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Isolation and Characterisation of Sulphur Oxidising Bacteria from Mangrove Soil of Mahanadi River Delta and Their Sulphur Oxidising Ability

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Abstract This study was conducted to isolate sulphur oxidising bacteria from mangrove soil of Mahanadi river delta, Odisha, India and evaluate their sulphur oxidation ability. Results showed that in total thirty sulphur oxidising bacteria were isolated from six different location of mangrove soil. From the qualitative screening it was found that out of the thirty bacterial isolates, twelve isolates could efficiently reduce the pH of the medium upto 4.2 from the initial pH 8.0. Their sulphate ion production abilities were in the range of 125 mg/ml- 245 mg/ml. Their sulphur oxidase activities were in the range of 11.6 to 126.83 U/ ml/ min. From morphological and biochemical characterisation, most of the isolates were identified as *Micrococcus spp., Bacillus spp., Pseudomonas spp.* and *Klebsiella spp.*

Keywords: *mangrove forest, sulphur oxidising bacteria, sulphate ion, biochemical characterisation*

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1. Introduction

Mangrove is a tropical coastal biome, located in the transition zone between land and sea, where the vegetation is dominated by a particular group of plant species [1]. This ecosystem is characterized by periodic tidal flooding which makes environmental factors such as salinity and nutrient availability highly variable, resulting in unique and specific characteristics [2]. Mangrove soils are sulphidic and variable, since their chemistry is regulated by a variety of factors such as texture, tidal range and elevation, redox state, bioturbation intensity, forest type, temperature and rainfall [3].

Bacteria are the major participants in the carbon, sulfur, nitrogen and phosphorous cycles in mangrove forest [4]. In this anoxic mud of marine estuaries and coastal sediments the anaerobic, heterotrophic metabolism of sulfate-reducing bacteria is responsible for most of the production of hydrogen sulfide (H_2S). Sulphate reducing bacteria use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide. Subsequently, the sulphide can be oxidized by sulphur oxidising bacteria to produce sulphate. [5]. As the original source of reduced sulfur compounds, H_2S hence, supports abundant populations of sulfur-oxidizing bacteria at the oxic-anoxic interface [6].

Sulphur is now considered the fourth major plant nutrient after N, P and K, and is one of the sixteen nutrient elements which are essential for the growth and development of plants, especially in the agricultural crop production [7]. The majority of sulphur taken up by plant roots is in the form of sulphate (SO_4), which undergoes a series of transformations prior to its incorporation into the original compounds [8]. The soil microbial biomass is the key driving force behind all sulphur transformation. Beside their important contribution in agriculture these microbes also play significant role in removal of toxic H_2S from the environment. Most of the known sulphur oxidising bacteria (SOB) belongs to the genera *Thiobacillus, Thiothrix, Thiomicrospira, Achromatium* and *Desulfuromonas* [9]. However, oxidation of sulfur compounds is not restricted to the true sulfur bacteria; this process also occurs in heterotrophic bacteria isolated from soil and marine environment [9]. *Thiobacilli* are reported not to be present in significant numbers in most agricultural soils [10,11]. In this context, Chapman [10] presumed that aerobic heterotrophic S-oxidizing bacteria were more important S^0 oxidizers than *Thiobacilli* in Scottish soils. It was reported that both *Thiobacillus* spp. and aerobic heterotrophic S-oxidizing bacteria oxidized reduced S^0 intermediate compounds, for instance thiosulfate and the intermediate compounds consequently were oxidized to sulfate [12]. Most of the heterotrophic bacteria belong to the genera *Pseudomonas* [13],

Xanthobacter [14], *Escherichia coli* strains [15] are mostly involve in sulphur oxidation.

A phylogenetic and functional description of sulphur oxidising bacterial diversity in the mangrove ecosystem has not been addressed to the same extent as that of other environments. To date, only a few obligately heterotrophic bacteria have been studied in detail and adequately described that are able to generate metabolically useful energy from the oxidation of reduced sulfur compounds. A more thorough description of the sulphur oxidising bacterial diversity and distribution in a mangrove would improve our understanding of sulphur geochemistry as well as microbial metabolism of suphur in that ecosystem. Keeping the above in vision the present investigation is aimed to isolate, characterize and estimate the sulphur oxidising ability of sulphur oxidizing bacteria from mangrove soil of Mahanadi river delta, Odisha, India.

