Partial Purification and Characterisation of Sulphur Oxidase from *Micrococcus* sp. and *Klebsiella* sp. Isolated from Mangrove Soils of Mahanadi River Delta, Odisha, India

B. C. Behera¹, S. K. Singh², M. Patra³, R. R. Mishra³, B. K. Sethi³, S. K. Dutta⁴, H. N. Thatoi^{1,*}

¹Department of Biotechnology, North Orissa University, India

²Department of Biotechnology, Pondicherry Central University, India

³Department of Biotechnology, MITS School of Biotechnology, India

⁴Department of Zoology, North Orissa University, India

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Abstract As knowledge of sulphur oxidising bacterial communities in mangrove sediments is very sparse and sulphur oxidizers have wide application such as in treatment of wastewater containing high sulphate levels which causes unwanted H₂S production by sulfate reducing bacteria (SRB), oxidation of elemental sulphur to plant available sulphate for plant growth promotion, bio leaching, biocontrolling agent etc. Hence, in the present study an attempt has been made to explore the diversity of sulphur oxidizing bacteria from mangroves of Mahanadi delta, Odisha to evaluate their biotechnological potential. Two sulphur oxidising bacteria (SOB-7 and SOB-8) were isolated from mangrove soils of Mahanadi river delta, based on the change of colour of the thiosulphate broth medium from purple to colour less by reducing the pH. Based on morphological, biochemical and 16S rRNA gene sequencing the two strains (SOB-7) and (SOB-8) were identified as *Klebsiella* sp., and *Micrococcus* sp. respectively. The gene bank accession number of the strains are KR632644 (SOB-7) and KR632643 (SOB-8). The strain *Klebsiella* sp. and *Micrococcus* sp showed sulphate ion production ability of 243 mg/ml and 240 mg/ml respectively with decrease in pH from 7.0 to 4.0. Among these two isolates, SOB-8 showed higher sulphide oxidase production ability (126.83 U/ml) than the isolate, SOB-7 (126.0 U/ml). Maximum sulphide oxidase by both the strains were obtained at temperature 45°C, peptone as nitrogen source, thiosulphate concentration of 10 mg/ml. pH 9.0 for the isolate SOB-8 whereas pH 7.0 for the isolate, SOB-7. Partially purified enzyme of both the isolate showed higher activity at same substrate concentration of 1.5mg/ml, but varies with pH and temperature.

Keywords Bromophenol Blue, Mangrove Ecosystem, Sodium Thiosulphate, Sulphur Oxidase, Sulphate Ion

1. Introduction

Sulphur is 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 [1]. Sulphur is required because of its structural role in the amino acids cysteine and methionine and it is present in number of vitamins, such as thiamine, biotin and lipoic acid, as well as in coenzyme A [2]. The majority of sulphur taken up by plant roots is in the form of sulphate (SO₄), which undergoes a series of transformations prior to its incorporation into the original compounds [3]. The soil microbial biomass is the key driving force behind all sulphur transformation. Biological oxidation of hydrogen sulphide to sulphate is one of the major challenges of the global sulphur cycle [4]. Sulphur oxidizing bacteria present in soil oxidize various sulphur compounds and improve soil fertility. It results in the formation of sulphate, which can be used by plants, while the acidity produced by oxidation helps to solubilise plant nutrients [5]. Sulphide oxidase is the key enzyme produced by these microorganisms, responsible for sulphide ions oxidation [6].

Beside their important contribution in agriculture sulphur oxidising bacteria also play significant role in removal of toxic H₂S from the environment. Sulfur containing volatile compounds such as hydrogen sulphide, methanol, dimethyl sulphide and dimethyl disulfide, which are major malodorous components generated from domestic animal waste, sewage treatment facilities and chemical factories. Sulphur oxidizing microorganisms have the ability to

deodorize these malodorous sulphur components. [7].

