Journal of Environmental Biology
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October 2007, 28(4) 819-823 (2007)
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Isolation of *Thiobacillus* sp from aerobic sludge of distillery and dairy effluent treatment plants and its sulfide oxidation activity at different concentrations

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(Received: December 09, 2005 ; Revised received: May 15, 2006; Accepted: June 17, 2006)

Abstract: *In the present study two strains of Thiobacillus sp were isolated from aerobic sludge of distillery and dairy effluent treatment plant using standard methods of isolation and enrichment. Experiments were conducted using isolated cultures in batch bioreactor with initial sulfide concentration of 75 and 150 mg/l. The effect of initial sulfide concentration on the activity of isolated Thiobacillus sp was studied. Sulfide oxidizing capacity was also determined at different initial sulfide concentrations. The results from the study indicate the possible isolation of Thiobacillus cultures from native source and application in the full-scale reactor.*

Key words: *Sulfur oxidizing bacteria, Sulfide, Isolation, Thiobacillus sp, Dairy and distillery sludge*
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Introduction

Major emission sources of the sulfides into the environment include effluents from distillery, paper and pulp, viscous rayon, tanneries, petrochemical and photographic industries (Kuenen and Roberson, 1988). Anaerobically treated sulfate, sulfite and thiosulfate rich effluents lead to the formation of sulfides in the environment (Rinzema and Lettinga, 1988; Kanna *et al.,* 2005). There is great need to develop processes for sulfide removal from wastewater because of its toxicity, corrosive properties, bad odour and high oxygen demand (WHO, 1981; Nath *et al.,* 2005; Singh, 2007). Sulfide emitted into the environment is of two forms, free sulfides in the form of H_2S and dissolved sulfides in the form of HS^- (Suzuki, 1974). Removal of sulfides from the industrial waste streams is presently being done by chemical methods, which are expensive as well as environmentally not benign.

Organisms belonging to the group of colorless sulfur bacteria oxidize sulfide to elemental sulfur under oxygen limiting conditions. Based on this feature many researchers worked for biological oxidation using various types of microorganisms (Gadre, 1989). The advantage of this biological sulfide oxidation system is that, no chemicals are required except oxygen (Buisman *et al.*, 1990). Use of chemoautotrophs for the oxidation of sulfide is advantageous due to their simple nutritional requirement (Chen and Morris, 1972). In this process chemoautotrophic *Thiobacillus* sp belongs to general class of sulfur oxidizing bacteria (SOB) is used for sulfide oxidation. Certain strains of the sulfur oxidizing bacteria belonging to the genus *Thiobacillus* can oxidize free sulfide to elemental sulfur. During the process they derive energy for growth from the oxidation of reduced sulfur compounds but they are rather sensitive to concentration of sulfide and survive only if the sulfide concentration is low (Buisman *et al.*, 1991). The two most important bioconversions of sulfide oxidation system are (Visser *et al.*, 1997).

$$2HS^- + O_2 \longrightarrow 2S^o + 2OH^- \longrightarrow (1)$$

$$2HS^- + 4O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \longrightarrow (2)$$

In biological sulfide oxidation end product will be produced based on the oxygen concentration. Under oxygen limiting conditions, that is at O_2 concentration below 0.1 mg/l, sulfur is the end product of the sulfide oxidation (Kuenen, 1975), while sulfate is formed under circumstances of sulfide limitation. The formation of sulfur is preferred because it is insoluble and can be easily recovered from the water stream (Kethum, 1995). The formation of end product is not only dependent on the sulfide concentration but also on the amount of oxygen supply to the reactor. This is evident from the following general scheme of biological sulfide oxidation system (Isamu, 1999).

$$S^{2-} \longrightarrow S^o \longrightarrow SO_3^{2-} \longrightarrow SO_4^{2-}$$

Present study was intended to isolate chemoautotrophic sulfide oxidizing bacteria from different native sources and to test their sulfide oxidation capacity at different initial sulfide concentration.

