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RESEARCH ARTICLE

SULPHUR OXIDATION BY FLUORESCENT PSEUDOMONADS ISOLATED FROM GROUND NUT RHIZOSPHERIC SOIL AND THEIR VARIABILITY IN SULPHATE PRODUCTION

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ABSTRACT

This study was conducted to identify sulphur oxidizing ability of fluorescent pseudomonads isolated from groundnut rhizosphere of Rayalaseema districs of Andhra Pradesh and evaluate their sulphur oxidation ability. Out of the 55 (JS1 …. JS55) isolates obtained, 14 were screened based on their efficacy to reduce the pH of the growth medium from 8.0 to ≤ 5.0. The selected isolates were characterized and related to the genus *pseudomonas.* Their sulphate ion production abilities were in the range of 5mg/ml – 60 mg/ml. JS7, JS16, JS24, JS40, JS49 and JS54 gave the lowest pH and the highest total sulphate in liquid medium after one week. These isolates were checked for optimum growth conditions for efficient sulphur oxidation and their ability to produce sulphate ion. In glucose amended medium broth, bacterial strains JS7 and JS16 produced maximum sulphates i.e. 55.22 mg/ml and 53.14 mg/ml compared to other sugars.

INTRODUCTION

Sulphur is considered as the fourth essential nutrient, it contributes high yield and quality of crops in agriculture. It is a component of the essential amino acids like Cysteine and Methionine and plays an important role in the formation of proteins, vitamins and enzymes. Crop plants have become gradually more dependent on the soil to need for S (Kertesz and Mirleau, 2004). Sulphur also improves the condition of sodium soil and reins some of plant pathogens (Kertesz *et al.,* 2004). Legumes habitually require almost equal amount of phosphorus and sulphur. When phosphorus and sulphur are present below the critical level in the soil, plant growth and quality of production are affected adversely (Dube and Mishra., 1970). Insufficient Sulphur availability leads to decreased yields and reduced Sulphur content in the plant products under extreme deficiency causes severe losses in crop yield (Zhao, Hawkesford *et al.*, 1999). Most of the S in soils is leap to organic molecules, and therefore not readily available to plants. Sulphur oxidation is the main important step of sulphur cycle, which improves soil fertility. Use of S oxidizers enhances the rate of natural oxidation of S and production of sulphates and makes them available to plants at their decisive stages, resulting in increased plant yield (Wainright, 1984). The majority of sulphur taken up by plant roots is in the form of sulphate (SO_4), which undergoes a series of transformations, former to its inclusion into the original compounds (Katyal, Sharma *et al.,* 1997). The soil microbial biomass is the key driving force behind all sulphur transformation. In microbial oxidation of inorganic S compounds, the reactions often impersonate chemical models, the intermediates formed chemically interact with each other making the pathway complex (Suzuki, 1999). The transfer of sulphur between the inorganic and organic compounds caused by the activity of the soil biota, predominantly the soil microbial biomass, which has the greatest potential for both mineralization and also for successive transformation of the oxidation state of sulphur. It consequences in the formation of sulphate, which can be used by the plants, while the acidity produced by oxidation helps to solubilize plant nutrients and improves alkali soils (Hitsuda *et al.,* 2005).

The sulphur oxidizing bacteria comprise a heterogeneous group of organisms which contribute to the ability to oxidize reduced or partially oxidized inorganic sulphur compounds. The sulphur oxidizing microorganisms are primarily the gram negative bacteria currently classified as species of *Thiobacillus, Thiomicrospira* and *Thiosphaera*, but heterotrophs, such as some species of *Paracoccus, Xanthobacter, Alcaligens* and *Pseudomonas.* (Kuenen and Beudeker, 1982). Reduced form of S in the fertilizers should be oxidized by bacteria to sulphate (available form of S) (Wainright, 1984; Grayston and Germida, 1991; Scherer, 2001). While several studies have reported on the enhancement of sulphur availability, rock phosphate solubilization, and the plant growth promotion of sulphur-oxidizing bacteria

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(Grayston and Germida, 1991; Stamford *et al*., 2002; El-Tarabily *et al*., 2006; Anandham *et al*., 2007a, 2008a). The main objective of this study is to identify *pseudomonas* to select the efficient isolates for higher sulphate production and evaluate optimum growth conditions for efficient sulphur oxidation.

MATERIALS AND METHODS

Soil Sample Collection

Fluorescent *Pseudomonas* were isolated from the rhizosphere soil collected from different ground nut growing areas of Rayalaseema region. Bacteria were isolated by serial dilution plate method (Pramer and Schmidt, 1956). From the final dilutions of 10^{-5} and 10^{-6}, one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature (28±2°C) for 24 hours. The colonies were viewed under UV light at 360 nm. Colonies with characteristics fluorescent *Pseudomonas* spp. were isolated individually and purified by streak plate method (Rangaswami, 1993) on King's B medium. The pure cultures were maintained on king's B agar slants at 4°C. Fluorescent *Pseudomonas* spp. was characterized on the basis of morphological biochemical and physiological tests as prescribed in Bergey's manual of systematic bacteriology (Kreig and Holf, 1984).

