoreproduced by *Pseudomonas* was determined by CASShuttleassay. The isolated strain of *Pseudomonas spp*. Showed 71% Siderophore production.

Plackett-Burman experiment

TABLE 2: % Siderophore obtained in each run of Plackett-Burman experiment

	A	B	C	D	E	F(D)	G	
	Time	Temp	pH	Amino acid	Succinic acid	Water Type	N_2 Source	% siderophore production
	H-12 L-03	H-40 L-28	H-07 L-05	H-01 L-0.1	H-04 L-01	H-DI L-I	H-01 L-0.1	
Experiments								
1	12	40	07	0.1	04	I	01	99.80
2	03	40	07	01	01	DI	0.1	75.33
3	03	28	07	01	04	I	01	88.74
4	12	28	05	01	04	DI	0.1	60.61
5	03	40	05	0.1	04	DI	01	52.48
6	12	28	07	0.1	01	DI	01	78.88
7	12	40	05	01	01	I	0.1	67
8	03	28	05	0.1	01	I	0.1	28

TABLE 3: Analysis of the yield

	A	B	C	D	E	F(D)	G
	Time	Temp	pH	Amino acid	Succinic acid	Water Type	N_2 Source
	H-12	H-40	H-07	H-01	H-04	H-DI	H-01
	L-03	L-28	L-05	L-0.1	L-01	L-I	L-0.1
∑H	336.29	296.61	342.75	291.68	299.8	267.3	318.54
∑L	244.55	256.23	208.09	298.16	249.21	282.8	230.94
Difference	61.74	38.38	134.66	-6.48	50.39	-15.5	87.6
Effect	15.435	9.595	33.66	-1.62	12.64	-3.87	21.9
Varience	3811.82	1473.02	18133.31	12.96	159.76	7.74	7633.96
F -test	492.48	190.31	2342.76	1.67	20.64	--	98.62
Rank	2	3	1				4

On the basis of F value obtained top four variables were selected for individual parameter optimization (pH, Time, Temperature and N2 source).

INDIVIDUAL PARAMETER OPTIMIZATION FOR SELECTED VARIABLES

Effect of pH on siderophore production

To check the effect of pH on Siderophore production, the bacteria were grown in Succinate medium at different pH (5.0, 5.5, 6.0, 6.5, and 7.0) at 37^0C with constant shaking at 120 rpm for 12 h. As shown in Fig. maximum siderophore production was observed at pH 6.0 (91.11%) followed by pH 7, 6.5, 5.5, and 5.This result suggests that the slight acidic pH favors more production of siderophore than neutral or alkaline pH. But if we increase acidity the Siderophore production decreases linearly.

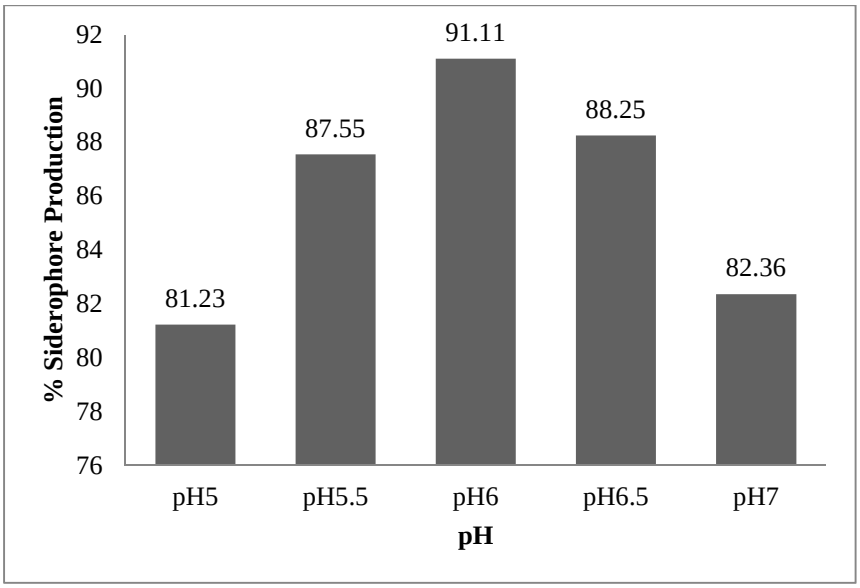


FIGURE 2: Effect of different pH on Siderophore production

Effect of N_2 source on siderophore production

To check the effect of Nitrogen source on Siderophore production, the bacteria were grown in Succinate medium of pH 6 at different conc. of N_2 source (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 gm) at 37^0C with constant shaking at 120 rpm for 12 h. As shown in Fig. maximum siderophore production was observed in medium containing 0.4 gm. N_2 source (89.56%).High or low conc. of N_2 source in production medium than the optimized value may retard the maximum production of siderophore.