Materials and Methods

Collection of microbial source: Isolation of sulfide oxidizing bacteria was done from aerobic sludge collected from dairy and distillery industry wastewater treatment plant. The aerobic sludge samples were collected and screened for the removal of big particles. The sludge was kept under aerobic conditions by continuous aeration

in order to prevent growth of any anaerobic bacteria for a period of 7 days at a temperature of 30±2°C.

Isolation of *Thiobacillus* sp: The aerated sludge was kept for activation by mixing *Thiobacillus* sp enrichment media (Vishniac and Santer, 1957) having composition NH_4Cl, 1.0; K_2HPO_4, 0.6; $CaCl_2.2H_2O$, 0.2, $FeCl_3.H_2O$, 0.02, $Na_2S_2O_3.5H_2O$, 10; Trace element solution of 10 ml contains $CaCl_2.2H_2O$, 100mg, $ZnSO_4.7H_2O$, 88 mg, $CuSO_4.5H_2O$, 40, $MnSO_4$ 15 mg, $Na_2B_4O_7$, 10, $(NH_4)_6Mo_7O_{24}.4H_2O$ 5 mg, in one liter of double distilled water for a period of 7 days. After 7 days of activation period, media was replaced by fresh media. The process was repeated for five transformations in order to ensure the suppression of growth of any anaerobic bacteria in the sludge and to activate only sulfide oxidizing bacteria.

After acclimatizing the sludge for a period of one month, the sludge was used as source for the isolation of *Thiobacillus* sp The same media defined previously was used for the isolation studies with 15% agar and sterilized at 120°C and 15 lbs pressure for a period of 20 minutes. After reaching to room temperature the sterilized media poured into petriplates. After solidifying the media at room temperature the plates were streaked with loop full of previously activated aerobic sludge. The enrichment plates were incubated at 30±2°C in dark for a period of 7 days to eliminate any carbon fixing photosynthetic contaminants. After seven days of incubation fresh media was prepared and 2 to 3 colonies, grown on petriplates was inoculated and kept in incubator. This process was repeated for 3 times. 100 ml liquid broth was prepared in 250-ml conical flask and sterilized at 120°C and 15 lbs for a period of 20 minutes. The flasks were inoculated with colonies grown on plates and kept in orbital shaker at 250 rpm for a period of 7 days. After seven days fresh media is prepared and inoculated with (20% v/v) into freshly prepared media under same conditions as mentioned earlier. This process was repeated for 4 times in order to get pure cultures of *Thiobacillus* sp. Later the cultures are named as IICT-SOB-DAIRY-201 for the cultures isolated from dairy effluent treatment plant sludge and IICT-SOB-DIST-210 for the cultures isolated from distillery spent wash treatment plant sludge.

Enumeration and characterization: The two 5 days old isolated cultures, were viewed under scanning electron microscopy for various characteristics like size and shape. The results of gram staining for both the isolated *Thiobacillus* sp from their native sources were negative. Standard plate count method was used for the colony count at different serial dilution ranging from 10^{-1} to 10^{-10} and the cell count was in the range of $7x10^5$ and $4x10^7$ cells/ml for IICT-SOB-DAIRY-201 and IICT-SOB-DIST-210 respectively.

Sulfide oxidation activity test: To test sulfide oxidation activity for the two isolated IICT-SOB-DAIRY-201 and IICT-SOB-DIST-210 cultures at different initial sulfide concentrations was carried out in a batch reactor. Isolated *Thiobacillus* cultures were maintained in a maintenance media having composition of $Na_2S_2O_3.5H_2O$, 10 gm; $MgCl_2.6H_2O$, 0.1 gm, NH_4Cl, 01 gm; $CaCl_2$, 0.1 gm; KH_2PO_4, 3 gm in one liter doubled distilled water and adjusting pH to 5 using 1N HCl solution.