Mangrove 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 [8]. These are tropical coastal biome, located in the transition zone between land and sea, where the vegetation is dominated by a particular group of plant species [9]. 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 [10]. Bacteria are the major participants in the biogeo-chemical cycles in mangrove forest [11]. 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. [12]. As the original source of reduced sulfur compounds, H_2S hence, supports abundant populations of sulfur-oxidizing bacteria at the oxic-anoxic interface [13]. 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 [14]. 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 sulphur 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. Materials and Methods

2.1. Isolation of Sulphur Oxidising Bacteria

The mangrove area in the Mahanadi delta ($20^{\circ} 15''$ to $20^{\circ} 70'N$ latitude and 87° to $87^{\circ} 40'E$ longitude) extends from south eastern boundary of Mahanadi river to river mouth of Hansua (a tributary of Brahmani) in the north, from the north eastern end of Mahanadi river up to Jamboo river in east. The soil samples were collected from different location of mangrove forest such as Jumbo, Kharnasi, Triveni, Nuagada, Atharabanki and Mangrove forest at Indian Farmer fertilizers 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 labeling, stored in ice box

and brought to the laboratory for further analysis. In the laboratory, the samples were stored at $4 \pm 0.1^{\circ}C$ 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 containing [15] 10 g of bacto-peptone, 1.5 g of K_2HPO_4 , 0.75 g of ferric ammonium citrate and 1.0 g of $Na_2S_2O_3 \cdot 5H_2O$ and agar 15 g per liter. The initial pH was adjusted to 7.0 using 1 M HCl before sterilizing by autoclave. The plates were incubated at $30^{\circ}C$ for 24 h. The morphologically distinct isolated colonies appeared on the plate were picked up by wire loop and re-streaked on the other sulphur-oxidizer medium agar plate for purity conformation. For qualitative screening of distinct sulphur oxidising bacteria, the isolated bacteria were further grown on the thiosulphate broth [16] containing: 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4 , 0.2 g $NaHCO_3$ and 0.1 g NH_4Cl in 1000 ml distilled water, with pH 8.0. Bromophenol blue was used as the indicator. The cultures which changed the colour of the thiosulphate broth from purple to colour less by reducing the pH after incubation for 3 days at $30^{\circ}C$ were selected for further characterisation and evaluation of their sulphate ion and sulphide oxidase activity.

2.2. Identification of the Bacterial Strain

Culture characteristics such as colony appearance, spore formation, and motility of each strain were tested according to the standard methods. Morphology of the bacterial isolates was checked with scanning electron microscope (Zeiss, Sigma). We also tested for catalase and urease production, indole production, nitrate reduction, citrate utilization, and acid-gas production from sugar. In addition, we tested for acetyl methyl carbinol production by the Voges-Proskauer (V-P) reaction. Hydrolysis of tributyrin, Tween-80, cholesterol, gelatin, casein, pectin and chitin by the culture was also determined. The results were compared with Bergey's Manual of Determinative Bacteriology [17].

16S rRNA gene of two most efficient isolates were amplified using universal 27F forward primer ($5' AGGCCTAACACATGCAAGTC-3'$) and 1492R reverse primer ($5'GGGCGGWGTGTACAAGGGC-3'$) described by Das et al. [18]. A PCR product of 16S rRNA (1500bp) was purified using QIA quick gel extraction kit, QIAGEN, GmbH (Germany) and nucleotide sequence were determined using the big dye terminator v 3.1 cycle sequencing kit in an automated 3130xl genetic analyzer system (Applied Biosystems, HITACHI, USA) and submitted to gene bank. The resultant sequences were aligned using Seqscape software (Applied Biosystems, USA) to get full length sequence. The BLAST programs of the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) were used to search databases for similar nucleotide and genus identification. A phylogenetic tree was constructed using MEGA 4.0 software using the neighbor joining DNA distance algorithm.

2.3. Sulphate Ion Determination

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 supernatant mix vigorously [19]. A resulting, white turbidity due to barium sulphate formation was measured at 450 nm with spectrophotometer (Systronics 119). Potassium sulphate (K_2SO_4) was used as standard to construct a sulphate calibration curve [20].

