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Continuous Operation of Fluidized Bed Bioreactor for Biogenic Sulfide Oxidation Using Immobilized Cells of *Thiobacillus* sp.

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Abstract: In the present study, obligate autotrophic *Thiobacillus sp.* was isolated from aerobic sludge distillery effluent treatment plant and the experiments were conducted in a fluidized bed bioreactor for the biological oxidation of sulfide using Ca-alginate immobilized *Thiobacillus sp.* All the experiments were conducted in continuous mode at different sulfide loading rates 0.018, 0.02475, 0.03375, 0.03825 and 0.054 and different hydraulic retention times 5, 3.67, 2.67, 2.35 and 1.67 h by varying flow rates 2.4×10^{-4}, 3.3×10^{-4}, 4.5×10^{-4}, 5.1×10^{-4} and 7.2×10^{-4}. Sulfide conversions higher than 90% were obtained at almost all sulfide loading rates and hydraulic retention times. All the experiments were conducted at constant pH of around 6 and temperature of 30±5°C.

Key words: Autotrophic bacteria, sulfide oxidation, *Thiobacillus sp.*, fluidized bed reactor, immobilization

INTRODUCTION

Major emission sources of sulfide into the environment include anaerobic treatment of wastewater containing partially oxidized components e.g. thiosulfate, sulfite or sulfate leads to the reduction of these compounds to sulfide also called as biogenic sulfide. Besides, effluents from various processing industries like distillery, paper and pulp, viscous rayon, tanneries, petrochemicals, photographic contains sulfides (Kuenen and Roberrson, 1992). There is great need to develop processes for sulfide removal from wastewater because of its toxicity, corrosive properties, bad odor and high oxygen demand (WHO, 1981). 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 sulfide from the industrial waste streams is presently being done by chemical methods, which are expensive as well as environmentally not benign. Under the present circumstances sulfide oxidation using biotechnological methods is the best suitable alternative. Organisms belonging to the group of colorless sulfur bacteria oxidize sulfide to elemental sulfur under circumstances of 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 no chemicals are required except oxygen (Buisman *et al*., 1990). 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 only survive if the sulfide concentration is low (Buisman *et al*., 1991). Use of chemoautotrophs for the oxidation of sulfide is advantageous due to their simple nutritional requirement (Chen and Morris, 1972).

The two most important bioconversions of sulfide oxidation system are (Visser *et al*., 1997).

$$2HS^- + O_2 \rightarrow 2S^\circ + 2OH^- \quad (1)$$

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

Various researchers have been studied the oxidation of sulfide in reactors like CSTR, packed bed reactor using isolated strains (Janssen *et al*., 1997; Cees *et al*., 1990;

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Cees *et al*., 1991). During biological sulfur cycle sulfide converts into sulfide by sulfur oxidizing bacteria. Organisms will oxidize sulfide until it runs out (Reaction 1) and then begin utilizing elemental sulfur (Reaction 2). This is logical, since more energy can be acquired from oxidizing sulfide compared to elemental sulfur. Since sulfur cannot dissolve in water it is easy to eliminate from the environment. In biological sulfide oxidation based on the oxygen concentration end product will be produced. Under oxygen limiting conditions, that is at O_2 concentration below 0,1 mg L^{-1}, 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 depending on the sulfide concentration but also on the amount of oxygen supply to the reactor. This is evident by from the following general scheme of biological sulfide oxidation system (Isamu, 1999).

$$S^{2-} \rightarrow S^{\circ} \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$$

Many processes have been practiced traditionally, embodying the basic principle of microbial conversions offered by cells bound to surfaces. Waste treatment in trickling filters and ethanol oxidation to produce vinegar are but a few examples of such processes. Immobilization of cells is the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrates, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. Therefore it is expected that the microenvironment surrounding the immobilized cells do not necessarily their free-cell counterparts experience the same.

Immobilization commonly is accomplished using a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose, etc. In these cases, the cells are immobilized by entrapment in the pertinent gel by a drop-forming procedure. When traditional fermentations are compared with the microbial conversions using immobilized cells, the productivity obtained in the latter is considerably higher, obviously partly due to high cell density and immobilization-induced cellular or genetic modifications. Nevertheless, a few critical parameters such as the cost of immobilization, mass transport limitations, applicability to a specific end-product, etc. are to be carefully examined before choosing any particular methodology.

