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Characterization and Optimization of Culture Conditions for Sulphur Oxidizing Bacteria after Isolation from Rhizospheric Mustard Soil, Decomposing Sites and Pit House

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Abstract**—**Sulphur oxidizing bacteria (SOB) have marked their significant role in perspectives of maintaining healthy environment as researchers from all over the world tested and apply these in waste water treatment plants, bioleaching of heavy metals, deterioration of bridge structures, concrete and for bioremediation purposes, etc. Also, these SOB are well adapted in all kinds of environment ranging from normal soil, water habitats to extreme natural sources like geothermal areas, volcanic eruptions, black shale and acid rock drainage (ARD). SOB have been isolated from low pH environment of anthropogenic origin like acid mine drainage (AMD) and bioleaching heaps, hence these can work efficiently in different environmental conditions. Besides having many applications in field of environment science, they may be proven to be very beneficial in area of agriculture as sulphur is the fourth major macronutrients required for the growth of plants. More amount of sulphur is needed by pulses and oilseed crops with respect to the cereal grains. Due to continuous use of land for overproduction of more demanding sulphur utilizing crops and without application of sulphur fertilizers, its concentration is decreasing day by day, and thus, sulphur deficiency is becoming a great problem as it affects the crop productivity and quality. Sulphur is generally found in soils in many forms which are unavailable for plants (cannot be use by plants) like elemental sulphur, thiosulphate which can be taken up by bacteria and converted into simpler forms usable by plants by undergoing a series of transformations. So, keeping the importance of sulphur in view for various soil types, oilseed crops and role of microorganisms in making them available to plants, we made an effort to isolate, optimize, and characterize SOB. Three potential strains of bacteria were isolated, namely SSF7, SSA21, and SSS6, showing sulphate production of concentration, i.e. 2.268, 3.102, and 2.785 mM, respectively. Also, these were optimized for various culture conditions like carbon, nitrogen source, pH, temperature, and incubation time, and characterization was also done.

Keywords**—**Sulphur oxidizing bacteria, isolation, optimization, characterization, sulphate production.

I. Introduction

SULPHUR is vital element for plant and animals. It is a major inorganic element which is necessary for the whole biological kingdoms because it is an important constituent of three amino acids, viz. methionine (21% S), cysteine (26% S), and cystine (27% S), which are main building block of proteins, many enzymes, vitamins, lipids, carbohydrates, and other biomolecules. Sulphur (S) has been recognized as the fourth major nutrient after nitrogen (N), phosphorus (P), and potassium (K) [1]. Sulphur is also responsible for the formation of chlorophyll, glucosides, and glucosinolates (mustard oils), sulfhydryl (SH−), and activation of enzymes that gives pungency to the onion and oils [2]. In the present era of population explosion worldwide, we are pressurized to apply more and more chemical fertilizers to soil to improve yield and quality of crop so that the continuously increasing demand of cereals, oilseeds, and pulses can be fulfilled. Sulphur fertilizer is especially important in oilseed crops because these crops require more sulphur than cereal grains. Generally, oilseed crops need about the same amount of sulphur as, or even sometimes more than phosphorus for the fulfilment of demanding amount and proper quality of product [3]. Due to overproduction and application, in intensive crop rotations especially in case of oil crops, more sulphur is used by plants, and the situation becomes alarming when we take away the crop remainings along with the product from the field. This results into deficiency of sulphur in soil and poor soil health, if the taken-away sulphur is not recovered through fertilizer. Also nowadays, researchers are becoming aware of this problem, and the necessity of sulphur in field of agriculture is recognized as its role in production and yield is very important [4]-[9]. The deficiencies of sulphur in soils of tropical and subtropical regions have been recognized for many years, and more than 70 countries including India have sulphur deficient soils [10]. Out of 142 million hectares of arable land in India, at least 57 million hectares (about 41%) of total suffer from various degrees of sulphur deficiency [11].

