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Characterization and Standardization of Cultural Conditions of Sulphur-Oxidizing Bacteria Isolated from Soil Samples

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Abstract: *Sulphur-oxidizing bacteria are the important organism of sulphur cycle. SOB is the dominating prokaryote with the ability to oxidize sulphur in to absorbable form sulphate. The ability to metabolize organic and inorganic form of sulphur make them organism of interest in soil. In nature SOB are the versatile microorganisms found in normal to acidic/alkaline soil, water habitat to extreme conditions of geothermal areas, acid drains and volcanic eruptions. The amount of sulphur oxidation depends on the physico-chemical conditions. The quantity of sulphate ion produced by SOB is drastically affected by cultural conditions. Attempts are made to standardize the cultural conditions and same was optimized for five SOB. The incubation time was optimized to 2 days at 30^{0}C with media pH 8.0. The carbon source improving sulphate ion production is glucose and nitrogen source is ammonium chloride.*

Keywords: Sulphur, Sulphate ion, SOB, Oxidize, cultural conditions.

I. INTRODUCTION

Sulphur is the fourth important element followed by Nitrogen, Phosphorous and Potassium(Joshi *et al.,* 2020). It is the secondary essential element for crop production and 13th abundant element present in earth's crust. Sulphur is one of the important inorganic element for biological kingdom due to its presence in three essential amino acids namely methionine, cystine and cysteine, they are the major components of biological molecules(Chaudhary *et al*., 2017). Sulphur is the important macronutrient for plants, microorganisms and humans (Prasad and Shivay, 2018). In crop production and plants sulphur has various oxidizing functions and involved in protein synthesis. The essentiality of sulphur in plant nutrition is recognized from the equidistant of last century. In earth's crust it occurs as organic and inorganic sulphur, the only form of sulphur available for plant and crop uptake. Due to increasing population demand, high amount of fertilizers are applied in soil. The imbalance between input and output of soil sulphur leads to sulphur deficiency. ICAR reported 46% deficiency of sulphur in soil samples of 18 states of India (Layek *et al*., 2014). Microorganisms are the key for the availability of sulphur in soil. Bacteria especially sulphur-oxidizing bacteria are the active pool for sulphur turnover in soil. Principally they are the gram negative bacteria classified in *Thiobacillus*, *Thiomicrospira* and *Thiosphaera*, in nature some heterotrophs like *Pseudomonas, Xanthobacter, Alcaligens, Bacillus* etc… has capability to grow chemolithotrophicallyon inorganic sulphur (Vidyalakshmi*et al.,* 2009).SOB and other bacteria can oxidize sulphur, hydrogen sulphide,sulphite, thiosulphate and several polythionate under alkaline (Sorokin *et al.,* 2001) acidic or neutral environment (Harrison 1984).Pure SOB and heterotrophic bacteria of soil oxidizes sulphur into sulphate the only available form of sulphur for plant uptake. These SOB and heterophs have potential to solve sulphur deficiency in soil.

II. MATERIALS AND METHOD

2.1 Soil Sample Collection

The soil samples were collected randomly using augar from five different soybean growing agricultural fields of Indore, M.P. India 22.796^{0}N and 75.85^{0}E. The samples were collected in sterile bags and transferred to laboratory.

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2.2 Isolation of Sulphur-oxidizing Bacteria

The bacteria were isolated using Starkey broth (Starkey and Collins, 1923) and Thiosulphate broth (Vidyalakshmi and Sridar, 2007).10 g Lit^{-1} of elemental sulphur was added in Starkey broth and sterilized for three consecutive days; thiosulphate broth doesn't have elemental sulphur. 5g sugar per liter of media was added for isolation of heterotrophs. Bromocresol purple was added as indicator and pH of media was adjusted to slightly alkaline (8.0±0.2) condition. 10g of soil sample was added in 90ml of broth and incubated, purified by transferring in to fresh media at every fortnight interval. The serially diluted culture was used to obtain pure colonies on thiosulphate agar plates.

