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RESEARCH ARTICLE

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IDENTIFICATION AND CHARACTERIZATION OF TOLUENE DEGRADING SULPHUR-OXIDIZING BACTERIA FROM ACID MINE DRAINAGE SAMPLE OF BARJORA COAL MINE, WEST BENGAL, INDIA.

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Abstract

Two different neutromesophilic sulphur oxidizing bacteria, strain SOB1 and N2 were isolated from acid mine drainage water sample of Barjora Coal Mine with thiosulfate as electron donor and CO_2 as carbon source. Heterotrophic growth was checked in thiosulphate agar media supplied with glucose (5 mM), fructose (5 mm), acetate (20 mM), peptone (0.1 %, w/v) and yeast extract (0.1 %, w/v). Strain SOB1 and strain N2, both were facultative chemoautotroph. These two bacteria were able to degrade toluene. The organisms were strictly aerobic. Growth were observed between pH values of 5-7.5 with an optimum growth at pH 6-7. The temperature limit for growth was 25-35˚C with an optimum growth between 28-30˚C. Heavy metal tolerence tests were carried out where strain SOB1 and N2 were able to grow in the presence of arsenic, copper, chromium, cadmium, cobalt, lead and zinc. The bacterium showed resistance against several antibiotics such as ampicillin, tetracycline, rifampicin, kanamycin and streptomycin. Based on the almost complete sequence of the 16S rRNA gene, strain SOB1 was found to form a polygenetic cluster with *Bacillus pumilus*, *Bacillus sufensis* and *Bacillus cereus*. Strain N2 formed phylogenetic cluster with *Enterobacter hormaechai* and *Enterobacter sp.* which showed 99% similarities.

Introduction:-

Coal deposited in coal mine contains high amounts of "pyritic-sulphur" and organic sulphur, which is exposed to the environment with the combined action of water and oxygen where oxidation takes place with the production of sulphuric acid. Acidophilic and neutrophilic iron and sulphur-oxidizing microorganisms greatly enhance this acid-

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producing oxidation. These organisms forms a chemoautotrophically-based biosphere in the subsurface, finally sustained by electron donors derived from sulphide minerals, CO_2, O_2, and N_2 derived from air, phosphate liberated by water rock interaction. The rate of AMD formation is increased by some microbial activities whereas; the AMD site is remediated by others. In addition to AMD-SRB, an understanding of the in situ sulphur oxidizing bacteria (SOB) of the AMD sites is also important. The wide variety of sulphur oxidizing bacterial (SOB) organisms can be used in ore processing and are a source of novel biomolecules (especially enzymes) for industrial processes. DNA-based studies of organisms populating in different mining environments have provided insights into the diversity of acidophilic and neutrophilic metal-tolerant species (Nordstrom, 2000).

Poly cyclic aromatic hydrocarbons (PAH) are a group of chemical compounds, consists of numerous carbon atoms fused together to form multiple ring structure. These compounds are neutral non polar molecules, mainly found in fossil fuels. Most PAH compounds are insoluble in nature. The solubility of PAHs decreases as the molecular mass increases. For e.g. two and three ring PAHs are soluble in water so it is more available for biological uptake and degradation. In contrast four or five ring structure is insoluble in water and mainly present in solid form in the environment making it less accessible for biological uptake and degradation (Cerniglia, 1992). So based on these characteristics PAHs can be divided into two category i.e. high molecular weight PAHs and low molecular weight PAHs. Low molecular weight PAHs are relatively volatile water soluble so it can't easily be degraded compare to high molecular weight PAHs. These are toxic environmental pollutants, often mutagenic or carcinogenic so it has a hazardous effect on human health. These are produced when insufficient oxygen or other factors result in incomplete combustion of organic matter. Soil is an important pool for polycyclic aromatic hydrocarbons (PAHs) as its high partition coefficient between air and soil (Haritash, 2009).

The physiological characteristics of sulphur-oxidizing bacteria are very diverse and highly dependent on various environmental factors such as pH, temperature, NaCl concentration, utilization of organic compounds, and availability of sulphur compounds (i.e., S^{2-}, S^0, and $S_2O_3^{2-}$). However, either thiosulphate or sulphide is generally used as an electron donor to enrich and isolate sulphur-oxidizing bacteria, even though the most abundant sulphur pool is elemental sulphur (S^0). Potential acid sulphate soils are rich in pyrite (FeS_2) (OWA., 2005). Thiosulphate is used by most of the sulphur-oxidizing bacteria, including chemolithotrophic bacteria, but also by chemoorganotrophic bacteria which oxidize thiosulphate to tetrathionate and use thiosulphate as a supplement but not as the sole energy source, e.g. some *Pseudomonas sp.* and *Halomonas sp.* species (Sorokin et al., 2005).