2.4. Sulphide Oxidase Assay

The sulphide oxidase activity was determined by measuring the product of enzymatic reaction, sulphate (SO_4^{2-}) in the reaction mixture following the standard method of Hirano et al. [21]. The reaction was initiated by addition of 0.5 ml of sodium sulphide (Na_2S) solution into the reaction mixture that contain 4.5 ml of 0.1 M sodium acetate buffer (pH 5.6) and 1 ml crude enzyme supernatant. The Na_2S solution was prepared by dissolving 0.06 g Na_2S in an alkaline solution consisting of 0.16 g NaOH, 0.02 g EDTA $\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (sodium ethylene diamine tetra acetic acid), 2 ml glycerol and 40 ml distilled water. The Na_2S solution was freshly prepared prior to use. The mixture was incubated for 30 minutes at 30°C and the reaction was subsequently terminated by the addition of 1.5 ml NaOH (1.0 M) followed by thorough mixing. Concentration of sulphate ion formed during sulphide oxidase assay was detected by the reaction of equal volume of barium chloride solution (10% w/v) and reactant and the absorbance was measured at 450 nm using spectrophotometer. The measurement of sulphate ion in the sample was based upon the formation of barium sulphate after addition of barium chloride which leads to the white turbidity. The amount of turbidity formed is proportional to the sulphate ion concentration in the sample. One unit of sulphide oxidase activity was defined as amount of enzyme required to produce 1 μmol sulphate per hour per ml (U/ml).

2.5. Optimisation of Growth Medium Parameters for the Production Of Sulphide Oxidase

Optimisation of sulphide oxidase production was carried out by inoculating the bacterial culture in the growth medium with respect to different environmental conditions such as pH, temperature, thiosulphate concentration and nitrogen source. Enzyme activities were measured following the standard method of Hirano et al. [21].

2.6. Partial Purification of Sulphide Oxidase

Partial purification of sulphide oxidase was carried out by ammonium sulphate precipitation followed by dialysis. The gradient ammonium sulphate precipitation (70%) was carried out with chilled cell free culture broth. The precipitate was collected by centrifugation after two hours incubation at 4 °C and dissolved in 0.2 M phosphate buffer

(pH 7.0). The enzyme extract was dialyzed overnight against the same buffer at 4°C and the dialyzed enzyme was used for further studies. To estimate the molecular weight of the partially purified enzyme, SDS-PAGE was done using 5% stacking gel and 10% resolving gel according to the method of Laemmli [22] and electrophoresis was done with 15 mA fixed current. Quantification of protein content of crude and partially purified phosphatase was done following the method of Lowry et al. [23], with Bovine serum albumin as a standard (Sigma, Germany).

2.7. Characterisation of Partially Purified Sulphide Oxidase

For characterization, the partially purified enzymes were subjected to different parameters such as pH, temperature and substrate concentration and sulphide oxidase activity was measured following the standard method of Hirano et al. [21].

3. Results

3.1. Isolation of Sulphur Oxidizing Bacteria

Two sulphur oxidising bacteria were isolated from mangrove soil sample of Mahanadi delta, Odisha using sulphur oxidizer-agar medium. Morphologically distinct bacterial isolates forming distinct colony on agar medium were isolated and inoculated on thiosulphate broth medium containing bromophenol blue (BPB) as an indicator. These bacterial isolates were able to change the colour of the BPB in thiosulphate broth medium by reducing the pH of the medium from initial pH 8.0. Both the isolates, SOB-7 (4.0) and SOB-8 (4.1) were found to decrease the pH of the medium more efficiently.

3.2. Identification of the Bacterial Strain

Based on morphological (Figure 1) and several biochemical test such as, gram stain, spore formation, motility, catalase, urease, indole, nitrate reduction, citrate utilization, acid-gas production, Voges-Proskauer (V-P) reaction, hydrolysis of tributyrin, tween-80, cholesterol, gelatin, casein, pectin and chitin, the bacterium, SOB-7 was tentatively assigned to the *Klebsiella* sp. and SOB-8 as *Micrococcus* sp. Further confirmation of genus was done by BLAST analysis data of the 16S rRNA gene sequence which showed similarity of SOB-7 with the genus *Klebsiella* sp. and SOB-8 as *Micrococcus* sp. Both sequences were submitted to gene bank. The gene bank accession number of the strain SOB-7 is KR632644 and SOB-8 is KR632643. Phylogenetic tree were constructed by comparing nucleotide sequences of 16S rRNA gene of the isolate, SOB-7 with different *Klebsiella* sp. and of the isolate, SOB-8 with different *Micrococcus* sp. We found that the isolate SOB-7 is most closely related to *Klebsiella* sp. (Figure 2) and SOB-8 related to *Micrococcus* sp. (Figure 3).

Figure 1. SEM photograph of (left) *Klebsiella* Sp. and (right) *Micrococcus* sp.

Figure 2. Phylogenetic tree of *Klebsiella* sp. (SOB-7) isolated from mangrove soil of Mahanadi river delta.