Media for Identification of Sulphur oxidizing *pseudomonas*

Sulphur-oxidizer medium for identification of S oxidizing *pseudomonas* contained (per liter) 10 g of Bacto-Peptone, 1.5 g of K_2HPO_4, 0.75 g of ferric ammonium citrate and 1.0 g of $Na_2S_2O_3.5H_2O$. The pH was adjusted to 8.0 using 1 M HCl before sterilizing by an autoclave. Bromo cresol purple was the indicator used. Agar was added to a final concentration of 15 g per liter. Isolation of sulphur-oxidizing bacteria was performed by using direct streaking on the sulphur-oxidizer medium.

Microbiological media for pH reduction by *pseudomonas*

The media employed for the pH reduction ability by fluorescent *pseudomonas* include Starkey broth (Starkey, Collins, 1923) composed of 3.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.5 g $(NH_4)_2SO_4$, traces of $FeSO_4$ in 1000 ml distilled water with pH 8.0; The thiosulphate broth contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl, 5g glucose in 1000 ml distilled water, with pH 8.0. Bromo cresol purple was the indicator used. The broth dispensed in tubes, under aseptic conditions. The broth dispensed in tubes, under aseptic conditions. The tubes were incubated incubator at 28° C for 7 days.

Sulphate ion production ability by *pseudomonas*

The thiosulphate broth dispensed in tubes, under aseptic conditions. The tubes were incubated in the incubator at 28°C for 7 days. The bacterial isolate which were found to able to reduce maximum pH and colour of the broth medium were further selected for their sulphate ion determination ability test.

Standards preparation for sulphate estimation

Potassium sulphate (K_2SO_4) was used as standard to construct a sulphate calibration curve according to Kolmert *et al*. Standard sulphate solutions were made by dissolving K_2SO_4 in deionized water to known concentrations in the range 0 to 3 mM. The amount of turbidity formed is proportional to the sulphate concentration.

Crude extract separation from production medium

The broth culture was centrifuged at 10,000 rpm for 15 minutes at 4˚C and supernatant was collected. The pellet was discarded that contained the bacterial cell fractions. The supernatant was collected and was further used for sulphate estimation.

Sulphate ion production ability

The amount of sulphate ion (SO_4^{2-}) produced during growth of sulphur-oxidizing bacteria on thiosulphate broth medium was determined spectrophotometrically. Sulphate was measured by adding 1:1 barium chloride solution (10% w/v) with bacterial culture supernatant followed by mixing the suspensions vigorously (Cha, Cha *et al.,* 1999). A resulting white turbidity due to barium sulphate formation was measured at 450 nm. The value obtained was compared with the sulphate standard curve.

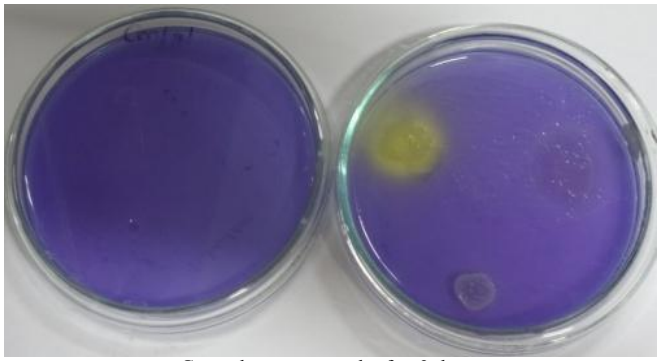
Optimization of growth conditions for efficient sulphur oxidation

Oxidation of sulphur was determined in thiosulphate medium broth at pH 8 and 28±2^{0}c temperature. The amendments of different sugars and variation in temperature were made to find out optimum conditions for efficient solubilization. Six best efficient sulphur oxidizing bacterial strains were used for these studies. For measuring the effect of carbon sources, rhizobacterial isolate was inoculated into 25 ml of modified thiosulphate medium broth in which glucose (15 mg/ml)was replaced with either of three different sugars i.e., galactose, xylose and arabinose, respectively. All the inoculated flasks were incubated at 28±2^{0}c for 5 days. The amount of sulphate ion in broths was estimated after incubation in comparison with a set of uninoculated controls. To determine the effect of incubation temperature, Thiosulphate medium broths were inoculated with six selected sulphur oxidizing bacterial strains. Cultures were incubated at different temperatures i.e., 25, 35 and 45^{0}c along with 30^{0}c for 4 days. After 5days of incubation, released sulphate was determined in a similar manner as previously described.

