CHARACTERIZATION AND OPTIMIZATION OF SIDEROPHORE PRODUCTION FROM *Pseudomonas fluorescens* STRAIN ISOLATED FROM SUGARCANE RHIZOSPHERE

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ABSTRACT

Extensive screening for the siderophore producing bacteria from the sugarcane rhizosphere was carried out. Seven isolates were found to produce more than 85 % siderophore units. Amongst them S-11 was found be the most efficient siderophore producer (96 % SU). S-11 was further characterized and identified as *Pseudomonas fluorescens*. Physico-chemical parameters were evaluated for optimum for production of siderophores by *Pseudomonas fluorescens* strain. It was found to produce maximum siderophore at pH 7 and 29^{o}C. Tyrosine as carbon source was found to stimulate bacterial growth as well as siderophore production. Maximum siderophore yield was obtained with ammonium sulphate and urea as independent nitrogen source. Iron concentration up to 20 µM was found to optimum for siderophore production. Shake flask studies revealed that the siderophore production starts after 6 h of growth and reached to maximum productivity of 96 % SU after 24 h. The present study reveals *Pseudomonas fluorescens* strain as a promiscus candidate for crop improvement and protection due to its PGPR activities.

Key Words : Rhizospheric, Siderophores, % SU, PGPR. Carbon, Nitrogen

INTRODUCTION

Rhizosphere is a dynamic environment, which harbors diverse group of microorganisms. Some of the bacteria that directly or indirectly stimulate plant growth have been referred to as Plant Growth Promoting Rhizobacteria (PGPR)[1]. Different modes of action of PGPR are nitrogen fixation, phosphate solubilization, production of phytohormones and siderophores, production of antifungal compounds and induced systemic resistance. Recently, fluorescent pseudomonads have emerged as the largest and potentially most promising group of PGPR involved in plant growth promotion and plant disease control[2,3]. Siderophores are low molecular weight, non-ribosomal peptides, secreted under low iron stress conditions and capture iron from the environment[4]. Siderophores are also thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth[5-7].

Sugarcane is one of the major food crops providing about 75 % of sugar harvested for human consumption[8,9]. The prime goal is to sustain and enhance growth and yield of sugarcane. The present study describes rhizospheric bacterial diversity of sugarcane fields in the South Gujarat region, India. The rhizospheric isolates were evaluated for its potential of siderophore production and the most efficient isolates were optimized for siderophore production.

MATERIAL AND METHODS

Enrichment and isolation

Sugarcane rhizospheric soil samples from 50 different locations were collected from 12 different sugarcane fields, with *Saccharum officinarum* cultivar Co - 901332. The soil samples were subjected to enrichment using King's B medium[10]. After 48 h of incubation at 28^{o}C, at 120 rpm, the enriched flora was purified using King's B agar plates.

Screening for siderophore production

The rhizobacterial isolates obtained were screened for siderophore production using spectrophotometric method, which was further confirmed by CAS agar method and Universal Chemical Assay [CAS] [11,12].

Production, detection and estimation of siderophore

The potential isolates were further evaluated for the quantity of siderophore produced. The amount of siderophore was then calculated in terms of percent siderophore units (% SU) using the following formula:

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

where, Ar = absorbance of reference at 630 nm (CAS reagent); As = absorbance of sample at 630 nm.

Characterization of the most efficient isolate S-11

The most efficient isolate was further characterized on the basis of its morphological, cultural and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology. The morphological characteristics of the isolates studied included cell shape, size, arrangement of cells and gram's nature. The cultural characteristics studied were colony morphology, exopolysaccharide production and pigmentation, if any. The purified isolates were further subjected to the biochemical characterization for identification of organisms up to genus level. The biochemical tests performed were on the basis of Bergey's Manual of Systemic Bacteriology, II edition[13].

Siderophore production in different media

Different media preparations were evaluated for siderophore production. Apart from succinic acid medium, Cas-amino acid medium, Barbhaiya and Rao medium and nutrient broth were tried[14]. Each medium was separately inoculated and incubated. Following the incubation, growth was measured and siderophore content was quantified as per Payne[15].

Optimization of media

Various physico-chemical parameters were optimized for siderophore production using succinic acid medium. The parameters tested include: pH, cell mass, sugars, organic acids, amino acids, organic acids, nitrogen source and iron concentration.

Purification of siderophore

For purification, the most efficient isolate S-11 was grown in iron deficient succinic acid medium for 48 hours at 28^{0} C and 120 rpm. The broth was harvested by centrifugation. The cell free supernatant was then subjected to CAS assay to check for the presence of siderophores. The supernatant was further used in purification of the siderophore by ethyl acetate extraction. The pH of the supernatant was brought down to 2 - 2.5 with the help of strong mineral acids and then quickly subjected to extraction using ethyl acetate solvent. The organic fractions were collected and subjected to drying by evaporation. The dried crystals of siderophores were then suspended in sterile double distilled water.