Present study was intended to isolate chemoautotrophic sulfide oxidizing bacteria, *Thiobacillus* sp. Further experiments were conducted in a fluidized bed bioreactor using Ca-alginate immobilized cells of *Thiobacillus* sp.

MATERIALS AND METHODS

Collection of microbial source: Isolation of sulfide oxidizing bacteria is done from aerobic sludge collected from distillery industry wastewater treatment plant. The aerobic sludge samples of aforesaid industry were collected and screened for the removal of big particles. Then the sludge is kept in aerobic conditions by continuous aeration in order to prevent growth of any anaerobic bacteria for a period of 7 days at temperature of 30±2°C.

Isolation of *Thiobacillus* sp.: The sludge is then kept for activation by mixing Sulfide Oxidizing Bacteria (SOB), *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. The sludge is kept for aeration with above said medium for a period of 7 days and after 7 days of activation period, media is replaced by fresh media. The aforesaid process is repeated for five transformations. This is to ensure the suppression of growth of any anaerobic bacteria in the sludge and to activate only sulfide oxidizing bacteria.

After acclimatizing the sludge to *Thiobacillus sp.* media for a period of one month the sludge is used as source for the isolation of SOB of Thiobacillus sp. The media, which is used for the activation of SOB, has been used for the isolation studies. Solid media with 15% of agar is prepared and sterilized at 120°C and 15 lbs pressure for a period of 20 min. After reaching to room temperature the sterilized media poured in to Petri plates. After solidifying the media at room temperature the plates are streaked with loop full of previously activated aerobic sludge.

These enrichment plates are kept in incubator at a temperature of 30±2°C in dark to eliminate any carbon fixing photosynthetic contaminants for a period of 7 days. After seven days of incubation fresh media is prepared and the 2 to 3 colonies, grown in previous step is inoculated and kept in incubator, this process is repeated for 3 times. Then 100 mL liquid broth is prepared (without agar) and transferred into 250 mL conical flasks and sterilized at 120°C and 15 lbs for a period of 20 min. After

cooling the media to room temperature flasks are inoculated with the colonies grown in previous step. The flasks are kept in an 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 is repeated for 4 times in order to get pure cultures of *Thiobacillus* sp.

ENUMERATION AND CHARACTERIZATION

Then the two isolated SRB strains, 5 Days old cultures are viewed under scanning electron microscopy for various characteristics like size and shape.

Immobilization of *Thiobacillus* sp. cultures: The isolated *Thiobacillus* sp. cultures grown in 500 mL of thiosulfate media were centrifuged at 7500 rpm for 10 min. After centrifugation the bacterial cells were separated by decanting the supernatant aseptically and stored in a vial. The bacterial cell pellet is washed with sterile double distilled water for 3 times. The bacterial cell pellet was weighed and 20 mg of cells were added to sodium alginate solution. The 4% sodium alginate solution was prepared by adding 4 g of high viscous sodium alginate in 100 mL of *Thiobacillus* sp. growth media by continuous stirring till the sodium alginate is completely mixed in solution. The beads are prepared with a peristaltic pump using a pipe of 2 mm diameter in $CaCl_2$ solution. The 4% $CaCl_2$ crosslinking solution was prepared by dissolving 6 g of $CaCl_2$ in 150 mL of double distilled water. The beads were allowed in crosslinking solution for 3 h. Later beads were transferred to 150 mL of maintenance medium containing NH_4Cl, 4 g L^{-1}, $MgSO_4.7H_2O$, 1 g L^{-1}, KH_2PO_4, 2 g L^{-1} and 10 mL of trace element solution for over night. The whole process is carried out in aseptic conditions to prevent any possibility of contamination.

Fluidized bed reactor construction: Fluidized bed reactor made up of glass column has been used in the present experiments. The reactor specifications are given in Table 1.

Reactor start up and operation: Figure 1 shows the schematic setup of continuous fluidized bed bioreactor with immobilized cells of *Thiobacillus* sp. The reactor was filled with immobilized beads prepared in the previous step along with the maintenance media having composition NH_4Cl, 4 g L^{-1}, $MgSO_4.7H_2O$, 1 g L^{-1}, KH_2PO_4, 2 g L^{-1} and 10 mL L^{-1} of trace element solution and 20 g of $NaHCO_3$ as an initial buffer was transferred to the reactor.