RESULTS AND DISCUSSION

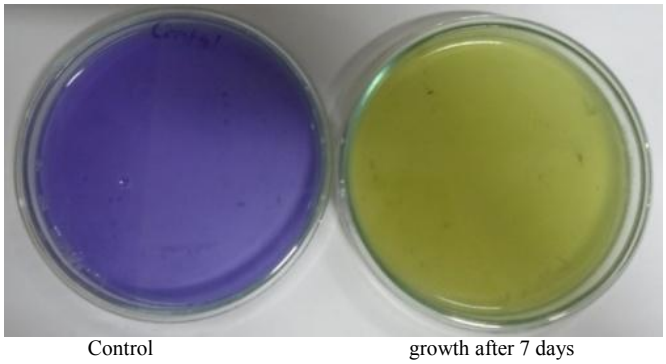
Isolation of Sulphur Oxidizing Bacteria

Total 55 fluorescent pseudomonads isolates were obtained from different rhizosphric samples of ground nut. Among them, 14 isolates were selected based on the better pH reduction ability on the bromo cresol purple containing sulphur oxidizing broth and agar medium by changing the colour of the media purple to yellow (Figure 1 and Figure 2).



Control growth after 3 days

Figure 1. Growth of Pseudomonas isolates on Sulphur oxidizing medium

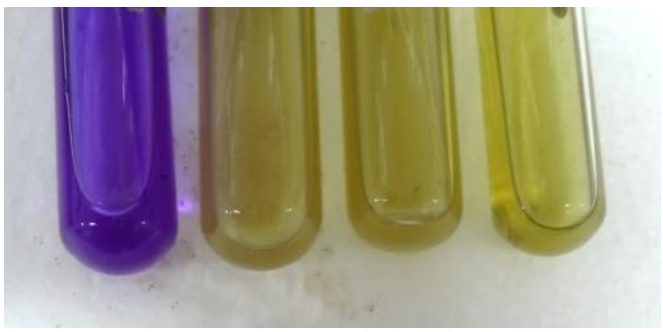


Control growth after 7 days

Figure 2. Growth of Pseudomonas isolates on Sulphur oxidizing medium

Control growth after 3 days

Figure 3. pH reduction in the growth media by Pseudomonas isolates



Control growth after 7 days

Figure 4. pH reduction in the growth media by Pseudomonas isolates

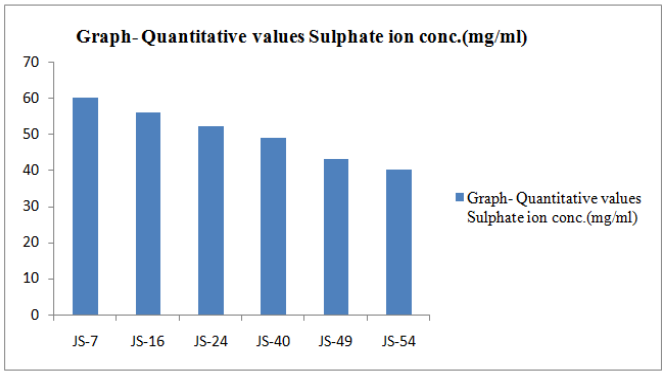
These bacteria were considered as efficient sulphur oxidizing bacteria and named as JS1. These isolates could reduce the pH up to 4.2 from the initial pH 8.0 of thiosulphate broth within 7 days of incubation. Reduction in pH of the growth medium was also reported by Donati *et al*. The pH reduction of the medium was due to the production of sulphuric acid. In a previous study, several thiosulphate oxidizing bacteria were isolated from the rhizosphere of crop plants and documented their thiosulphate oxidation path-way (Anandham *et al*., 2008b). Sulphur-based fertilizers decrease the pH of soil and, thus, increase the uptake of other plant nutrients, which contributes to increased yields. The results of our study are in agreement with that of Sullivan *et al*., Sutaliya *et al*., and El-Desuki *et al*. Smatanova *et al*.

Table 1. Reduction of pH in the growth media by sulphur oxidizing bacteria (SOB)

Name of bacterial isolate	pH Reduction in growth media	
	Starkey broth	Thiosulphate broth
JS-7	4.5	5.2
JS-16	4.7	5.5
JS-24	5.3	5.8
JS-40	5.7	6.2
JS-49	5.9	6.5
JS-54	6.3	6.8

Sulphate Ion Determination

The sulphate ion production ability of the bacterial isolates were in the following order, JS-7 > JS-16 > JS-24> JS-40> JS-49 and JS-54. It can be clearly from the graph that among the 6 no. of isolates JS-7 (60 mg/ml) showed maximum sulphate ion determination followed by the isolate JS-16 (56 mg/ml) and minimum sulphate ion was produced by the isolate JS-54 (45 mg/ml). Ravichandra *et al* reported the maximum sulphate ion production from 14-150 mg/ml by a *Thiobacillus spp.* The sulphate ion producing efficiency of the isolates revealed that all the *pseudomonas* significantly produce higher amount of sulphate ion from Na_2SO_3 supplied in the medium. Yesmin and Banerjee isolated S oxidizers among the rhizobacterial strains and that these bacteria were critical to meet plant S demand while utilizing elemental S.