2. Ease of Use

2.1. Soil Sample Collection

The soil samples were collected from different location of mangrove forest such as Jumbo, Kharnasi, Triveni, Nuagada, Atharabanki and Mangrove forest at Indian Farmer fertilisers Corporation (IFFCO). Top layer of soil (about 1 cm) was removed. In each site soil samples were collected from five different spots. Samples were mixed thoroughly and put in sterile polythene bags with proper labelling, stored in ice box and brought to the laboratory for further analysis. In the laboratory, the samples were stored at 4 ± 0.1°C in a refrigerator. For each soil sample, several sub-samples were taken, homogenized in sterile Milliq water containing 0.85% NaCl (w/v) and serially diluted and poured on sulphur oxidising agar plate medium.

2.2. Media for Isolation of Sulphur Oxidising Bacteria

Sulfur-oxidizer medium for isolation of SOB contained (per liter) [16] 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 7.0 using 1 M HCl before sterilizing by an autoclave. Agar was added to a final concentration of 15 g per liter. Isolation of sulphur-oxidizing bacteria was performed by using direct plating method. The serially dilluted sample (0.1 mL) were poured onto the sulfur-oxidizer agar medium and incubated at 30°C for 24 h. The well defined isolated colonies appeared on the plate were picked up by wire loop and streaked on the sulfur-oxidizer medium agar plate for purity conformation. For qualitative screening, the isolated bacteria from agar plate were further grown on the thiosulphate agar and broth [17] contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl in 1000ml distilled water, with pH 8.0. 0.0025 g of Bromo phenol blue (BPB) was used as an indicator [7]. The bacterial isolates in the broth and agar culture were incubated at30°C up to 264 h. pH of the broth inoculated medium were measured at 24 h interval. 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.

2.3. Sulphate Ion Production Ability

The amount of sulfate ion ($SO4^{2-}$) produced during growth of sulfur-oxidizing bacteria on thiosulfate broth medium was determined spectrophotometrically. Sulfate was measured by adding 1:1 barium chloride solution (10% w/v) with bacterial culture supernatant followed by mixing the suspensions vigorously [18]. A resulting white turbidity due to barium sulfate formation was measured at 450 nm with a systronics-119 spectrophotometer. The value obtained was compared with the sulphate standard curve. Potassium sulfate (K2SO4) was used as standard to construct a sulfate calibration curve according to Kolmert et al. [19]. Standard sulfate solutions were made by dissolving K2SO4 in deionized water to known concentrations in the range 0 to 3 mM. The amount of turbidity formed is proportional to the sulfate concentration.

2.4. Identification of the Isolates

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterisation. The parameters investigated included colony characteristics, shape, size, spore, motility, Gram's reaction, catalase production, urease production, Voges-Proskauer (V-P) reaction, Indole production, Nitrate reduction, citrate utilization, carbohydrate metabolism(acid-gas production), starch hydrolysis, Tributyrin (or vegetable oil) hydrolysis, Tween-80 hydrolysis, Cholesterol hydrolysis, gelatin hydrolysis, Casein hydrolysis, Growth at different pH and Temperature, Pectin hydrolysis and chitin hydrolysis test were carried out following the standard methods described in Bergey's Manual of Determinative Bacteria [20].

2.5. Statistical Analysis

Statistical analysis was performed by SPSS, version 10 for windows (SPSS Inc; Chicago, IL, USA).

3. Results and Discussion

3.1. Isolation of Sulphur Oxidising Bacteria

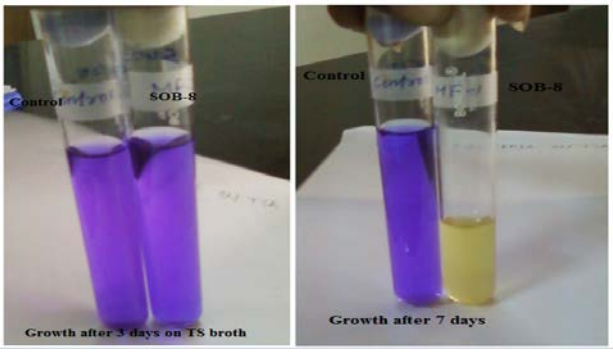

Figure 1. Screening of sulphur oxidising bacteria on thiosulphate broth supplied with bromophenol blue as an indicator

In total thirty isolates were obtained from different samples of the sulphur oxidising medium plate. Among them, 12 isolates were selected based on the better pH reduction ability on the bromophenol blue containing sulphur oxidising broth and agar medium by changing the

colour of the media purple to colourless (Figure 1 & Figure 2). These bacteria were considered as efficient sulphur oxidising bacteria and named as SOB 1-12. The sulphur oxidising bacterial isolates obtained from the mangrove soil could reduce the pH up to 4.3 to 4.2 from the initial pH 8.0 of thiosulphate broth within 11 days of incubation. Reduction in pH of the growth medium by sulphur oxidizing bacteria was also reported by Donati et al. [21]. The pH reduction of the medium was due to the production of sulphuric acid.