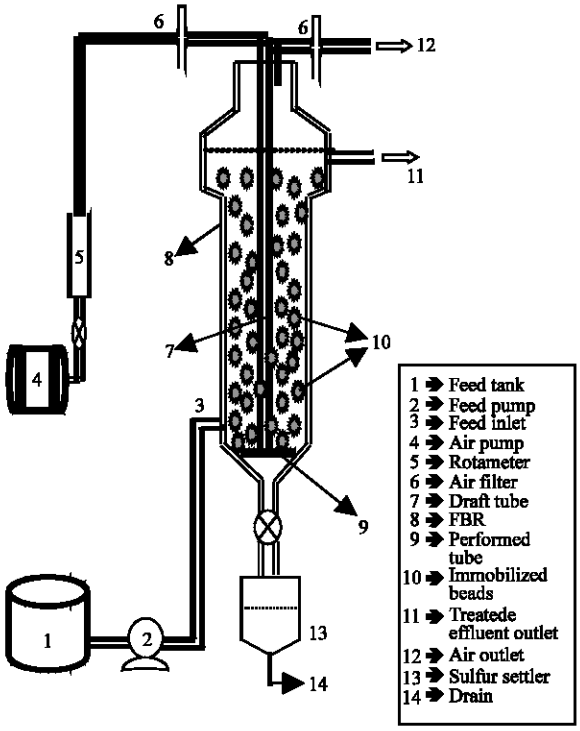

Fig. 1: Schematic setup of continuous fluidized bed bioreactor with immobilized cells of *Thiobacillus* sp.

Table 1: Fluidized bed reactor specifications and operational parameters

Unit/component	Dimension
Height of the FBR	54 cm
Internal Diameter of the FBR	6 cm
Effluent inlet and outlet port diameter	8 mm
Draft tube height	52 cm
Draft tube diameter	6 mm
Total volume of the reactor	1500 mL
working volume of the reactor	1200 mL
Packed bed height	16 cm
Fluidized bed height	48 cm
Minimum fluidization air flow rate	1.44×10^{-4} m^3 h^{-1}
Air filter, PTFE, GELMAN	0.2 μM
Peristaltic pump, Watson marlow	2-240 rpm
Feed pipe internal diameter	6 mm
Rotameter, Engineers India ltd	0.5-5 m^3 h^{-1}
Feed tank, Glass made	15 L
Air pump,	0.5×10^{-4} to 4×10^{-4} m^3 h^{-1}
Temperature	30±5°C
Initial pH	6.2
Average immobilized beads size	~2 mm

The packed bed height of the beads before starting the reactor was 16 cm. The reactor was fed with maintenance medium continuously by the help of peristaltic pump for a period of 4 days at a HRT of 5 h and during this period the beads were maintained in fluidized state by supplying sterile air at a flow rate of 1.44×10^{-4} m^3 h^{-1} with the help of air pump. After

acclimatizing the immobilized beads with maintenance medium for 4 days it was gradually replaced with synthetic effluent having initial sulfide concentration of 75 mg L^{-1} along with other nutrients as defined in previous section. The total replacement of maintenance medium was done after 20 h of reactor startup. The maintenance medium replacement with synthetic effluent was done at every five hrs intervals a step increment 20%.

Varying effluent inlet flow rates without changing initial sulfide concentration in the effluent varied the sulfide loading rates to the reactor. The reactor was operated at five different inlet flow rates, viz., 2.4×10^{-4}, 3.3×10^{-4} 4.5×10^{-4}, 5.1×10^{-4} and 7.2×10^{-4} m^3 h^{-1} which resulted in HRT's of 5, 3.63, 2.67, 2.35 and 1.67 h. This corresponds to the respective sulfide loading rates of 0.018, 0.024, 0.03375, 0.03825 and 0.054 g S m^{-3} h^{-1}. The samples are drawn at 24 h frequency and analyzed for pH, Temperature, DO, sulfate sulfide and thiosulfate.

Analytical methods: Standards methods (APHA, 1998) are used for the analysis of pH, Temperature, DO, sulfate, sulfide, sulfur and thiosulfate.

RESULTS AND DISCUSSIONS

Scanning Electron Microscopic (SEM) analysis of immobilized Ca-alginate beads: Sample of immobilized beads were taken from the reactor column in order to verify, by means of scanning microscopy analyses, whether the microbial immobilization had occurred in the Ca-Alginate matrix. Analyses were performed on cut section of the immobilized beads using an SEM at 10 kV and 7000 magnifications.