Optimization of conditions for efficient sulphur oxidation

Six bacterial strains i.e., JS-7, JS-16, JS-24, JS-40, JS-49 and JS-54 were selected based on sulphate ion production. These bacterial strains were further tested for optimization of

conditions for efficient sulphur oxidation under varying conditions of carbon sources, temperature. In glucose amended medium broth, bacterial strains JS-7 and JS-16 caused 55.22 and 53.14 mg/ml of sulphur oxidation (Table 2). When glucose was replaced with other sugars, it was found that sulphur oxidation was comparatively less in galactose, xylose or arabinose amended broth with all the six bacterial strains. Among the three sugars tested, sulphur oxidation by bacterial cultures was more in case of galactose than xylose and arabinose. Maximum sulphur oxidation was observed with strain JS-7 in glucose amended broth where as bacterial isolates JS-16 and JS-24 showed significant sulphur oxidation with all the four sugars.

Table 2. Sulphur oxidation by fluorescent pseudomonads at different carbon sources (Mean Triplicate values)

Bacterial isolates	Glucose (mg/ml)	Galactose (mg/ml)	Xylose (mg/ml)	Arabinose (mg/ml)
JS-7	55.22	41.50	38.12	29.56
JS-16	53.14	40.46	34.58	26.18
JS-24	46.20	37.24	32.00	23.80
JS-40	41.46	34.28	29.16	21.48
JS-49	36.00	32.64	26.10	19.42
JS-54	34.12	29.32	23.38	17.28

Values represented the amount of Sulphate produced by fluorescent *Pseudomonas* in thiosulphate broth amended with different sugars.

Different temperatures were used for growth and sulphur oxidation by selected bacterial cultures and it was found that bacterial strain JS-7caused maximum solubilization (53.52 mg/ml) at 30^0c and sulphur oxidation by this strain decreased at higher temperatures of incubation (Table 3). Other bacterial strains showed significant solubilization in the temperature range of 25^0c to 35^0c. Sulphur oxidation decreased at higher temperature of incubation i.e., 45^0c with all the bacterial strains.

Table 3. Sulphur oxidation by fluorescent pseudomonads at different temperatures (Mean Triplicate values)

Bacterial isolates	25^0c (mg/ml)	30^0c (mg/ml)	35^0c (mg/ml)	45^0c (mg/ml)
JS-7	50.12	53.52	47.32	38.32
JS-16	49.10	52.44	45.58	35.56
JS-24	42.22	45.28	40.08	34.10
JS-40	36.46	39.20	32.16	25.48
JS-49	32.08	33.62	27.12	12.36
JS-54	26.14	29.36	24.36	19.22

Values represented the amount of Sulphate produced by fluorescent *Pseudomonas* in thiosulphate broth when incubated at different temperatures.

Banerjee and Yesmin reported S-oxidizing rhizobacteria was effective to enhance canola production. Whipps stated that the importance of plant growth promoting rhizobacteria (PGPR) was well reputable and the beneficial effects of PGPR may be linked to biocontrol. PGPR increases plant growth indirectly either by the suppression of diseases caused by major pathogens or by reducing the deleterious effects of pathogen. These isolates antagonist to pathogenic fungi and ability to produce plant growth promotion by IAA, Ammonium and salicylic acid which gives systemic resistance.(Nirmala Jyothi Lukkani, Surendranatha Reddy. EC.). Soil microorganisms, like *Azospirillum* sp. (W. Zimmer and H. Bothe), *Enterobacter* sp., *Azotobacter* sp. and *Pseudomonas* sp. (A. Gamliel and J. Katan), have shown to promote plant growth (R. Arteca) by promoting the incidence of secondary roots, acting as protectors against pathogenic microorganisms by release of plant hormones and siderophores (B. Amstron, A. Gustafsson).

Conclusion

The present study emphasizes the importance and the role of sulphur oxidizing bacteria in the oxidation of sulphur in soil. These pseudomonads isolates can be incorporated to enhance sulphur oxidation in soil and to increase soil available sulphate. Also, the pH reducing property of sulphur oxidizing bacteria by the assembly of sulphuric acid can be utilized for reclamation of alkali soils. These results suggested that efficient sulphur oxidizing; plant growth promoting bacterial strains could be further exploited for plant growth improvement under field conditions.

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