Experiments were conducted for sulfide oxidation using two isolated cultures at 75 mg/l and 150 mg/l of initial sulfide concentration in glass reactors having total volume of 600 ml. Each glass reactor was filled with 200 ml of synthetic media consists of maintenance medium components medium define above except $Na_2S_2O_3.5H_2O$. The $Na_2S_2O_3.5H_2O$ was substituted with 75 mg/l and 150 mg/l sodium sulfide. The reactors were inoculated with 20% (v/v) of 5 day old isolated *Thiobacillus* species. All the reactors were supplied with sterile air at a flow rate of 0.75 l/min. Samples from the reactors were drawn at every 24 hr and analyzed for various parameters mentioned below. The operation batch reactor setup was conducted till the sulfide concentration depletes (168 hr).

Analytical methods: Standards methods (APHA, 1998) were used for the analysis of the sulfide, pH, sulfate temperature in the liquid media.

Results and Discussion

Sulfide oxidation by IICT-SOB-DAIRY-201 at different initial sulfide concentration: Fig. 1 shows the oxidation of sulfide and sulfate formation with time by IICT-SOB-DAIRY-201 at two different initial sulfide concentrations in a batch reactor. From the Fig. it is evident that, at both initial sulfide concentrations *i.e.* at 75 mg/l and 150 mg/l, sulfide was almost oxidized to 0 at the end of 168 hr of batch reactor operation. During entire period of batch reactor operation sulfate formation was recorded. In the first 48 hr of reactor operation sulfate formation was, 14 mg/l for 75 mg/l of initial sulfide concentration and 28 mg/l for 150 mg/l of initial sulfide concentration. During the same period sulfide oxidation rate was high compared to sulfate formation rate. The difference in sulfur balance during this phase was because of the sulfide which was immediately converting into elemental sulfur.

The formation of elemental sulfur was observed in the reactor in the form of light yellow precipitation. After 48 hr of batch reactor operation the sulfate formation was increased with the decrease in sulfide concentration in the reactor. At the end of the reactor operation, after 168 hr, the sulfate in the reactor was 40 mg/l for 75 mg/l of initial sulfide concentration and 38 mg/l for 150 mg/l of initial sulfide concentration. At both initial sulfide concentrations the isolated *Thiobacillus* sp had shown similar sulfide oxidation pattern, but sulfate formation was more in case of 75 mg/l of initial sulfide concentration when compared to 150 mg/l of initial sulfide concentration. From the results it was apparent that at higher initial sulfide concentrations, formation of sulfate was less by using the isolated culture, IICT-SOB-DAIRY-201.

Sulfide oxidation by IICT-SOB-DIST-210 at different initial sulfide concentration: Fig. 2 shows the sulfide oxidation and sulfate formation pattern in batch reactor with different initial sulfide concentrations by isolated IICT-SOB-DIST-210. The two batch