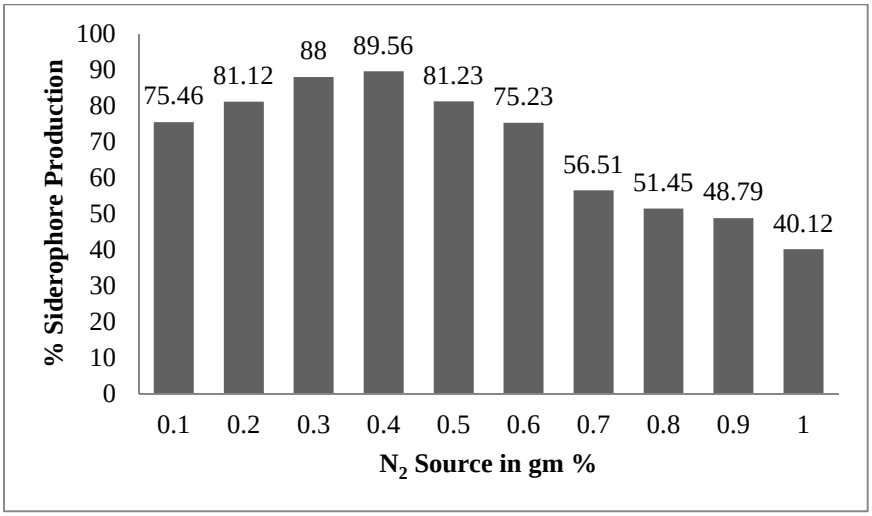


FIGURE 3: Effect of different N_2 source onSiderophore production

Effect of time on siderophore production

To determine the effect of incubation time the bacteria were grown at 37^0C at 120 rpm and the samples were removed at different time intervals. As shown in Fig. Siderophore production increases from 3 to 12 h, maximum production of were observed after 12 h of incubation (82.13%). Further incubation did not increase the siderophore yield.

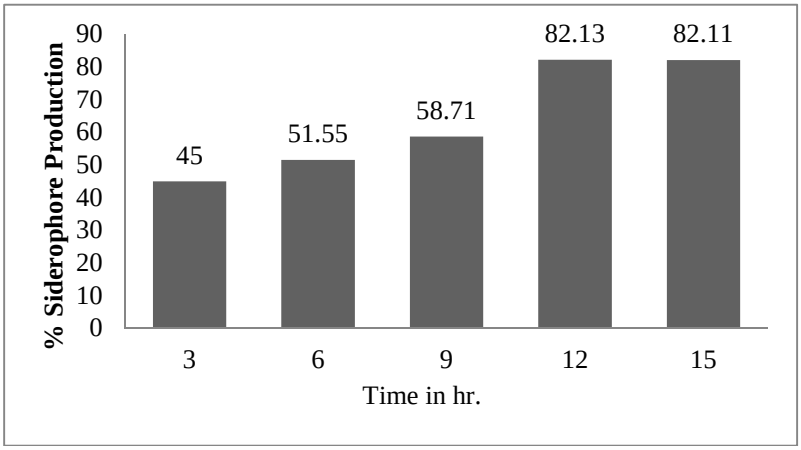


FIGURE 4: Effect of incubation time on Siderophore production

Effect of temperature on siderophore production

To determine the effect of different temperatures on siderophore production, the production medium was incubated at different temperature in the range of 30^0C, 33.5^0C, 37^0C, 40.5^0C, 44^0C, 47.5^0C, 51^0C and 54.5^0CAfter incubation for 12 h with pH 6 and constant shaking at 120 rpm on rotary shaker, it was found that maximum production of siderophore was observed at 37^0C.(96.15%). This result indicates that optimum incubation temperature required to produce maximum siderophore is 37^0C increase in temperature above 37^0C did not show further improvement in yield.

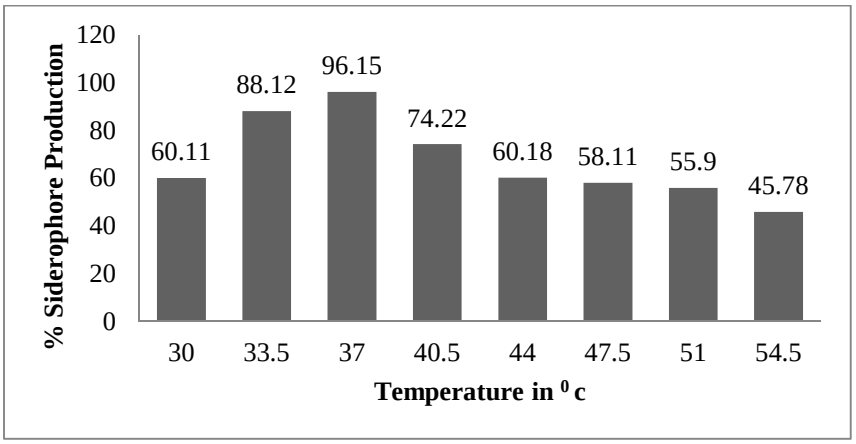


FIGURE 10: Effect of Temperature on Siderophore production

CONCLUSION

Instead of using classical approach for the formulation of media one can use full factorial method of Plackett-Burman design satisfactorily. By using classical approach method we can get the important parameter affecting the production, but it is a time consuming method and each parameter has to be optimized individually. In the present study we used Plackett- Burman design for determine the significant parameter of Siderophore production. From the results obtained we found that the parameters which significantly affect Siderophore production are medium pH, incubation time, temperature and N_2 source. The F value obtained ranked the selected parameter in the above order. Further the significant parameter obtained was optimized individually by classical approach. Siderophore production was found to be maximum after 12hours of incubation with the medium pH 6, temperature of incubation 37^0c and N_2 source conc.0.4 gm%

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