Figure 3. Phylogenetic tree of *Micrococcus* sp. (SOB-8) isolated from mangrove soil of Mahanadi river delta

3.3. Sulphate Ion Determination

The amount of sulphate ion produced and changes in pH taken place in the medium up to 264 h of incubation are presented. Productions of sulphate ion in the liquid medium by both the strains were accompanied by significant drop in pH values from an initial pH of 8.0. In the control no sulphate ion was detected as well no drop in pH was observed. However among the two bacterial isolates, the maximum sulphate ion production was observed by the bacterial isolate, SOB-7 (245 mg/ml) with maximum decrease in pH (pH 4.0) of the medium (Figure 4) followed by the bacterial isolate, SOB-8 (240 mg/ml) with decrease in pH value of 4.1 of the thiosulphate broth medium (Figure 5).

Figure 4. Comparative pH reduction, sulphate ion production and sulphide oxidase production ability of *Klebsiella* sp.

Figure 5. Comparative pH reduction, sulphate ion production and sulphide oxidase production ability of *Micrococcus* sp.

3.4. Sulphide Oxidase Assay

Experiments were carried out to determine the sulphide oxidase production ability (S.O. activity) of two bacterial isolates (SOB-7 and SOB-8) during 264 hours of incubation. Among these two isolates, SOB-8 showed higher sulphide oxidase production activity (126.83 U/ml) than the isolate, SOB-7 (126 U/ml).

3.5. Optimization of Growth Condition for Sulphide Oxidase Production

Optimization of growth condition for sulphide oxidase production was carried out by maintaining the culture medium at different pH, temperature, nitrogen sources and thiosulphate concentration. Effect of pH on enzyme production showed that very less sulphide oxidase produced

by both the isolates when the initial pH of the culture medium was maintained at pH 3.0 (Figure 6a). Maximum sulphide oxidase production (123.66 U/ml) was observed by the isolate SOB-7 when the initial pH of the culture medium was maintained at pH 7.0. In case of the isolate SOB-8, sulphide oxidase production was found to be maximum (121.33 U/ml) when the initial pH of the medium was maintained at pH 9.0. Decrease in enzyme activity was observed beyond their optimum pH.

Optimum temperature for the production of sulphide oxidase by both the strains was observed at 45°C (Figure 6b). The bacterial isolate, SOB-7 showed maximum sulphide oxidase activity of 125.0 U/ml, where as maximum sulphide oxidase activity of 120 U/ml was observed at temperature of 45°C by the isolate, SOB-8. Sulphide oxidase productions decreased by both the bacterial isolates with increase in

temperature beyond 45°C.

Effect of nitrogen source towards sulphide oxidase synthesis was studied by amended sulphur-oxidizer medium with varying nitrogen sources (Figure 6c). The initial supplementation of culture medium with peptone showed enhanced sulphide oxidase production (126.83 U/ml) by SOB-8 and (126.0 U/ml) by SOB-7 in comparison to other source used.

Concentration of thiosulphate in sulphur-oxidizer medium was varied in a range of 5–25 mg/ml (w/v). Sulphide oxidase production was significantly affected by the initial increased in thiosulphate concentration. Maximum sulphide oxidase productions of 125.1 U/ml by SOB-8 and 137.32 U/ml by SOB-7 were observed when 10 mg/ml of thiosulphate was incorporated in the medium (Figure 6d).

Figure 6. Effect of (a) pH, (b) Temperature, (c) Nitrogen source, (d) Thiosulphate concentration of the culture medium on sulphide oxidase production activity

Table 1. Partial purification of sulphide oxidase from the bacterial isolates, SOB-7 and SOB-8

Isolates	Total Volume (ml)	Protein mg/ml	Total Protein (mg)	Sulphide oxidase U/ml	Total Activity (U)	Specific Activity U/mg	Fold of Purification	Total Yield (%)
Culture extract of SOB-7	50	21.7	1085	126 ± 2.26	6300	5.80	1	100
70% (NH ₄) ₂ SO ₄ precipitation and dialysis of SOB-7	15	9.2	138	154.31 ± 2.3	2314.6	16.77	2.89	36.74
Culture extract of SOB-8	50	27	1350	126.83 ± 2.6	6341.5	4.69	1	100
70% (NH ₄) ₂ SO ₄ precipitation and dialysis of SOB-8	15	12.5	187.5	140 ± 6.32	2100	11.2	2.38	33.11