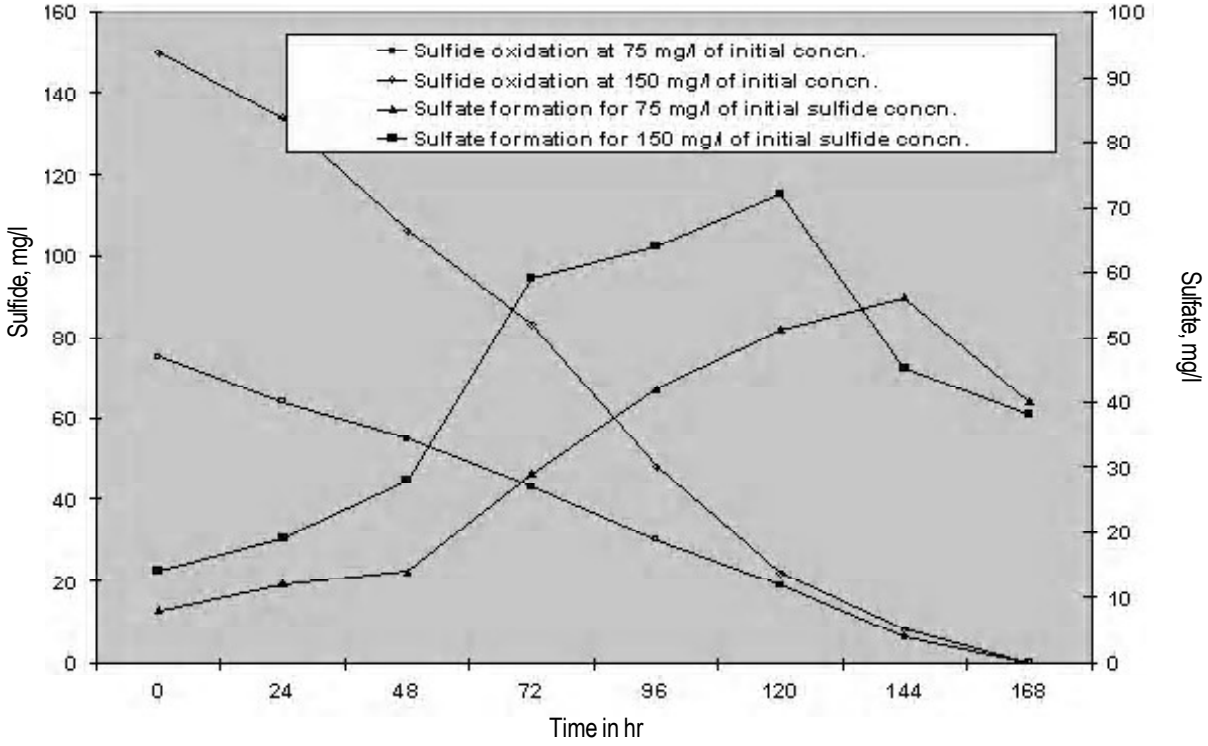

Fig. 1: Oxidation of sulfide at different initial sulfide concentration and formation of sulfate with time using isolated *Thiobacillus* sp IICT-SOB-Dairy-201