Characterization of siderophores

The siderophore produced by the most efficient isolate S-11 was further evaluated to characterize whether it was catecholate, hydroxamate or carboxylate type, respectively[16-18].

RESULTS AND DISCUSSION

Enrichment and isolation

Total of 63 different isolates were obtained after enrichment and isolation of rhizospheric soil samples. The isolates were designated as S-1 to S-63 and were further screened for the siderophore production potential individually.

Screening for siderophore production

Siderophore production is one of the important trait of PGPR and is driving much attention since last few decades due to applications of siderophores in various other fields apart from agriculture. In the same context, the isolates were screened for their siderophore production potential and it was found that 12 out of 63 isolates were positive for the siderophore production and were used for the further study. The positive isolates were S-1, S-11, S-13, S-16, S-19, S-23, S-26, S-33, S-41, S-43 and S-56.

Production, detection and estimation of siderophore

The potential siderophore producing isolates were further screened out by the quantity of siderophore produced. The assay revealed that out of twelve, seven isolates were producing more than 85 % SU under iron starvation conditions in succinic acid medium. Amongst these 7, the isolate S-11 was found to produce 96 % SU after 48 hours of incubation. It was further characterized and subjected to optimization of siderophore production under in vitro conditions.

Characterization of S-11

It was found that the most efficient isolate S-11 produced fluorescent green pigment. It was gram negative rod, arranged singly and was catalase and oxidase positive and showing growth at either of the extreme temperatures, 4^0C and 41^0C also, they displayed oxidative utilization of glucose. Thus, it was evident that the isolate S-11 was identified to be a strain of *Pseudomonas fluorescens* **(Table 1).**

Siderophore production in different media

Evaluation of various media for siderophore production was studied. It was found that the development of orange red color, on reaction with CAS reagent, was faster with succinic acid medium than in casamino acid medium and Barbhaiya and Rao medium. However, no color change was observed in nutrient broth, indicating absence of siderophore production. This may be due to nutrient broth containing complex organic compounds as beef extract that can serve as source of many micronutrients, such as iron, and hence could not induce siderophore synthesis. Similar results have been obtained with Pseudomonas[14].

Table 1: Characterization of the most efficient isolate S-11

Isolates	S-11
Morphological Characteristics	
Size	Long
Shape	Rods
Arrangement	Single
Gram reactivity	Negative
Cultural Characteristics	
Size	Large
Margin	Irregular
Elevation	Raised
Opacity	Transleucent
Pigmentation	Bluish green
Consistency	Soft
Biochemical Characteristics	
Sugar ferm	-
Glucose	-
Galactose	-
Fructose	-
Lactose	-
Ribose	-
Sucrose	-
Rhamnose	-
Mannose	-
Mannitol	-
Nitrate reduction	+
TSI agar test	-
Oxidative sugar utilization	
Oxidation	+
Fermentation	-
Indole test	-
Oxidase test	+
Citrate utilization	+
Growth at	
40 C	-
410 C	+
Catalase	+++

Key: + = Positive reaction, - = Negative reaction, d = variable reaction, (+) = acid and gas production, +++ = strong positive reaction.

Siderophore production

Selected strain of Pseudomonas was evaluated for their growth curve and siderophore production profile in shake flask studies. It was observed that there was a lag phase of 6 hours in growth. The siderophore synthesis started after 12 hours of incubation, which increased up to 28 hours and declined thereafter. Thus, maximum siderophore production was observed after 28 hours of incubation. Previous findings show similar results with Pseudomonas[14].

Optimization of siderophore production

Optimization of liquid culture conditions such as carbon and nitrogen sources, nutrients, pH, temperature, along with microbial physiology has profound impact on the qualities and quantity of metabolite production[22].

Influence of pH

pH plays an important role in the solubility of iron and thereby availability to the growing organism in the medium. It was found that S-11 showed maximum siderophore production i.e. % SU at pH 7. This may be because bacteria grow better at physiological pH and iron is present in insoluble form at neutral pH. Thus, siderophore production was found to be induced under iron stress conditions.