SEM of cross section through bead stocked with *Thiobacillus* sp. showing growth throughout particle not just at periphery. Figure 2 shows the cross sectional SEM image of Ca-alginate immobilized *Thiobacillus* sp. beads.

Enumeration and characterization: Standard plate count method is used for the colony count at different serial dilution ranging from 10^{-4} to 10^{-10} and the cell count is in the range of 7×10^5 for isolated *Thiobacillus* sp. The result of gram staining for the isolated *Thiobacillus* sp. was negative.

Sulfide oxidation in continuous Fluidized Bed Bioreactor (FBR): So far, little attempt was made to study the biological oxidation of sulfide in a fluidized bed reactor and almost there are no reports about the use of Ca-alginate immobilized cells of *Thiobacillus* sp. for sulfide oxidation in a fluidized bed reactor. An important reason for not using free cell reactor in sulfide oxidation process

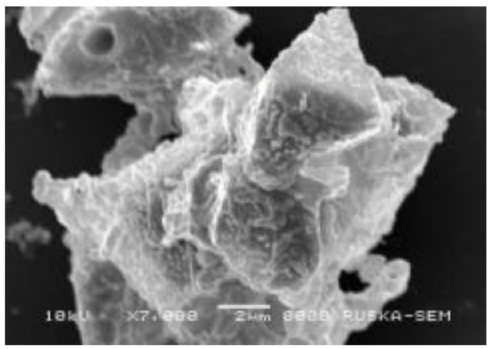

Fig. 2: Cross sectional SEM image of Ca-Alginate immobilized Thiobacillus

is to prevent the formation of sulfate by oxidation of elemental sulfur by free cells of *Thiobacillus* sp.

A laboratory scale fluidized bed reactor was operated in continuous mode using Ca-alginate immobilized cells of isolated *Thiobacillus* sp. The results of typical continuous FBR with immobilized cells of *Thiobacillus* sp. are shown in Fig. 3-5.

Figure 3 shows the sulfide oxidation pattern in the FBR during 38 days of operational period. During the entire operational period fluidization of immobilized beads was done by supplying sterile air at a constant flow rate of 1.443×10^{-4} m^3 h^{-1} and this flow rate was considered as minimum fluidization flow rate.

Initially the FBR was fed with 0.018 g Sm^{-3} h^{-1} at a flow rate of 2.4×10^{-4} m^3 h^{-1}. The HRT to the reactor was 5 hrs. The reactor has reached to a stable 90% sulfide oxidation on 9th day of reactor operation. At this stage sulfide loading rate to the reactor was varied, 0.02475 g Sm^{-3} h^{-1}, by changing the flow rate 3.3×10^{-4} m^3 h^{-1} with a change in HRT of 3.67 h from 5 h. The reactor has taken 7 days to reach a stable sulfide oxidation of more than 90% from 10th day to 17th day. From 18th day sulfide loading rate was further increased to 0.00375 g Sm^{-3} h^{-1} and operated till it reaches (25th day) a stable oxidation rate of more than 90%. Similarly sulfide-loading rate to the reactor further varied 0.03825 g Sm^{-3} h^{-1} and 0.054 g Sm^{-3} h^{-1} and attained a stable removal rate of more than 90% at 30th and 38th days, respectively.

Sulfide oxidation in the system has shown almost a similar pattern throughout the operational period and could attain a maximum of 92% at all sulfide loading rates and hydraulic retention times.

Sulfate and thiosulfate formation in FBR: Figure 4 shows the sulfate and thiosulfate formation in the FBR during

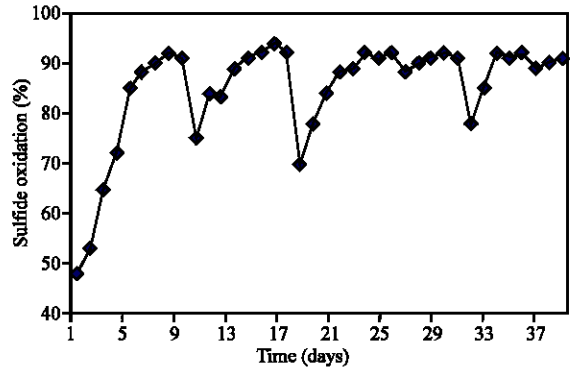

Fig. 3: Variation in % sulfide oxidation in FBR with immobilized cells of Thiobacillus sp. at different HRT's