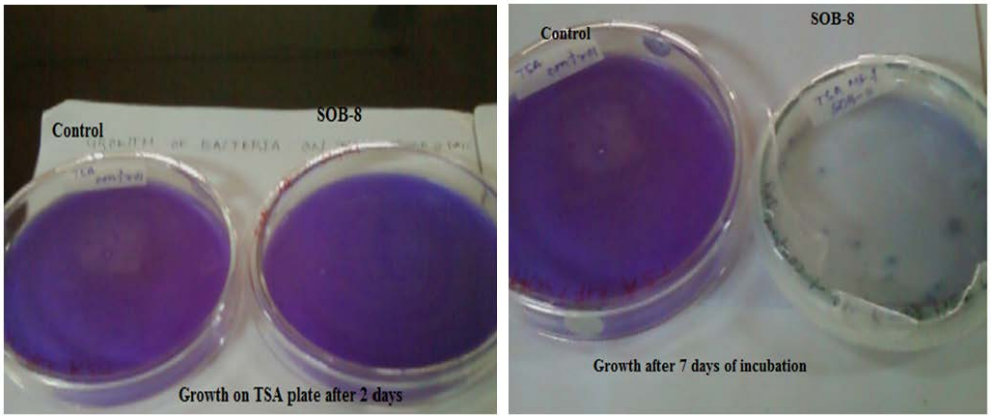

Figure 2. screening of sulphur oxidising bacteria on thiosulphate agar medium

Though this is the first report of isolation of sulphur oxidising bacteria from mangrove soil of Mahanadi river delta but earlier studies on isolation of sulphur oxidizing bacteria by various researchers also reveal their existence from various mangrove ecosystem [22,23,24].

3.2. Sulphate Ion Determination

The sulphate ion production ability of the bacterial isolates were in the following order, SOB-7 (245 mg/ml) < SOB-8(240 mg/ml) < SOB-5 and SOB-11(220 mg/ml)< SOB-12(205 mg/ml) < SOB-3 and SOB-10 (200 mg/ml) < SOB-9(188 mg/ml) < SOB-6 (185 mg/ml) < SOB-2 (184 mg/ml) < SOB-1(150 mg/ml) < SOB-4(125 mg/ml). It can be clearly seen from Figure 3 that among the 12 no. of isolates SOB-7 (245 mg/ml) showed maximum sulphate ion determination followed by the isolate SOB-8(240 mg/ml) and minimum sulphate ion was produced by the isolate SOB-4 (125 mg/ml). Ravichandra et al. [25] reported the maximum sufate ion production from 14-150mg/ml by a *Thiobacillus spp.* Similarly Babana et al. [26] reported the highest sulphuric acid concentration (243mg/l) by a strain ATTC55128 followed by (230 mg/l) by another strain, AHB436.The sulphate ion producing efficiency of the twelve isolates revealed that all the SOB significantly ($P \leq 0.01$) produce higher amount sulphate ion from Na_2SO_3 supplied in the medium.