2.3 Screening of Potential Isolates

A. Dye Reduction Test

The isolates were screened initially on the basis of their ability to reduce the colour of dye from purple to yellow. Isolates were inoculated in to thiosulphate broth containing bromocresol purple and incubated at 32^0C and observed for colour change.

B. Sulphate Ion Production

Spectrophotometric determination of sulphate ion by potential isolates was carried out. The isolates were inoculated in thiosulphate broth in triplicate and incubated for 15 days at 32^0C. After incubation the broth was centrifuged at 12000 rpm for 10 min to separate supernatant from bacteria. A 1:1 barium chloride solution (10% w/v) was added and suspension was mixed by inverting tubes. A white turbidity obtained due to barium sulphate formation was measured at 450nm in a spectrophotometer. The resultant values were compared with standard curve drawn by K_2SO_4 as standard. The concentration of K_2SO_4 was in the range 0.00 to 5.00 mM and turbidity was measured which is directly proportional to amount of sulphate ion.

2.4 Standardization of Cultural Conditions (Sulphate Ion Production)

The cultural conditions were standardized for different factors affecting the growth and production of sulphate ions.

A. Incubation Time

The potential isolates screened previously were inoculated and incubated for different time interval *viz.*1, 2, 3 and 4 days and optimized for maximum sulphate ion production.

B. Temperature

The potential isolates were incubated at different temperature *viz.* 20, 25, 30, 35 and 40^0C respectively and best growth temperature was optimized.

C. Media pH

Isolates were optimized for sulphate ion production at different pH range *viz.* 6, 7, 8, 9 and 10 in thiosulphate broth.

D. Carbon Source

The isolates were optimized for different carbon source for production of sulphate ion the different carbon source i.e. Sucrose, Mannose and Glucose. Thiosulphate broth with different carbon source was prepared and isolates were inoculated and incubated according to previously optimized conditions.

E. Nitrogen Source

Similar to carbon source three different nitrogen sources i.e. CH_4N_2O, NH_4Cl and KNO_3 were optimized for sulphate ion production in thiosulphate broth.

2.5 Characterization of Isolates

The potential isolates were characterized for Morphological, Biochemical and Physiological characters according to Bergey's manual(2005 and 2009). Different parameters were evaluated *viz.*colonial characteristics, Grams staining, Catalase test, Oxidase test, Citrate utilization, Urease test, MR-VP test, Indole test, H_2S Production, TSI and Starch hydrolysis.

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III. RESULTS AND DISCUSSION

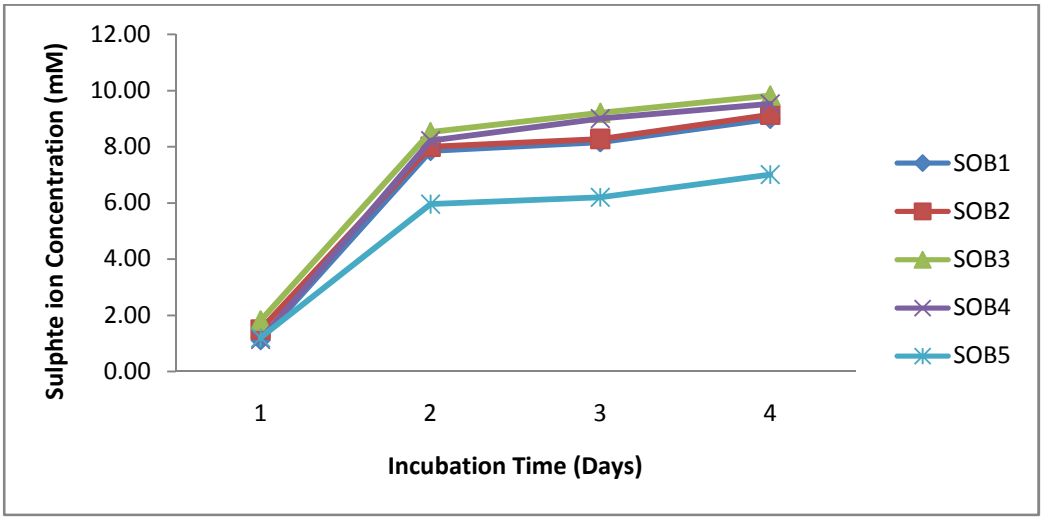
3.1 Isolation and Screening of Sulphur-Oxidizing Bacteria