Toluene is the part of polycyclic aromatic hydrocarbon, is a methyl substitution on the aromatic benzene ring. It distributed in water, soils, and industrial effluents (Witzig et al., 2006). This compound and some of its derivatives are toxic but microorganisms like *Bacillus*, *Pseudomonas* and *Actinomyces* were able to survive in high toluene concentrations and used toluene as a carbon source. Toluene degrading bacteria use different pathways for toluene consumption. The oxidative microbes degrade toluene via hydroxylation of the aromatic ring to a mixture of catechols and cresols (Chaillan et al., 2004)

In the present study, investigate was carried out to isolate neutrophilic, facultatively autophothic sulphur-oxidizing bacteria from the coal mine drainage water sample of open cast mining area of Barjora coal mine in Bankura district, West Bengal, India. General morphological and biochemical characterization was undertaken. Other characterization such as pH, temperature, heavy metal resistance activity, multi drug resistance activity and PAH degrading activity was also investigated.

Materials and Methods:-

Sample collection:-

Coal mine drainage water samples were collected from Barjora Coal mine, which is situated at Bankura district of West Bengal. This zone lies between 23˚23' and 23˚31' North latitude and between 87˚05' and 87˚20' East longitude (Roy et al., 2017). On the same day pH of the water was checked and it came around ±6.1.

Enrichment and Isolation of bacteria:-

Isolation of neutrophilic sulphur oxidizing bacteria from water sample of Barjora Coal Mine was carried out followed by the method is given in the paper of Owa et al. (OWA., 2005) with slightly modifications. 10 ml of water samples were mixed with 50 ml of sterile distilled water. The samples were shacked at room temperature for 30 minutes on a reciprocal shaker at 180 rpm. Thiosulphate agar media was prepared for isolation of neutrophilic sulphur oxidizing bacteria. The media contained 0.1 g L^{-1} of NH_4Cl, 0.05 g L^{-1} of KH_2PO_4, 0.02 g L^{-1} of $MgSO_4$, 0.4

g $^{L-1}$ of 1 g L^{-1} of $Na_2S_2O_3$ and 2% agar. For the isolation of neuphilic sulphur oxidizing bacteria the pH was adjusted to 6.5 with 0.1 M Sodium Hydroxide. Then the sample was diluted in 10 fold and 0.1 ml aliquot was spreaded on same agar plates. The samples were incubated at 30˚C. Isolated colonies were appeared in 10^{-4} dilution after four days of incubation. After cultivation all morphologically different single colonies were streaked in the mentioned agar media to get pure colonies.

Morphological and biochemical characterization:-
The isolated colonies were tested morphologically by gram staining and endospore staining. Glycerol stocks were prepared and their general biochemical characterization tests were performed. After morphological and biochemical characterization, two morphologically and biochemically different bacteria were selected for further studies.

Utilization of organic and inorganic electron donors:-
Heterotrophic growth was checked in Starkey agar media (Vlisedis, 1966)without pH indicator supplied with The same additions in Starkey medium with bromothymol blue containing glucose (5 mM), fructose (5 mm), acetate (20 mM), peptone (0.1 %, w/v) and yeast extract (0.1 %, w/v) for testing for an inhibitory effect of organic compounds on thiosulfate oxidation. The isolates were grown in LB (Luria Bertoni) agar media and nutrient agar media. Bacterial were grown in thiosulfate (20mM), elemental sulphur media to check the utilization of inorganic electron donor (Sievert, 2000).

Heavy metal tolerance test:-
Heavy metal tolerance tests were performed of two isolates followed by the method of *Roychowdhury et al*., 2016. Bacterial strains were streaked in LB agar media containing Arsenic (Sodium Arsenide), Cadmium (Cadmium Chloride), Cobalt (Cobalt Chloride), Copper (Copper Sulphate), Chromium (Potassium Di Chromate), Zinc (Zinc Sulphate), Mercury (Mercuric Chloride). The stock concentration of the heavy metal was 50µg/ml except Mercury, Chromium and Sodium Arsenide. Initial concentration of Mercury was 7.2µg/ml and initial concentration of Chromium and Sodium Arsenide was 12µg/ml.