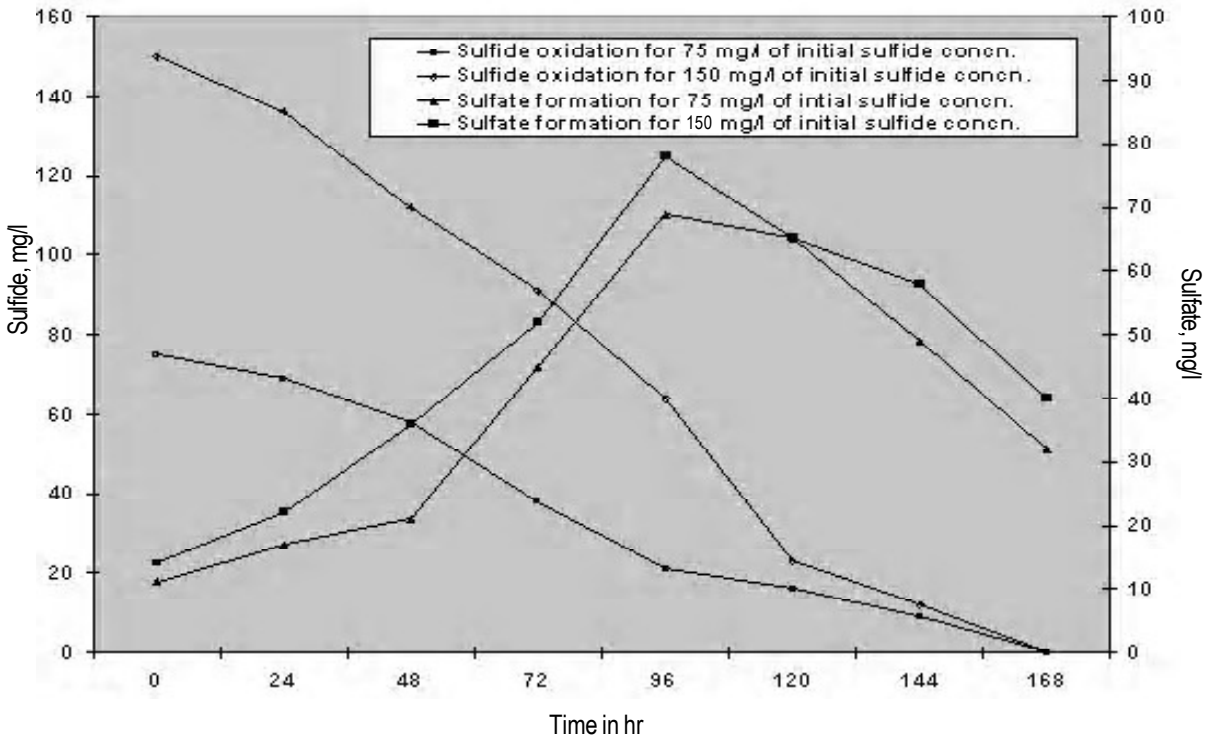

Fig. 2: Oxidation of sulfide at different initial sulfide concentration and formation of sulfate with time using isolated *Thiobacillus* sp IICT-SOB-DIST-201

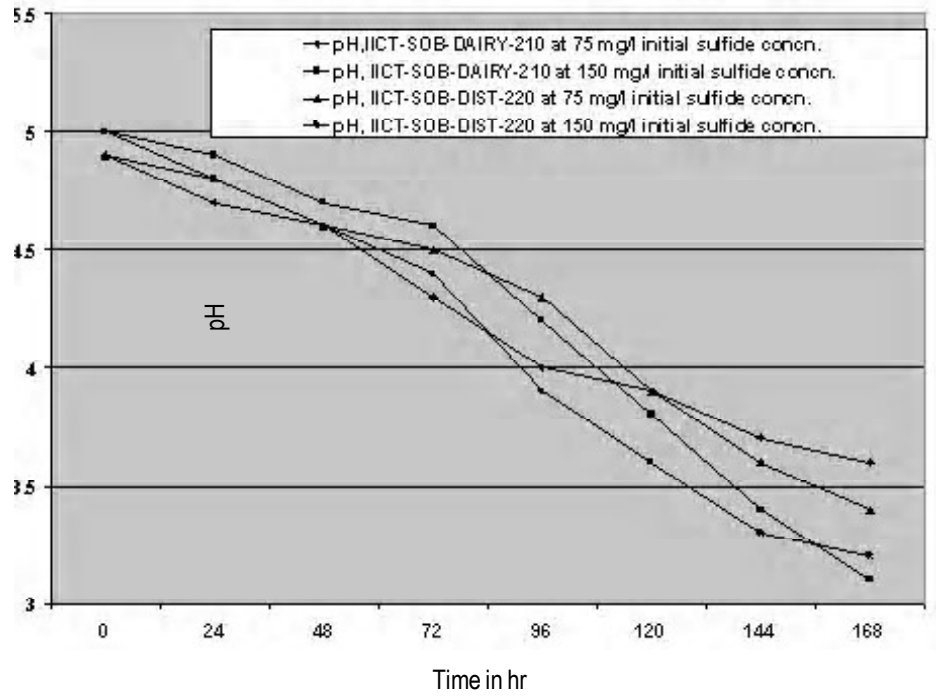

Fig. 3: Change in pH with time for the sulfide oxidation by IICT-SOB-DAIRY-201 and IICT-SOB-DIST-210 at 75 mg/l and 150 mg/l of initial sulfide oxidation

reactors were operated for a period of 168 hr with different initial sulfide concentrations of 75 mg/l and 150 mg/l respectively. The sulfide oxidation pattern for the IICT-SOB-DIST-210 at two different concentrations at the end of the experiment, after 168 hr is shown in Fig. 2. At the end of 168 hr of batch reactor operation, the sulfide was oxidized to zero by showing a similar oxidation pattern at both initial sulfide concentrations. But, sulfate formation pattern was varied for different initial sulfide concentrations. During the initial period of operation, sulfide oxidation was high compared to sulfate formation. The sulfate formation in both reactors, at the end of 168 hr, was 32 mg/l and 40 mg/l at 75 mg/l and 150 mg/l initial sulfide concentration respectively. The difference in sulfur balance during this phase was because of rapid oxidization of sulfide to elemental sulfur. The formation of elemental sulfur was observed in the reactor in the form of light yellow precipitation. After this period the sulfate formation was increased with the decrease in sulfide concentration in the reactor. When compared Fig. 1 and 2 the oxidation pattern of sulfide is similar for different initial sulfide concentrations using isolated *Thiobacillus* cultures.