3.6. Partial Purification and Characterisation of Sulphide Oxidase Activity

Partial purification of sulphide oxidase was performed by 70% of ammonium sulphate precipitation followed by dialysis and SDS-PAGE gel electrophoresis. After partial purification the enzyme of the isolate, SOB-7 showed some specific bands of approximately 72 kDa, 55 kDa, 50 kDa and 43 kDa. Partially purified protein of SOB-8 revealed some specific band of approximately 30 kDa and 25 kDa. (Figure 7a). Sulphide oxidase from the isolate, SOB-7 could be purified 2.89 fold with 36.74% yield, protein content 9.2 mg/ml and specific activity of 16.77 U/mg. Similarly sulphide oxidase from the bacterial isolate, SOB-8 was purified 2.38 fold with 33.11% yield, protein content of 12.5 mg/ml and specific activity of 11.2 U/mg (Table 1).

3.7. Characterisation of Partially Purified Sulphide Oxidase

In the present study to determine the suitable condition for maximum enzyme activity, the reaction mixture of the partially purified enzyme was studied under different conditions of pH, temperature and concentration of substrate.

The effect of pH on partially purified sulphide oxidase activity was examined by maintaining the reaction mixture at various pH ranging from pH 3.0 to 10.6 in different buffer

solution. The optimal pH for maximum sulphide oxidase activity by the isolate, SOB-7 was found at pH 7.0 (136.66 U/ml), whereas pH 9.0 was found to be optimum for maximum sulphide oxidase activity (131.88 U/ml) by the isolate, SOB-8 (Figure 7b).

The effects of temperature on partially purified sulphide oxidase activity of both the isolates were examined at various temperatures ranging from 25°C to 65°C. Optimal temperature for partially purified sulphide oxidase activity of both the isolate was found to be 45°C, beyond which steady decrease in enzyme activity was observed (Figure 7c). The maximum sulphide oxidase activity by the bacterial isolate, SOB-7 was found to be 145.0 U/ml, where as SOB-8 showed maximum enzyme activity of 140.0 U/ml at 45°C.

Substrate specificity of the purified enzyme was performed by assaying the activity of the purified enzyme against different concentrations of Na₂S solution (0.25-2.5 mg/ml) to the reaction mixture. The enzyme activities of both the isolates increased up to a certain substrate concentration and remain almost same thereafter. It was observed that the partially purified enzyme of both the isolate, SOB-7 (154.31 U/ml) and SOB-8 (137.89 U/ml) showed higher activity at 1.5 mg/ml of substrate concentration and remained almost same thereafter (Figure 7d).

Figure 7. (a) SDS-PAGE of partially purified sulphur oxidase from SOB-7 and SOB-8, effect of (b) pH, (c) temperature, (d) substrate concentration on partially purified sulphide oxidase activity.

4. Discussion

Two sulphur oxidising bacterial isolates (SOB-7 and SOB-8) were isolated from mangrove soil of Mahanadi delta, based on their pH reduction abilities where the blue color is faded to color less due to reduction of pH. Studies on isolation of sulphur oxidizing bacteria by various researchers reveal their existence in mud soil, canal water, fresh water sources [24] and also from various mangrove ecosystems [14]. Purple sulphur bacteria, a phototrophic anaerobe requires sulphide which they oxidise to sulphate for their growth are widely distributed in sulphide rich reducing environment such as mangrove, mud flat and polluted water [25]. Thatoi et al. [26] reported the occurrence of *Pseudomonas* sp. that oxidises sulphur in the mangrove of Bhitarakanika, Odisha. Sulphur oxidising bacteria was also

reported from garden soil, activated sludge [27] and soil sulphur compost [28]. Heterotrophic isolates of thiosulphate producing bacteria, which acidified the medium moderately by 0.5 to 1.0 pH units, were obtained from marine sediments and hydrothermal vents [29].

In the present study, pH reduction of the medium was due to the generation of acid in the medium. Reduction in pH of the growth medium by sulphur oxidizing bacteria was reported earlier by Donati et al. [30].