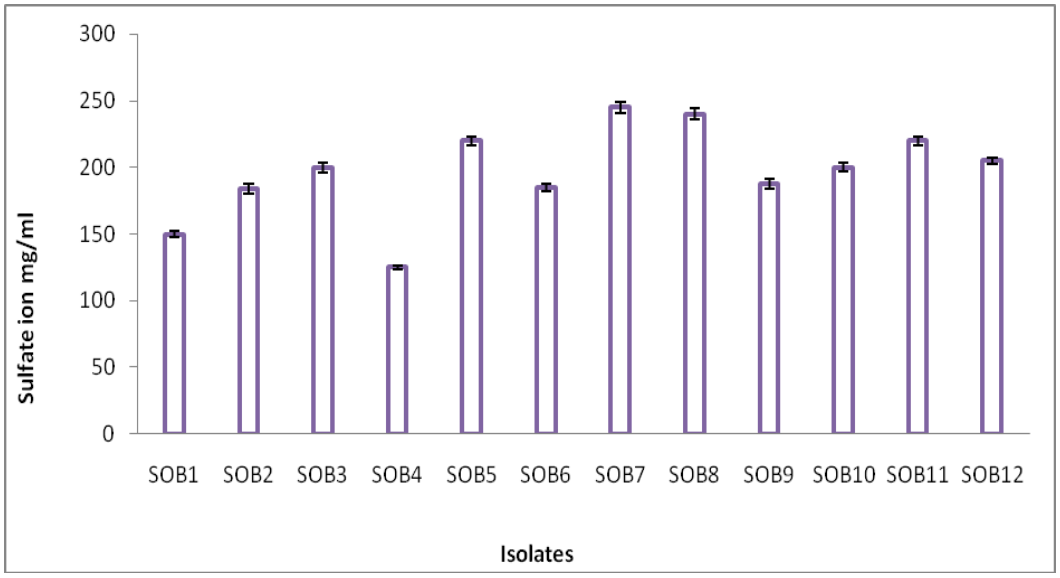

Figure 3. sulphate ion production by twelve sulphur oxidising bacterial isolates

3.3. Sulphur Oxidase Activity

Bacterial isolates were inoculated in the production medium to screen their sulphur oxidase production ability. Among the 12 isolates SOB-1produced (112.64 U/ml), SOB-2,(117.5 U/ml), SOB-3, (116.16 U/ ml), SOB-4 (78.16 U/ ml), SOB-5 (73.5 U/ ml), SOB-6 (105 U/ ml), SOB-7, (126 U/ ml), SOB-8 (126.83 U/ ml), SOB-9 (93.3 U/ml), SOB-10 (100.33 U/ml), SOB-11 (82.83 U/ml) and SOB-12 (108.5 U/ml) of enzyme activity (Figure 4). SOB-8 found to be highest sulphur oxidase producer. In general sulphur oxidase production abilities of all the isolates were within a range of 11.6 to 126.83 U/ ml/ min. Rohwerdert and Sand [27] reported sulphur dioxygenase activity of 5.0±1.7 nmol min^{-1} $mg^{-}1$ – 373 ± 90 nmol min^{-1} mg $^{-}1$ by *Acidithiobacillus* and *Acidiphilium spp.* Similarly Nakada

and Ohta [28] reported the crude sulphur oxidase extract activity of 11.7 units by *Bacillus spp.* Crude extract of thiosulphate oxidase from *P. aeruginosa* showed maximum activity of 130 U/ml, was also reported earlier by Schook and Berk [29].

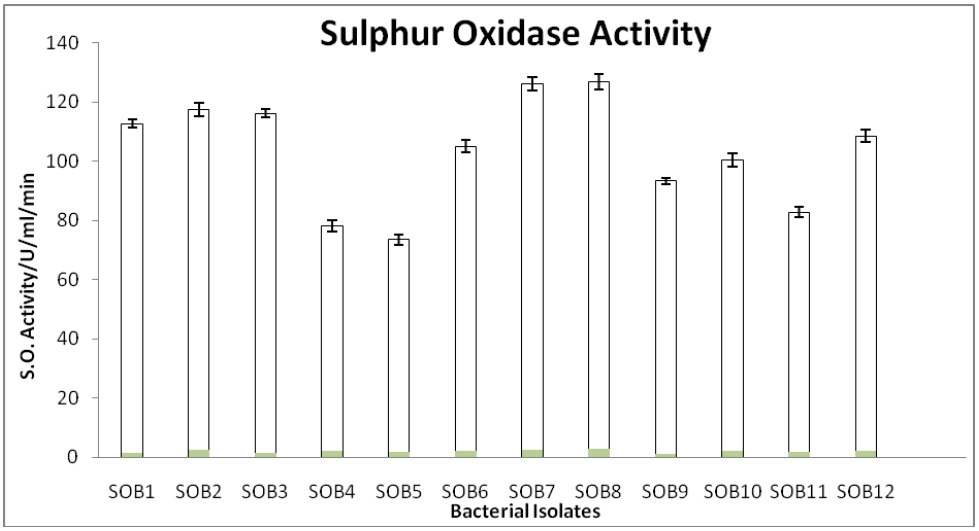

Figure 4. Sulphur oxidase activity of twelve sulphur oxidising bacteria

Table 1. Biochemical identification of Sulphur oxidising bacteria. SOB= Sulphur oxidising bacteria, ND= Not Detected