Variation in pH in a batch reactor using isolated IICT-SOB-DAIRY-201 and IICT-SOB-DIST-210 at different initial sulfide concentration: Fig. 3 shows the variation in pH in batch bioreactors during sulfide oxidation with time by isolated IICT-SOB-DAIRY-201 and IICT-SOB-DIST-210**.** Initially, the pH for 75 mg/l of initial sulfide concentration was 4.9 and 5 for 150 mg/l of initial sulfide concentration. As the reactor operation progress, the pH was decreased and at the termination of the reactor operation *i.e.* after 168 hr, it reached to 3.6 and 3.1 for 75 mg/l and 150 mg/l of initial sulfide concentration respectively for IICT-SOB-DAIRY-201. In the case of IICT-SOB-DIST-210 it was 3.4 and 3.2 at 75 mg/l and 150 mg/l of initial sulfide concentration respectively. Only a limited number of biological oxidation of sulfide to sulfur and sulfate reactions will precede in a sulfide-removing reactor and the microorganisms are capable of switching within two hr from sulfur to sulfate and then sulfuric acid formation (Janssen *et al*., 1995; Rao *et al*., 2006). During the batch operation initially the sulfide was oxidized to sulfur and as the concentration of sulfide decreases the microbes start using the elemental sulfur as energy source for their metabolism. During this period sulfate was formed and once sulfur deplete in the reactors the isolated cultures shift to sulfate for their survival and resulting in sulfuric acid formation. This is evidenced by the variation in pH during the batch bioreactor operation.

The study indicates that isolation and enrichment of SOB's from different sources is highly essential for enhanced performance of the reactor. At lower initial sulfide concentrations formation of sulfate will be more and at higher initial sulfide concatenations sulfate formation is less. In batch reactor when the sulfide concentration decreases more sulfate will be formed and ultimately the sulfate oxidizes and system turns to acidic phase. Further experiments can be done on continuous basis to estimate exact nature of isolated cultures to terminate operation to get sulfur as end product.

Biological waste water treatment system are most suitable for the removal of pollutants emited from industries like paper and pulp mill. Biological processes are more economical and eco friendly

than advance wastewater treatment due to its low running cost (Singhal *et al.,* 2005; Bishnoi *et al.,* 2006; Ugoji and Aboaba, 2004). Autotrophic *Thiobacillus* sp are used in the removal of hydrogen sulfide (gaseous phase) (Rao *et al.*, 2006) and oxidation of metallic sulfides of mineral ores (solid form) (Fomchenko *et al.*, 2003).

Acknowledgments

Authors are grateful to Dr. J.S. Yadav, Director, IICT, for his encouragement. One of the authors, P.Ravichandra, acknowledges CSIR, New Delhi, for senior research fellowship.

References

APHA.: Standards methods for examination of water and wastewater. 20th Edn. Washington D.C., USA (1998).

Bishnoi, N.R., R.K. Khumukcham and R. Kumar: Biodegradation of pulp and paper mill effluent using anaerobic followed by aerobic digestion *J. Environ. Biol.,* **27**, 405-408 (2006).

Buisman, C.J.N., B.G. Geraats, P. Ijspreet and G. Lettinga: Optimization of sulfur production in a biotechnological sulfide removing reactor. *Biotechnol. Bioeng.*, **35**, 50-56 (1990).