Sulphur undergoes a series of biological conversions before it gets fixed in organic forms, which are being carried out exclusively by microorganisms. The vast majority of sulfur is consumed by plant roots is as sulfate, a more oxidized form that can be easily uptaken by plants [12]. SOB have been isolated from various habitats like rhizosphere of paddy field, pulses rhizosphere, biogas slurry, sewage, tannery effluent and mine soil [13], AMD and black shale samples [14], from degrading bridge structures [15]. Many of the identified SOB belong to genera *Thiobacillus, Thiothrix, Thiomicrospira Beggiatoa,* and *Achromatiu* [16]. However, this process of sulphur oxidation is not only limited to the pure SOB, but this process also has been reported in many heterotrophic bacteria, which were found in soil and marine environment. Most of the heterotrophic bacteria belonging to the genera *Pseudomonas,*

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Xanthobacter and *Escherichia coli* strains are involved in sulphur oxidation with the continuous use of sulphur-free fertilizers and lesser amount of organic manures. The sulphur deficiency has appeared in many parts of Haryana, India [11], and SOB have the potential to solve the problem of sulphur deficiency. Keeping this in vision, the present investigation was carried out.

II. Materials and Methods

A. Collection of Soil Samples

The soil samples were collected from rhizospheric soils of mustard crop from various fields of Ludhiana (Punjab), Rewari, Gurgaon, Hisar, Karnal, Kurukshetra, Kiathal, Fatehbad, Bhiwani, Dadri, Sirsa of Haryana (India), decomposing sites, pit house (Deptt. Of Microbiology CCSHAU, Hisar) and municipal waste dumping site of Hisar, India.

B. Isolation of SOB

Bacteria were isolated by enrichment culture technique using Starkey broth [16] and modified Thiosulphate broth [17]. One litre of Starkey broth contained 3.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 0.5 g $(NH_4)_2SO_4$, traces of $FeSO_4$, 10 g Elemental sulphur, and pH was adjusted to 8. Thiosulphate broth contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, and 0.1 g NH_4Cl, 5.0 g Glucose per liter, and pH was adjusted to 8. 10 g of soil sample was added to 90 ml of the sterilized broth and was kept in shaker for enrichment for 15 days. Elemental sulphur at 10 g per litre was added to Starkey broth and was half-an hour steam sterilized for three consecutive days. 1 ml of the enriched culture broth was added to 9 ml of fresh broth and incubated at 30 ºC for 15 days. 1 ml of the serially diluted culture broth was taken and spreaded on sterilized thiosulphate agar plates. The plates were incubated at 30 ºC for three days. Isolated colonies were picked and then streaked on fresh thiosulphate agar plates and purified by streaking further on fresh medium plate. Then, they were streaked on thiosulphate agar medium, and individual colonies were picked and preserved on modified sodium thiosulphate slants [18].

C. Screening of Isolate by Dye Reduction Test

The screening of obtained isolates was done by inoculating on thiosulphate agar medium containing bromo cresol purple dye (0.004 gm per litre) of the media. The isolates were screened on the basis of ability to reduce the color of the dye from purple to yellow.

D. Sulphate Production Ability

The amount of sulphate ion (SO_4^{-2}) produced during growth of SOB on thiosulphate broth medium was determined spectrophotometrically. A loopful of 48 hrs. old culture of each isolate was inoculated into 10 ml of thiosulphate broth. All the inoculated tubes were incubated at 30 ºC for seven days. After seven days of incubation, the broths were centrifuged at 15000 rpm for 10 minutes to separate the supernatant from the cell growth. Sulphate production measured by pouring 1:1 barium chloride solution (10%, w/v) into isolates supernatant, and resulting suspensions were mixed properly. A resulting white turbidity due to barium sulphate formation was measured at 450 nm. The values obtained were compared with the sulphate standard curve which was plotted by using Potassium sulphate (K_2SO4) as standard. Sulphate solutions for standard were made by mixing potassium sulphate in deionized water of known concentrations in the range 0 to 3 mM, and then, the amount of turbidity formed was measured which is directly proportional to the concentration sulphate produced.

E. Optimization of Culture Conditions for Sulphate Production

Culture conditions for standardizing the isolation procedure for SOB were optimized for sulphate production with respect to the many affecting factors:

a. Temperature optimization- Isolates were incubated at different temperatures, i.e. at 20 ^oC, 25 ^oC, 30 ^oC, 35 ^oC, and 40 ^oC respectively and temperature, at which best growth were there, observed.
b. Optimization of pH – The same way thiosulphate medium of different pH, i.e. 4, 5, 6, 7, 8, and 9 consecutively was prepared, and optimum pH was noted down.
c. Incubation Period – Bacterial cultures were incubated at different time intervals of 1, 2, 3, 7, and 8 days and optimized by measuring maximum sulphate production.
d. Different Carbon source supplementations: The medium was replaced with sucrose, and mannitol carbon sources instead of glucose in the default one and sulphate production with these were measured.
e. Nitrogen source supplementations- As the same way in carbon source, three different nitrogen sources, i.e. ammonium sulphate, urea, and potassium nitrate in place of ammonium chloride were taken, and the best one was figured out.