Influence of cell mass

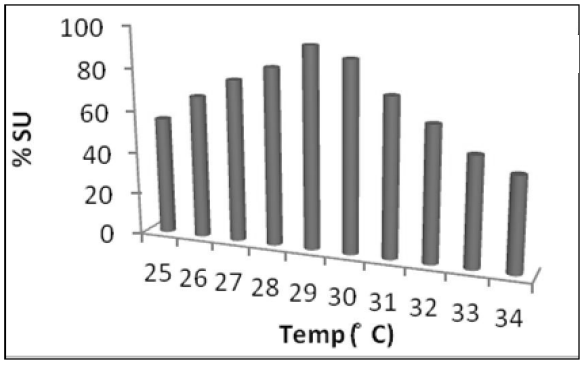
Amount of cell mass determines the growth and therefore production of secondary metabolites (such as siderophores). Study was conducted to evaluate the effect of cell mass on siderophore production. It was evident that isolate S-11 produced maximum siderophores i.e. 88 % at 1 mg % cell mass. Further increase in cell mass concentration showed no significant increase in growth and SU.

Influence of Sugars, Organic acids and Amino acids

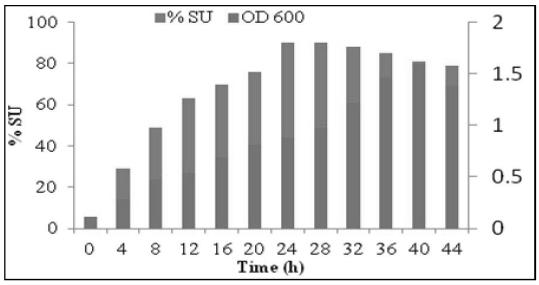
Nature of C compound determines the Fe requirement of the cell regulates siderophore production. The easily assimilable sugars contribute fast growth and thereby increased siderophore production. Studies were carried out to evaluate various organic compounds as C source for maximum siderophore production. It was observed that sugars showed adverse effect on siderophore production. This may be due to utilization of sugar for growth and not siderophore production. Organic acids, such as succinic, citric and lactic acids are major root exudates. It was evident that succinic acid was the most efficient C source with maximum siderophore production. Amongst the various amino acids tested, S-11 showed maximum siderophore production with tyrosine. It was evident that S-11 showed better siderophore production with amino acids than sugars and organic acids.

Influence of nitrogen source

During evaluation of various compounds as suitability of nitrogen source, it was found that urea was the best suited for siderophore production by S-11. Complex nitrogen source such as soy flour did not show any siderophore production. This may be due to very high iron content of soy flour. Urea as a nitrogen source has additional benefit of the possible exploitation of the isolates for the bioremediation of soils by reducing the amount or urea present in the soil.



A



B

Fig. 1 : Media Optimization of Siderophore production by S-11. A - Effect of Temperature on siderophore production and B - Siderophore production as a function of time.

Influence of iron

It has been reviewed that iron-stressed conditions lead to production of strong iron-chelating agents such as siderophores[19]. Hence studies were done to evaluate the influence of iron concentration on siderophore synthesis. As it is depicted from the observations, the isolate S-11 showed gradual decrease