Toluene degrading activity:-
Toluene degrading ability test was performed followed by the method of Roy *et al*., 2012. Initial concentration of toluene was 1x. MSM (Mineral Salt Media) medium was used for this test containing the mentioned PAH compound. MIC (The Minimum Inhibitory Concentration) was carried out to check in which concentration it inhibits its growth in presence of toluene.

Antibiotic resistivity test:-
Antibiotic sensitivity tests were carried out of the two pure isolated colonies. Bacterial strains were streaked in LB agar media containing tetracycline, rifampicin, ampicillin, streptomycin and choramphenicol and they were incubated at room temperature in a shaker at 180 rpm for 24 hours in presence of respective antibiotics as 1x concentration. Four different types of antibiotics disks were used to check multi drug resistivity test. The disks were brought from Himedia laboratory. Each disk contain six different types of antibiotics. The catalog numbers are – Hexa G minus 2 (Hx007), – Hexa G minus 3 (Hx008), – Hexa G minus 5 (Hx010) and – Hexa G plus 6 (Hx022).

DNA extraction from colonies and PCR amplification for screening:-
25 ml of bacterial cells were harvested by 2 centrifugation steps at 5000-7000 × g, 20 minutes at 4˚C. Most supernatant was removed and remaining supernatant with palette was collected into 2 ml tubes. Again centrifuged and supernatant was removed. 600 µl of sterile distilled water was added and centrifuged after mixing. Supernatant was discarded and 200-300 µl of sterile distilled water was added and the tubes were kept at water bath for 10 minutes at 86˚C. Then the mixture was centrifuged and the supernatant was collected for PCR.

16s rRNA gene sequencing and phylogenetic analysis of a pure culture:-
The nearly full-length 16S gene was amplified by PCR with the bacterial universal primer set 27f and 1492r (Jeremy A. F. 2008). The sequences obtained were compared with those available public databases (NCBI Genbank and DDBJ) with the BLAST system. Multiple sequence alignment was carried out using the software Clustalw (Julie D.Thompson, 1994; http://clustalw.ddbj.nig.ac.jp/).

Nucleotide sequence accession number:-
The 16S rRNA gene sequences of strain SOB1 and N2 have been deposited in the GenBank nucleotide sequence databases under the following accession no. i.e. KT363764 for SOB1 and KY228386 for N2.

Results and Discussions:-

Enrichment and Isolation:-
Two pure unique isolated cultures were obtained by sub culturing single colonies of the lowest dilution from enrichments. After sequencing of the 16S rRNA genes of these isolates, it was obvious that the bacteria were identical. The name of the cultures was given as SOB1 and N2. Both strains were able to grow in both liquid and plate thiosulphate medium and also could grow in modified starkey medium which was enrichment medium for sulphur-oxidizing bacteria. Thiosulphate was used as sole energy source as in general, thiosulphate has been used as the electron donor for isolation of sulphur-oxidizing bacteria (Durand et al., 1993). The bacteria were able to grow in both commercially available Luria Broth agar (LB) and Nutrient agar media as well as could able to grow in fructose (5 mM), glucose (5 mM), acetate (20 mM), lactate (10 mM), peptone (0±1 and 0±01 % w/v) and yeast extract (0±1 and 0±01 % w/v). So, it could able to grow in presence and/or organic compounds. Based on the almost complete sequence of the 16S rRNA gene, strain SOB1 was found to form a polygenetic cluster with *Bacillus pumilus*, *Bacillus sufensis* and *Bacillus cereus*. . Strain N2 formed phylogenetic cluster with *Enterobacter hormaechai* and *Enterobacter sp.* which showed 99% similarities.

Morphology:-
Strain SOB1 was a Gram-positive, non-spore forming, rod-shaped bacterium and strain N2 was gram negative and rod shaped motile bacterium. Cells were able to grow between 3 and 37˚C with optimum growth at 29˚C. Growth observed at pH 6.5-7.5, with optimum growth atpH 7.