A total of 30 distinguished bacterial morphotypes were isolated from soil samples of five different agricultural field of Indore M.P. India. 10 isolates were found to be autotrophic as they are growing in media devoid of sulphur and 20 were found to be heterotrophs as they grow in glucose containing media. Among 30 isolates 5 were chemolithoautotrophs utilizing elemental sulphur as their metabolic energy source and synthesizing organic carbon from carbon-di-oxide via Calvin cycle. The remaining population is chemolithoheterotrophs require glucose as carbon source. The isolated bacterial morphotypes were primarily screened on the basis of their ability to reduce dye. Isolates oxidizes elemental sulphur and thiosulphate in to sulphate and resulting acidity changes colour of media from purple to yellow due to presence of bromocresol purple. A total of 21 isolates were selected on the basis of their ability to produce acidity in media in turn change in colour of media. Out of 21 isolates 5 isolates were screened on the basis of their ability to produce extra cellular sulphate ion in media the maximum was in SOB3 followed by SOB4, SOB2, SOB1 and SOB5. The isolates showed formation of $BaSO_4$ in tubes leads to higher turbidity in turn higher absorbance. Chaudhary*et al.,* (2017) isolated SOB from rhizospheric soil of mustard and screened on the basis of dye reduction on soild media and further by their ability to produce sulphate ions. Sulphur-oxidizing bacteria were isolated from bioleaching pulp of zinc and copper concentrate and screened (Behera*et al.,* 2014). Presence of sulphur-oxidizing bacteria in agricultural soil is also demonstrated by Joshi *et al.,* (2020).

3.2 Standardization of Cultural Conditions (Sulphate Ion Production)

For validation of isolation process 5 screened isolates *viz.*SOB1, SOB2, SOB3, SOB4 and SOB5 were evaluated for standardization of cultural conditions. Bacterial morphotypes were optimized for sulphate ion production with different growth affecting parameters *viz.* Incubation time, temperature, media pH, carbon source and nitrogen source. The first increase in sulphate ion was recorded at 2nd day of incubation and minimum was at the end of 1st day of incubation. All the isolates showed optimum incubation time of 2 days. The incubation temperature for all the five isolates was optimized to 30^{0}C. All the isolates showed an increase in sulphate ion production with increase in temperature from 20 to 30^{0}C and reduction was noted in between 35 to 40^{0}C. The maximum sulphate ion production was recorded at 30^{0}C. Chaudhary*et al.,*(2017) reported 25^{0}C as optimum temperature for sulphur-oxidizing bacteria that shows SOB produces sulphate ions according to their habitat. Isolates showed maximum sulphate ion at pH 8 and reduction was observed at pH value 6 and 10. The result showed that isolates grow in slightly alkaline conditions. The carbon sources were amended in culture media and it was recorded that maximum sulphate ion production was with glucose followed by mannose and sucrose.The sulphate ion production was maximum in case of NH_4Cl followed byCH_4N_2O, and KNO_3. All the isolates have the ability to grow and metabolize in all form of nitrogen.

Figure 1: Standardization of Cultural Conditions A) Incubation Time B)Incubation Temperature C) Media pH D) CarbonSource E)Nitrogen Source.