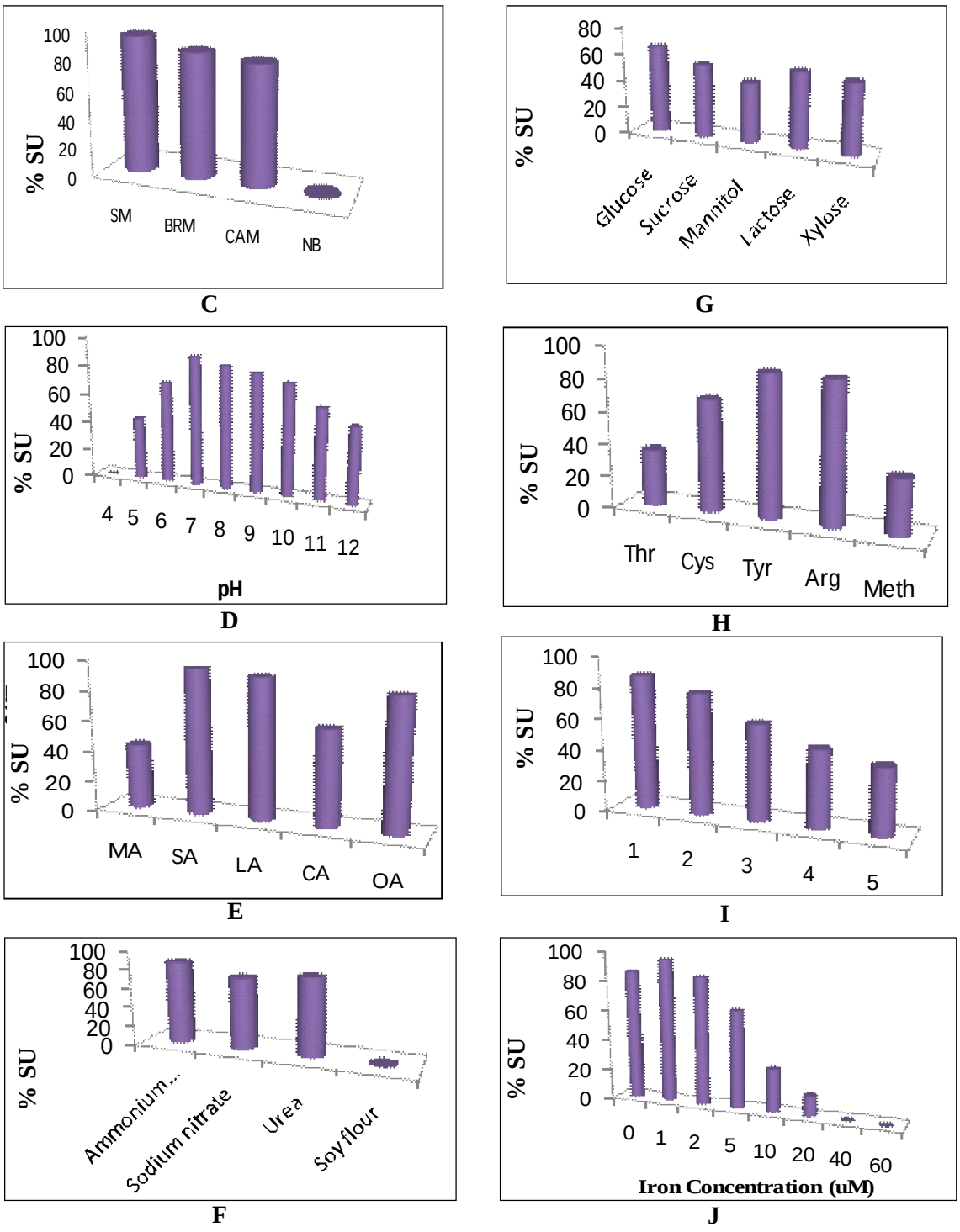


Fig. 2: Media Optimization of Siderophore production by S-11. C - Effect of different media; D - Effect of pH; E - Effect of organic acids; F - Effect of nitrogen source; G - Effect of various sugars; H - Effect of Amino acids; I - Effect of inoculum size and J - Effect of iron concentration

in siderophore production and was found to be completely repressed at higher concentrations. Maximum siderophore production was obtained at 1 µM concentration. reported the repression of siderophore production with increasing concentration of iron[20]. Reported the transcription of iron-regulated gene is under the negative control of fur protein (repressor) with Fe2+ as an essential co-repressor[21,22].

Characterization of siderophore

Characterization of the type of siderophore produced by the most efficient isolate S-11 revealed presence of different types of siderophores. It was evident that S-11 produced mixed siderophores, i.e. catecholate and hydroxamate **(Table 2)**. Although few reports are available on the biotechnological applications of siderophores, it is clear that they may have applications in many fields of human endeavour including healthcare products, environment and industry. The siderophores can thus be exploited for betterment of human life. But it has been reported that most aerobic bacteria produce siderophores of mixed type[4].

Table 2: Characterization of siderophore produced by S-11

Isolate no.	S-11
No. of peaks	02
Peak (nm)	450.00 495.00
Absorbance at peak	2.404 3.431
Arnow assay	+
Csaky assay+	
Vogel's assay	-

Hence, study of microbial system is required to channel the formation process to produce solely mono-siderophore but not the mixture and determine its aptitude in iron acquisition and anything else.

CONCLUSION

PGPR are increasingly used for crop improvement and protection. In the same context, present study was focused for isolation and characterization of PGPR from sugarcane rhizosphere. Siderophore production was considered for the present study and the most efficient isolate was characterized and evaluated for optimum conditions for maximum siderophore production. The morphological, cultural and biochemical characterization of the most efficient isolate S-11 revealed that it was *Pseudomonas fluorescens*. Iron stressed conditions were found to be necessary for optimum siderophore production for the most efficient isolate. Media optimization studies showed maximum siderophore production at pH 7 and 29^{o}C. Tyrosine as carbon source was found to stimulate bacterial growth as well as siderophore production, whereas ammonium sulphate and urea were promising nitrogen sources. Shake flask studies revealed that the siderophore production starts after 6 hour of growth and reached to maximum productivity of 96 % SU after 24 h. The results are promising for design of potentially active siderophore producing S-11 strain based formulation which would be beneficial for sugarcane crop improvement and crop protection. The potential of this strain could be investigated in detail and field application shall be studied for its biocontrol potential.

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