F. Identification of Isolates

The bacterial cultures were characterized on the basis of Bergey's Manual of Determinative Bacteriology for various morphological, biochemical and physiological characteristics as per procedure described in manual. The parameters investigated include Gram's reaction, colony characteristics, their shape, colour and shape of cells, cell arrangement, Indole production, Methyl red test, Citrate utilization test, Voges-Proskauer reaction, Oxidase test, Catalase production, Acid production, H_2S production, Cellulose hydrolysis, and Starch hydrolysis.

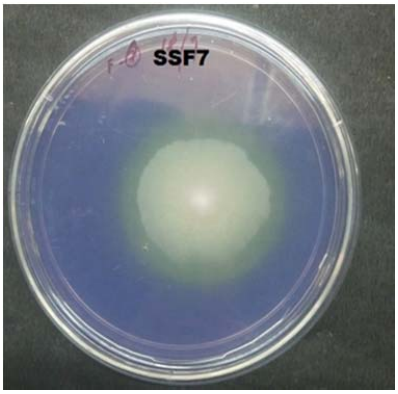
III. Results and Discussion

A. Isolation and Screening of SOB

A total of 30 mutually distinct bacterial isolates, which were isolated from soil samples collected from various sites, were screened for bromo cresol purple dye reduction test using Thiosulphate agar medium plates and broth containing bromo cresol purple dye and incubated at 30 ºC for 15 days. By observing their zone of clearance (Fig. 1), all the screened

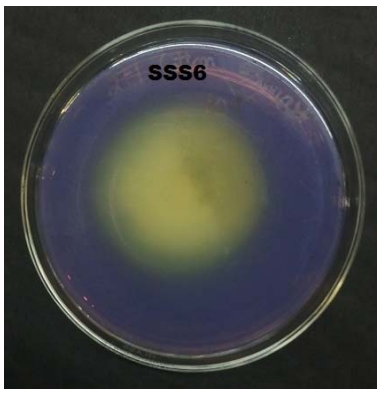
bacterial isolates were inoculated in thiosulphate broth containing bromo cresol purple dye. The bacterial isolates, which showed positive dye reduction on thiosulphate agar plates, showed positive test in broth also and reduced the colour of the dye.

In a similar study, SOB have been isolated from bioleaching pulp of zinc and copper concentrates [19]. In another study [20], there has also reported screening of SOBl isolates isolated from mangrove soil of Mahanadi River, on the basis of bromo phenol dye reduction test and pH reduction due to sulphuric acid production.



(a)

(b)



(c)

Fig. 1 Dye reduction test by bacteria in thiosulphate agar plate a) SSF7, b) SSA21 AND c) SSS6

B. Sulphate ion Determination

Selected bacterial isolates which were screened by dye reduction test were further tested using barium chloride test. Bacterial isolate SSF7, SSA21 and SSS6 showed maximum sulphate production, 2.268, 3.102, and 2.785 mM, respectively. These three bacterial isolates were considered as promising bacterial isolates and used for further studies.

C. Optimization of Culture Conditions for Sulphate Production

Three bacterial isolates SSF7, SSA21, and SSS6 were used for optimization of conditions (Table I) for sulphate production using different carbon sources, nitrogen sources, pH, incubation time and temperature. Carbon sources such as glucose, sucrose, and mannitol were amended in the broth. All three isolates showed highest sulphate production with glucose followed by sucrose and mannitol. The bacterial isolates produced significant amount of sulphate with all four nitrogen sources taken; urea, ammonium sulphate, ammonium chloride, and potassium nitrate. Maximum sulphate production was with ammonium chloride.