Characters	SOB1	SOB2	SOB3	SOB4	SOB5	SOB6	SOB7	SOB8	SOB9	SOB1	SOB1	SOB1
Shape	cocci	Rod	Rod	Rod	Rod	Rod	Rod	cocci	Rod	Rod	Rod	Rod
Cell	0.7-2.3	0.76-	0.9-	0.7-0.8	0.8-1.1	1.0-	1.3-1.5	0.7-2.3	0.8-	1.55-	1.55-	0.8-0.9
Spore	-	-	-	-	-	-	-	-	-	+	+	-
Motile	-	+	+	+	+	+	-	-	+	-	-	+
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	-	+	-	-	-	+	+	-	+	+	-
Gram stain	+	+	-	-	-	+	-	+	+	+	+	-
PHB	-	-	-	-	+	-	-	-	-	-	-	+
Catalase	+	-	+	+	+	+	-	+	+	+	+	+
MR	-	+	+	-	-	+	-	-	-	-	-	-
VP	-	+	-	-	-	+	+	-	-	-	-	-
Citrate	-	+	+	+	+	+	+	-	+	+	+	-
Protease:												
Gelatin	-	+	-	-	+	+	-	-	+	-	-	-
Casein	-	+	-	-	+	+	-	-	+	-	-	-
Lipase:												
Tributyrin	-	+	-	+	+	-	-	-	-	-	-	+
Tween 80	-	+	-	+	+	+	-	-	-	-	-	+
Lecithinase egg	-	-	+	-	-	-	-	-	-	+	+	-
Chitinase		+	-	-	-	+	-		-	-	-	-
Argine	-	+	+	-	-	+	-	-	+	-	-	+
Strach hydrolysis	-	-	+	+	-	+	-	-	+	-	-	-
Oxidase	+	+	-	+	+	+	-	+	+	+	+	+
Nitrate reduction	-	-	-	+	+	+	+	-	-	+	+	-
Acid from:												
Glucose	-	+	+	+	+	+	+	-	+	-	-	+
Fructose	-	+	+	+	+	+	+	-	-	-	-	+
Mannose	-	+	+	+	+	+	+	-	-	-	-	+
Gas production												
Glucose	-	-	-	+	+	-	+	-	-	-	-	-
Indole	-	-	-	-	-	-	+	-	-	-	-	-
Growth at pH 5-	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 40°C	+	+	+	+	+	+	+	+	+	+	+	+

3.4. Identification of the Isolates

These twelve selected bacterial isolates subjected to various morphological and biochemical characterisation described in Table 1, with a view to identify them. The isolates were undulate, convex, and circular having gummy and sticky consistency. Microscopic observation of the isolates revealed that most of them are rod shaped, motile. The isolates were found variable towards Gram's stain. Based on various morphological and biochemical tests the isolates were identified as *Micrococcus spp.*

(SOB-1), *Bacillus pumilus* (SOB-2), *Pseudomonas spp.* (SOB-3), *Pseudomonas spp.* (SOB-4), *Pseudomonas spp.* (SOB-5), *Bacillus subtilis* (SOB-6), *Klebsiella spp.* (SOB-7), *Micrococcus spp.* (SOB-8), *Bacillus megaterium* (SOB-9), *Bacillus spp.* (SOB-10), *Bacillus spp.* (SOB-11) and *Pseudomonas spp.* (SOB-12). Thatoi et al. [22] reported the occurance of *Pseudomonas spp.* that oxidising sulphur in the mangrove of Bhitarakanika, Odisha, India. Most of the heterotrophic bacteria, involve in sulphur oxidation belong to the genera *Pseudomonas* [13], *Xanthobacter* [14], *Escherichia coli* strains [15] were also reported earlier. Sulphur oxidising activity of *Micrococcus spp.* and *Bacillus spp.* were also well reported earlier from Indian Terai soil- a Himalayan foot hill soil of the order mollisol [30].

4. Conclusion

The present study emphasizes the importance and the role of sulphur oxidizing bacteria in the oxidation of sulphur in soil. It was concluded from the present study that all the tweleve bacterial isolates decrease the pH of the culture medium and efficiently produce sulphate ion in the medium. The pH reducing property of sulphur oxidizing bacteria by the production of acid can be utilized for reclamation of alkali soils. Use of these SOB as bio-inoculants can be incorporated to enhance sulphur oxidation in soil and to increase soil available sulphate to minimize the S-fertilizer application. They can also be apply to reduce environmental pollution and promotes sustainable agriculture.

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