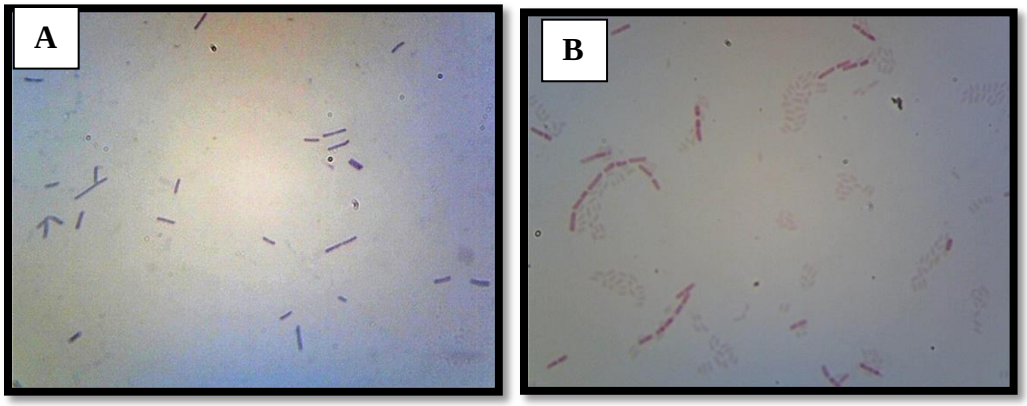

Figure 1:- A: Strain SOB1 was a Gram-positive, non-spore forming, rod-shaped bacterium B: strain N2 was gram negative and rod shaped motile bacterium.

Heavy metal tolerance and antibiotics resistivity test:-
Strain SOB1 and N2 showed growth in presence of multiple heavy metals such as Copper, Zinc, chromium, Arsenic, Cobalt and lead. MIC of heavy metals is given in the following table (table 1).

Bacterial Strains	$CuSO_4.5H_2O$	$ZnSO_4.H_2O$	$K_2Cr_2O_7$	$NaAsO_2$	$HgCl_2$	$CoCl_2$	$Pb(NO_3)_2$
SOB1	2 mM	1.5 mM	2 mM	20 mM	0.2 mM	1 mM	10 mM
N2	2.7 mM	0.5 mM	4.5 mM	10 mM	0.5 mM	3mM	7 mM

Antibiotic resistivity test showed that the bacterium SOB1 was able to grow in the presence ampicillin, tetracycline, rifampicin, and streptomycin, penicillin G, chloramphenicol. It was unable to grow in the presence of vancomycin. Strain N2 was able to grow in presence of ampicillin, tetracycline, rifampicin, penicillin G and vancomycin and was unable to grow in presence of streptomycin and chloramphenicol.

Toluene degradation ability:-

It was observed that strain SOB1 and N2 was able to grow in presence of toluene at initial concentration as it gave thick growth or turbid growth in MSM broth containing toluene. Strain SBO1 gave MIC of toluene at 2 x concentrations and N2 showed MIC of toluene and 4 x concentrations.

Growth curve:-

Growth curve of strain SOB1 and N2 was prepared in SOB medium containing thiosulphate, toluene at the same concentration in which they gave MIC. Here positive control was kept where growth was observed in Luria-Bertani (LB) medium without toluene or thiosulphate. This is a comparative study of the growth of both strains in different medium. The growth phase varied from LB medium to toluene. The growth rate was fast in LB medium compared to thiosulphate and toluene. Lag phage remained for almost 8 hours in SOB and toluene medium. After 8 hours the optical density increased that means the growth shifted towards log/exponential phase. It took almost about 48 hours to reach stationary phase for both strains. In LB medium after 3 hours log phage started and after 30 hours growth shifted towards stationary phase.