In the present study, to screen out the potential sulphur oxidising bacteria, medium was amended with sodium thiosulphate and the sulphate ion production ability of both the isolates was measured. The isolates, SOB-7 and SOB-8 were found to produce maximum sulphate ion i.e. 245 mg/ml and 240 mg/ml respectively after 216 h of incubation. Ravichandra et al. [31] reported the maximum sulfate ion production of 14-40 mg/ml by a *Thiobacillus* sp.

Further optimisations of sulphide oxidase production by these two strains were carried out. Among these two isolates, SOB-8 showed comparatively highest sulphide oxidase production ability (126.83 U/ml) after 216 h of incubation followed by the isolate, SOB-7 (126 U/ml) at 240 h of incubation. Our finding is comparable to the findings of others. Crude extract of thiosulphate oxidase from *P. aeruginosa* showed maximum activity of 130 U/ml, reported by Schook and Berk [32]. Rohwerder and Sand [33] reported sulphur dioxygenase activity of 5.0 ± 1.7 nmol min⁻¹ by *Acidithiobacillus* and *Acidiphilium* sp.

On the basis of their morphological, physiological, biochemical test and 16S rRNA sequencing, both the isolates were identified as *Klebsiella* sp. (SOB-7) and *Micrococcus* sp. (SOB-8). Sulphur oxidising activity of *Micrococcus* sp. were well reported earlier from Indian Terai soil- a Himalayan foot hill soil of the order mollisol [34].

The rate of growth and metabolism of bacteria depend very much on the composition of the medium and the prevalent environmental conditions. Various bacterial strains differ in their nutritional requirements and cultural conditions for optimal growth and enzyme production. In the present study, an attempt has been made to optimize the production of sulphide-oxidizing enzyme from these two bacterial isolates of Mahanadi delta by manipulating the physical and environmental factors such as pH, temperature, inoculum size, thiosulphate concentration and different nitrogen sources.

The bacterial isolate SOB-7 showed maximum sulphide oxidase activity (123.66 U/ml) at pH 7.0. whereas the isolate, SOB-8 showed maximum sulphide oxidase activity (121.33 U/ml) at pH 9.0. The optimum pH for sulphur-oxidizing bacteria was varied depending on the microbial habitat. The halo alkaliphilic *Thioalkalivibrio* isolated from soda lakes showed optimum sulphide oxidase at pH 10.0 [35] while *Thiobacillus caldus* found in environment such as coal spoil heaps was able to grow down to pH 1.0 [36]. However, Mohapatra et al. [6] had reported in their work that the optimum pH for sulphide oxidase production by *Arthrobacter* sp. strain FR-3 in GY medium was at pH 7.5.

Temperature is one of the most important environmental factors affecting growth and activity of microorganisms [37]. Optimum temperature for sulphide-oxidase production by both the strain was found to be 45 °C and it starts to decrease steadily beyond that temperature. This might be due to denaturation of enzyme that leads to changes in the configuration of enzyme active site and loss of catalytic properties [38]. Hence both the isolates SOB-7 and SOB-8 were found to be mesophilic in nature and most of the proteins from mesophiles are inactivated at temperatures above 45°C [38].

Nitrogen is a major element in proteins, nucleic acid and several other constituents in the cell. Nitrogen can be found in nature as both organic and inorganic forms. In the present study peptone was found to be the best nitrogen sources for sulphide oxidase production. Asgher et al. [39] stated that

organic sources like yeast extract and peptone usually have stimulating effect on enzyme activity. The result of this study is in good agreement with Asgher et al. [39] where stimulation of sulphide oxidase activity can be seen when peptone and yeast extract was supplied in the medium. Most enzymes including amylase, protease and nitrate reductase are repressed by ammonium salts [40-41]. In this study, ammonium salts also showed an inhibitory effect towards sulphide oxidase production considerably. In the present study when fermentation was carried out with ammonium chloride as a nitrogen source it yields very low sulphide oxidase production by both the isolates. Mohapatra et al. [6] also observed that ammonium salts repressed the production of sulphide oxidase by the *Arthrobacter* sp.