TABLE I
STANDREDIZATION OF CULTURE CONDITIONS FOR SULPHATE PRODUCTION

ANALYSIS	Sulphate reduction		
	SSF7	SSA21	SSS6
Carbon sources			
Glucose	3.379	3.267	4.576
Sucrose	1.021	1.030	1.854
Mannitol	1.040	1.127	1.674
Nitrogen sources			
Ammonium chloride	3.367	3.247	4.289
Ammonium sulphate	1.303	1.292	2.867
Urea	2.484	2.417	3.848
Potassium nitrate	1.504	1.661	2.001
pH			
4	3.39	3.84	4.455
5	3.90	3.70	4.967
6	4.24	3.96	4.567
7	4.01	3.43	3.987
8	3.52	3.30	3.781
9	3.16	3.12	3.67
Incubation temperature			
20ºc	2.040	0.781	3.903
25ºc	3.999	3.847	4.434
35ºc	2.146	3.398	3.377
45ºc	3.099	3.050	3.011
Incubation time			
1day	0.52	0.491	0.701
2 days	0.89	0.750	0.976
3 days	1.20	0.935	1.045
7 days	4.399	3.871	4.897
8 days	4.222	3.781	4.333

To determine the effect of pH on sulphate production, the selected bacterial isolates were grown under different pH conditions ranging from 4 to 9, and the maximum sulphate production by bacterial isolate SSF7 and SSA21 was at pH 6, and that of SSS6 was at pH 5. In a similar study, [21] they

reported that the sulphur oxidation rate of bacterial isolate *Thiobacillus* sp. ASWW-2 increased with increasing pH values from 2 to 4, but it decreased with increasing pH value in the range of pH 5 to 8. The maximum sulphur oxidation rate, 0.61 g-S_2O_3/L.d, was obtained at pH 4. Different incubation temperatures 25 to 45 ºC for the growth of bacterial isolates were selected, and 25 ºC temperature was found to be optimum temperature. Incubation period of seven days showed maximum sulphate production by measuring activity at 1, 2, 3, 7, and 8 days of incubation.

TABLE II
CHARACTERIZATION BY MORPHOLOGICAL AND BIOCHEMICAL ANALYSIS

Characters	Isolates		
	SSF7	SSA21	SSS6
Morphological analysis			
Gram's stain	+	-	-
Colony shape	flattened	flattened	dome shaped
Colony colour	white	creamy	creamy
Shape	rods	Slightly curved rods	rods
Biochemical analysis			
Oxidase	+	+	+
Oxidase	+	+	+
Indole	-	-	-
Catalase	-	+	+
Acid production	–	–	
H_2S production	-	-	-
MR-VP	-	-	-
Cellulose hydrolysis			
Starch hydrolysis	+	+	+

D. Sulphide Oxidase Activity

It was found that the sulphide oxidase activity of the bacterial isolate SSF7, SSA21, and SSS6 were 33.2, 30.7, and 28.5 U/ml, respectively. In a similar study, [22] it has been reported that out of 60 isolates, 11 strains produced sulphide oxidase and sulphide oxidase activity of the promising strain SO02 was 6.83 U/ml, and the strain also showed maximum specific activity of 2.34 U/mg.

E. Characterization of Selected Isolates

The promising bacterial isolates were subjected to various morphological and biochemical characterization described in Table II, with a view to identify them. Bacterial isolates were grown at 28±2 °C for 24h on medium slants. For the identification of these isolates, their morphological, physiological and biochemical characters were studied as per procedures described in Bergey's Manual of Determinative Bacteriology [23]. Bacterial isolates SSF7 was found to be Gram positive having white colony colour, cells were thick rod shaped and were scattered in arrangement whereas; isolate SSA21 and SSS6 were Gram negative creamy colour colony, and cells were scattered. On the basis of the morphological and biochemical characters, it was concluded that bacterial isolates SSF7, SSA21, and SSS6 probably belong to the genus *Xanthobacter*, *Pseudomonas* and *Pseudomonas*, respectively.

IV. CONCLUSION

The present study reveals the importance of sulphur oxidising bacteria. The *Xanthobacter* and *Pseudomonas* isolates can be used as bioinoculants to enhance sulphur oxidation in soils which will in turn increase the amount of available phosphate in soil. As a result, the requirement of sulphur fertilizers will be reduced. Also, the pH reducing property of sulphur oxidising bacteria can be utilized for reclamation of alkali soil.

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