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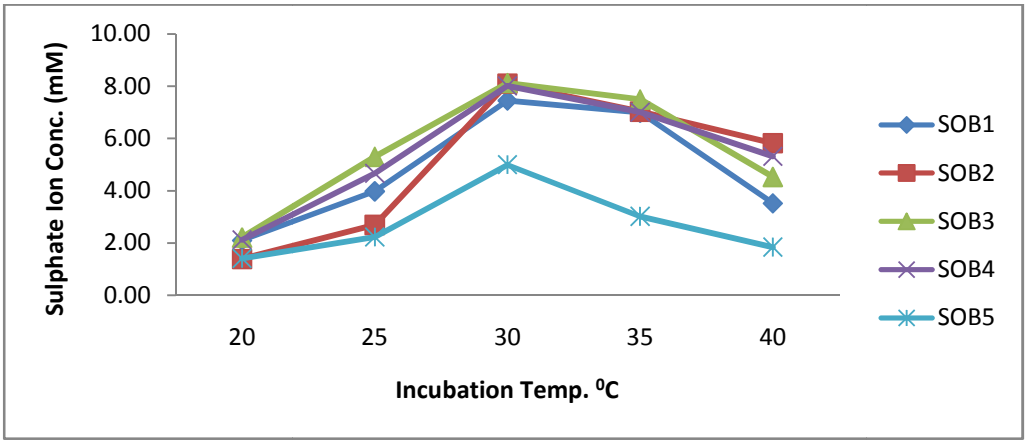
(A)



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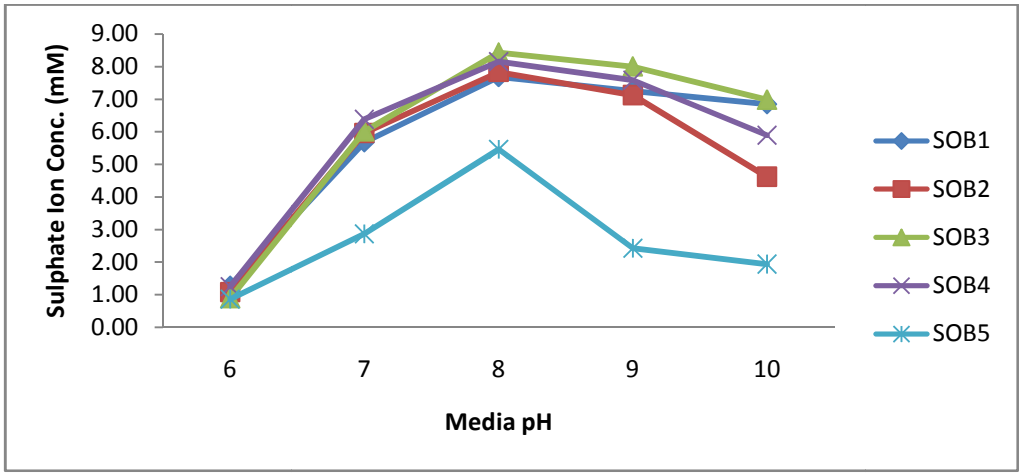

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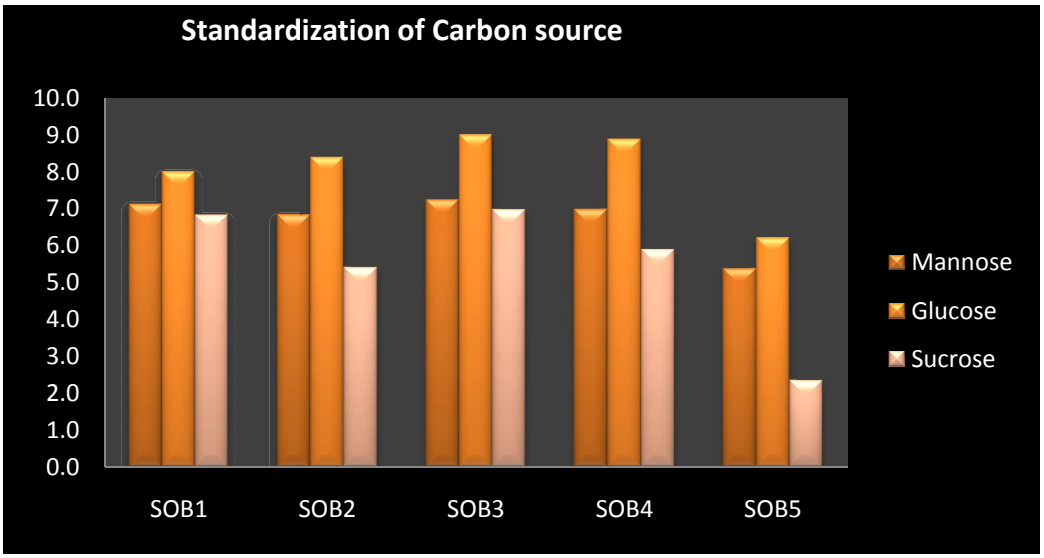