It should be noted that activity of sulphide oxidase enzymes involved in the metabolism of inorganic sulphur compounds varied depending on type of sulphur compounds added to the medium. In the present study, sulphide oxidase activity significantly affected by the increased in thiosulphate concentration from 05 - 10 mg/ml (w/v) of the medium and gradually declined thereafter. This may be due to the inhibition of thiosulphate utilization at high concentration which leads to less consumption of thiosulphate by both the bacteria. According to Spring et al. [42], concentration of thiosulphate above the optimum concentration inhibits the bacterial growth and thiosulphate utilization by the bacteria. According to Eccleston and Kelly [43], very low thiosulphate concentration allowed very high oxidation rate. Higher thiosulphate concentration only resulted in loss of thiosulphate oxidizing capacity and decrease in cell mass production. This might be due to the thiosulphate toxicity at high concentration that can repressed cell growth and also thiosulphate oxidizing capability of the bacteria [44]. Skirnisdottir et al. [45] observed that the conversion of thiosulphate to sulphate by *Hydrogenobacter* sp. was reduced with the increase thiosulphate concentration. The above description also affirms the present finding that high concentration of thiosulphate was not suitable for the production of sulphide oxidase by both strains.

A number of sulphide oxidases have been purified from autotrophs, and their oxidation mechanisms have been investigated. For instance, the purified sulphide oxidoreductase from *Thiobacillus ferrooxidans* [46-47] performs an important role in energy production, and *Chlorobium thiosulphatophilum* [48] has a cytochrome c-553, that plays a sulphide oxidation role in electron transport. However no such reports on sulphide oxidases have been published on microorganism from mangrove environment. So, this is the first attempt made to partially purify and characterise sulphide oxidase from microorganism from mangrove soil of Mahanadi river delta, Odisha, Indi. The partially purified sulphide oxidase of SOB-7 in SDS-PAGE exhibits four consecutive bands where as the bacterial isolate, SOB-8 exhibits two consecutive bands. According to Mohapatra et al. [6], the purified sulphide oxidase was showed to be monomer with a molecular weight of 43 kDa. This molecular weight was

found to be higher compared to the purified sulphide oxidase from the *Bacillus* sp. BN53-1 which is 37 kDa [49]. Further the partially purified enzymes were characterised in different pH, temperature and Na₂S concentration.

To maintain the hydrogen ion concentration or pH of an enzyme is most important because a change in pH can change the charge properties of the substrate so that it affects the catalysis of the E+S complex. From the observation it was found that in case of the isolate SOB-7, optimum pH for sulphide oxidase activity is 7.0 after which it showed decreasing in binding with the substrate hence decrease in activity. Similarly the maximum sulphide oxidase activity of the isolate SOB-8 was observed at pH-9.0. Nakada and Ohta [49] reported optimum activity of the purified sulphide oxidase at at pH-8.0.

Temperature plays a major rule in activation of the enzyme to bind the substrate. Beyond the optimum temperature the protein structure denaturised resulting the thermal deactivation or deceleration in enzyme kinetics. The optimum enzyme activities of different enzyme system such as amylase, protease, sulphide oxidase, phosphatase, sulphide oxidase etc. are dependent to the organism and the habitat from where they are isolated. In the present study sulphur oxidising bacteria were isolated from a tropical mangrove forest hence most of the microbes are mesophilic. Both the bacterial isolates showed optimum activity at 45°C which was also observed after partial purification. Nakada and Ohta [49] reported optimum activity of the purified sulphide oxidase at 40°C.

In the present investigation the ranges of different concentrations of Na₂S solution were added to the reaction mixture (0.25-2.5 mg/ml) and observed that both the isolates SOB-7 and SOB-8 showed increased activity from 0.25 to 1.5 mg/ml of Na₂S, after which it showed saturated activity with the enzyme. Hence the rate of formation of product depends on the activity of the enzyme itself, and adding more substrate will not affect the rate of the reaction to any significant effect.

5. Conclusions

The present study emphasizes isolation and identification of two efficient sulphur oxidizing bacteria such as *Klebsiella* sp., and *Micrococcus* sp. from mangrove soil of Mahanadi river delta, Odisha India. Both the bacterial isolates could efficiently oxidise the reduced sulphur compound by decreasing the pH of the culture medium and efficiently produced sulphate ion. Sulphide oxidase activity of both the isolates were optimized and characterized after partial purification which could probably help taxonomists, agriculturalists and even some industrialists in their own researches. Use of these bacteria as bio-inoculants may enhance sulphur oxidation in soil and increase soil available sulphate to minimize the S-fertilizer application. They may have also probable use to reduce environmental pollution and promotes sustainable agriculture.

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