Buisman, C.J.N., P. Ijspreet, A. Huf, A.J.H. Janssen, R. Ten Hagen and G. Lettinga: Kinetic parameters of mixed culture oxidizing sulfide and sulfur with oxygen. *Biotechnol. Bioeng.,* **38**, 813-820 (1991).

Chen, K.Y. and J.C. Morris: Kinetics of oxidation of aqueous sulfide by O_2. *Environ. Sci. Technol.,* **6**, 529-537 (1972).

Fomchenko, N.V., O.V. Slavkina and V.V. Biryukov: Biological oxidation of sulfide raw material using a culture of *Thiobacillus ferrooxidans* under various conditions of leaching. *Appl. Biochem. Microbiol.*, **39**, 82-86 (2003).

Gadre, R.V.: Removal of hydrogen sulfide from biogas by chemoautotrophic fixed film reactor. *Biotechnol. Bioeng.*, **34**, 410-414 (1989).

Isamu, Suzuki: Oxidation of inorganic sulfur compounds: Chemical and enzymatic reactions. *Can. J. Microbiol.*, **45**, 97-105 (1999).

Janssen, A.J.H., R. Sleyster, C. Van der Kass, A. Jochemsen, J. Bontsema, and G. Lettinga: Biological sulfide oxidation in fed batch reactor. *Biotechnol. Bioeng.*, **47**, 327-333 (1995).

Kannan, V., R. Ramesh and C. Sasikumar: Study on ground water characteristics and the effects of discharged effluent from textile units at Karur District. *J. Environ. Biol.,* **26**, 269-272 (2005).

Kethum, P.A.: Microbiology: Concepts and Applications. John Wiley and Sons, Oakland, USA (1995).

Kuenen, J.G.: Colour less sulfur bacteria and their role in the sulfur cycle. *Plant Soil*, **43**, 49-76 (1975).

Kuenen, J.G. and L. A. Roberson: The use of natural bacterial populations for the treatment of sulfur compounds containing wastewater. *Biodegradation*, **3**, 239-254 (1992).

Nath, Kamlesh, Sonia Saini and Yogesh Kumar Sharma: Chromium in tannery effluent and its effect on plant metabolism and growth. *J. Environ. Biol.,* **26**, 197-204 (2005).

Rao, A.G., P. Ravichandra and Annapurna Jetty: Effects of humidification and inlet hydrogen sulfide concentration on the performance of biofilter operated with mixed agricultural residue as filter material. *Chem. Biochem. Eng. Qual.*, **20**, 171-178 (2006).

Rinzema, A. and G. Lettinga: Anaerobic treatment of sulfate containing waste water. *In*: Biotreatment systems (*Ed.*: D.L. Wise). Vol. 3, CRC Press, Boca Raton, FL. pp. 65-109 (1988).

Singh, Pratibha: Sequential anaerobic and aerobic treatment of pulp and paper mill effluent in piliot scale bioreactor. *J. Environ. Biol.*, **28**, 77-82 (2007).

Singhal, V., A. Kumar and J.P.N. Rai: Bioremediation of pulp and paper mill effluent with *Phanerochaete chrysosporium. J. Environ. Biol.,* **26**, 525-529 (2005).

Suzuki, I.: Mechanism of inorganic oxidation and energy coupling. *Annu. Rev. Microbiol.*, **28**, 85-101 (1974).

Ugoji, E.O. and O.O. Aboaba: Biological treatments of textile industrial effluents in Lagos metropolis, Nigeria. *J. Environ. Biol.,* **25**, 497-502 (2004).

Vishniac, W. and M. Santer: The Thiobacilli. *Bacteriological. Rev.*, **21**, 185-213 (1957).

Visser, J.M., L.A. Roberstson, H.W.V. Verseveld and J.G. Kuenen: Sulfur production by obligately chemolithoautotrophic *Thiobacillus* species. *Appl. Environ. Microbiol.*, **63**, 2300-2305 (1997).

WHO.: H_2S, Environmental Health Criteria, 19, WHO, Geneva (1981).