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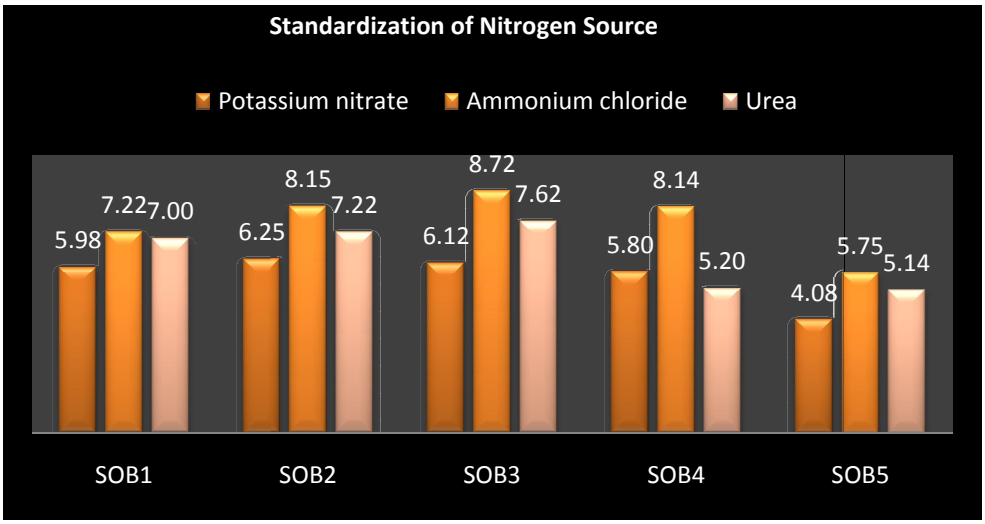

(E)

3.3 Characterization of Isolates

For the primary identification of isolates morphological and various biochemical tests were performed it was noted that microscopically all the isolates are rods except SOB2 (short Rods). SOB2, SOB3 and SOB4 are gram negative and SOB1 and SOB5 is gram positive. All have scattered arrangement except SOB5 is streptobacilli in appearance. All isolates have small to large colony with irregular and circular shape. The results of biochemical characters are depicted in Table 1. In the conclusion of biochemical and morphological character likely genus of SOB1, SOB2, SOB3, SOB4, and SOB5 probably closest to *Bacillus spp*., *Strenotrophomonas spp*., *Kosakonia spp*., *Enterobacter spp*. and *Bacillus Spp*.

Table 1: Biochemical Characteristic of selected bacterial isolates

S. No.	Biochemical Test	SOB1	SOB2	SOB3	SOB4	SOB5
1	Catalase	P	P	P	P	P
2	Oxidase	P	N	N	N	N
3	Citrate Utilization	P	P	P	P	P
4	Urease	p	P	P	P	P
5	Indole	N	N	N	N	N
6	MR	P	N	N	N	N
7	VP	P	P	P	P	P
8	H_2S Production	N	N	N	N	N
9	TSI	Acidic But Alkaline slant	Acid Production	Alkaline slant Acidic But	Alkaline slant Acidic But	Acidic slant & But
10	Starch Hydrolysis	N	N	N	N	N

IV. CONCLUSION

The present investigation reveals presence and role of sulphur-oxidizing bacteria in oxidation of sulphur. The availability and potentiality of the isolates can be exploited as strong environmental friendly and economic alternative to fulfill sulphur deficiency. The use of isolates as bioinoculants will increase sulphate ions in turns increase acidity which also help in phosphate solubilization. The acidity produced by SOB will also help in nutrient uptake and improve soil fertility. Oilseed crop require high amount of sulphur isolates obtained in present studies has the potential to fulfill the gap.

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