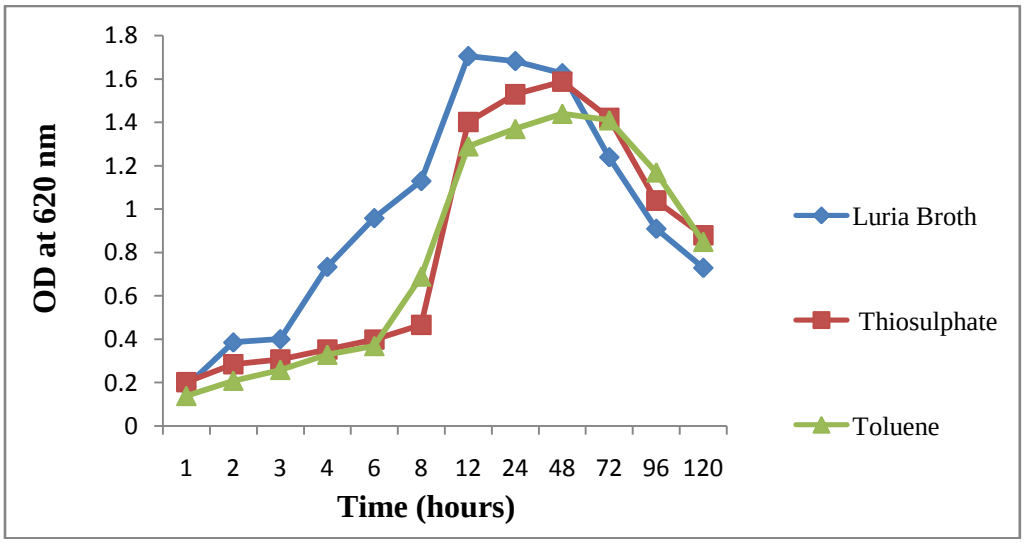

Figure 2:- graphical representation of growth curve of strain SOB1 in three different medium such as SOB broth containing thiosulphate, MSM broth containing toluene and Luria broth as positive control.

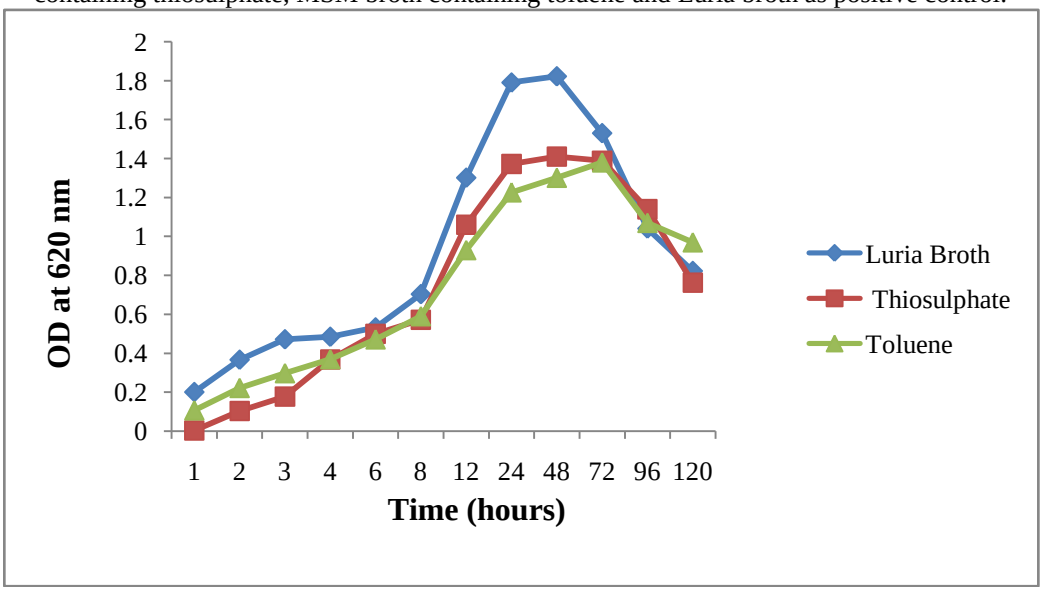

Figure 3:- graphical representation of growth curve of strain N2 in three different medium such as SOB broth containing thiosulphate, MSM broth containing toluene and Luria broth as positive control.

Phylogenetic tree:-

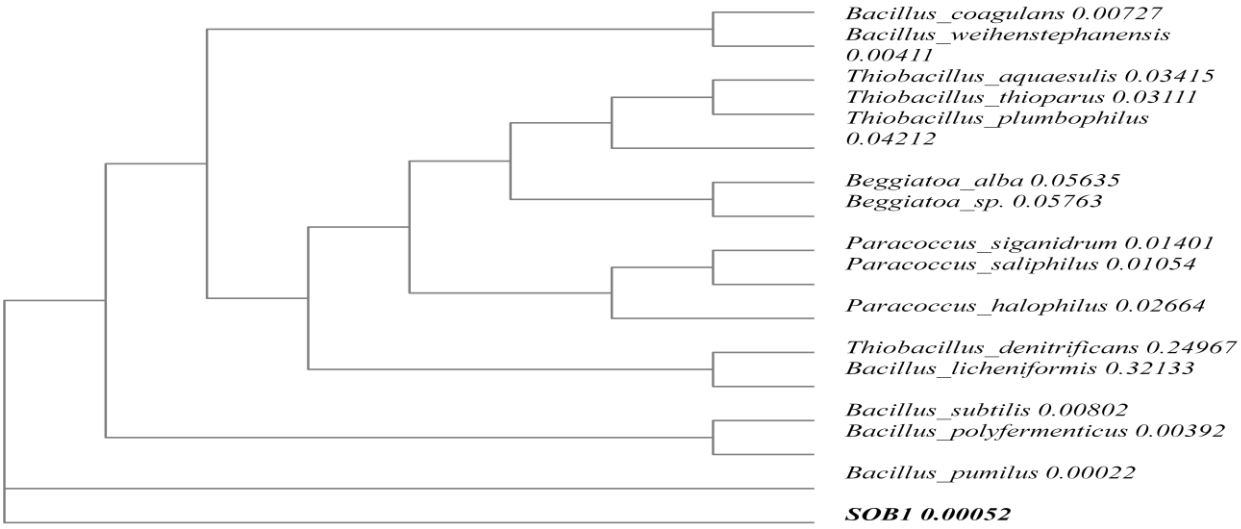

Figure 4:- Phylogenetic tree of strain SOB1, which was found to form a polygenetic cluster with *Bacillus pumilus, Bacillus sufensis* and *Bacillus cereus*.

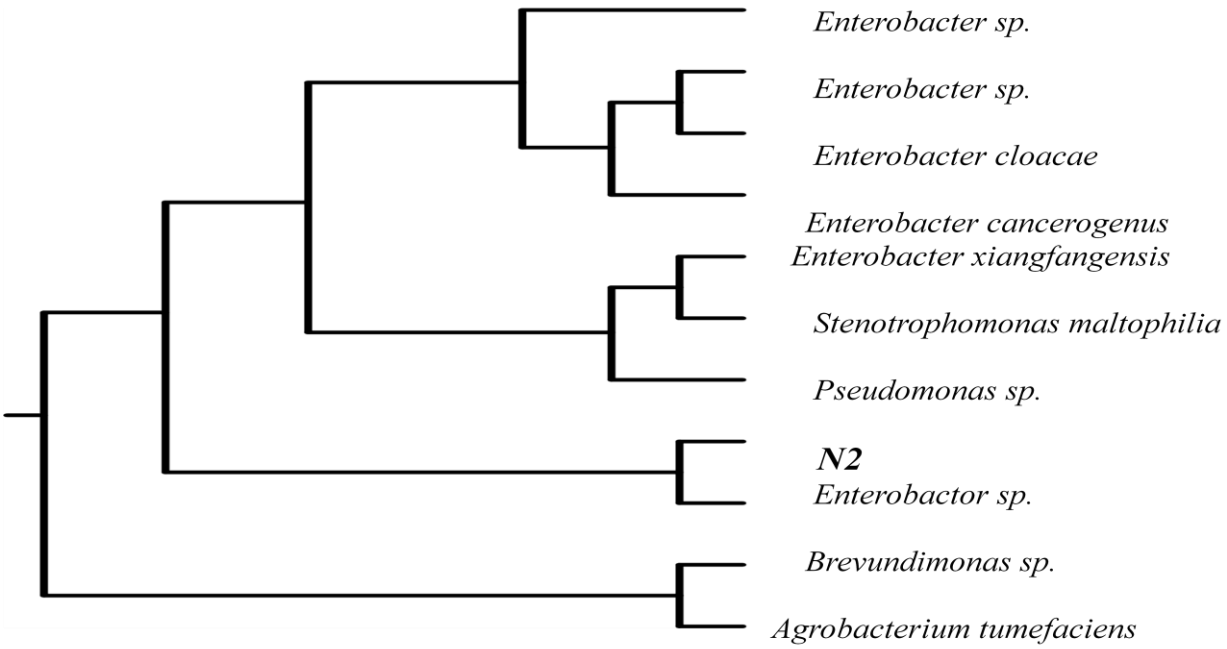

Figure 5:- Phylogenetic tree of N2, which formed phylogenetic cluster with *Enterobacter hormaechai* and *Enterobacter sp.* which showed 99% similarities.

Conclusion:-

In conclusion two morphologically different toluene degrading sulphur-oxidizing bacteria, strain SOB1 and N2, were successfully isolated from Barjora coal mine of West Bengal in India with thiosulfate as energy source and CO_2 as carbon source. They are mesophilic, multi heavy metal and multi drug resistant. Based on the almost complete sequences of the 16S rRNA gene, strain SOB1 showed maximum similarities with *Bacillus sp.* and strain

N2 showed highest similarities with *Enterobacter sp.*. 16s rRNA sequences of SBO1 and N2 were deposited to NCBI and they are now available in NCBI with KT363764 and KY228386 accession numbers.

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