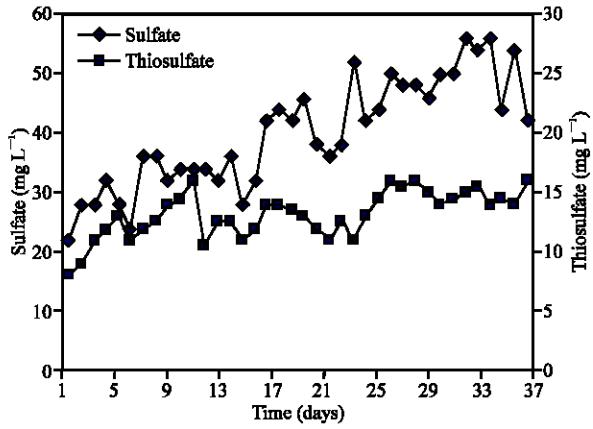

Fig. 4: Sulfate and thiosulfate formation in FBR with immobilized cells of Thiobacillus sp. at different HRT's

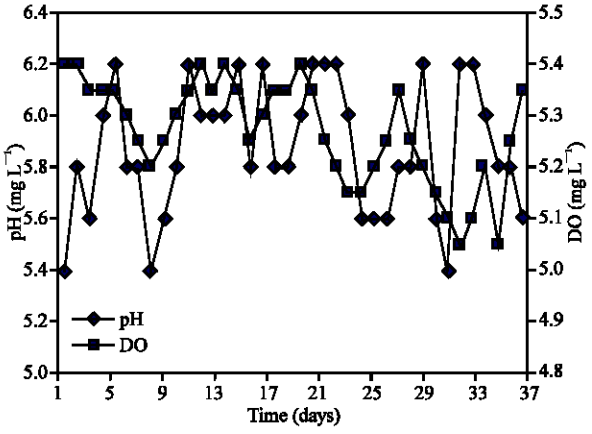

Fig. 5: Variation in pH and DO in FBR with immobilized cells of Thiobacillus sp. during entire period operation

entire period of operation. The sulfate and thiosulfate pattern was almost followed a uniform trend for the entire 38 days of operation at all sulfide loading rates and hydraulic retention times. Formation of sulfate in a biological sulfide oxidation process is all depends on the amount of oxygen supplied and it follows the reaction explained in the previous steps.

The biological formation of thiosulfate will not occur in sulfide oxidizing system but oxidation sulfide under the oxygen limiting conditions. However it can be excluded that the formation of the thiosulfate resulted from the sulfide auto oxidation process. The sulfate and thiosulfate formation in the reactor was in the range of 16-30 and 11-29 mg L^{-1}, respectively.

Since all the experiments were conducted at constant O_2 supply rate, the sulfate and thiosulfate formation can best studied under varied dissolved oxygen conditions. Further, thiosulfate formation may increase at higher sulfide loading rates and lower hydraulic retention time because of chemical oxidation of sulfide.

Variation in pH and DO in the reactor: Figure 5 shows variation pH of the system and change DO in the reactor. Since the air supply was done at a constant flow rate the DO in the system was maintained almost constant. Uniform pH pattern in the reactor shows the least possibilities of formation of sulfide in to sulfuric acid by aerobic oxidation.

Sulfur formation: At the end of the reactor operation the content in the system was drained from the bottom and filtered for elemental sulfur. The filterate was dried in oven at 80°C for 2 h and weighed. The sulfur formed for the entire 38 days of reactor operation was around 8 mg (dry weight). The elemental sulfur was pale yellow in colour having crystalline structure.

CONCLUSIONS

Isolation, enrichment and acclimatization of autotrophic Thiobacillus sp. from different sources is essential for enhanced performance of the sulfide oxidation process. Sulfide is the primary source for the *Thiobacillus* sp. in sulfide oxidation process and elemental sulfur is the immediate oxidative product. If sulfide concentration depletes in the system the *Thiobacillus* sp. start feed on elemental sulfur for its food source which results in high sulfate formation. Continuous operation fluidized bed reactor with immobilized cells of *Thiobacillus* sp. reduces the possibilities of sulfate formation during the sulfide oxidation process.

Although a complete sulfur balance was not made on the reactor system the results of sulfide oxidation, sulfate, sulfur and thiosulfate formation shows that most of the sulfur (65-75%) in the system is converting into elemental sulfur.

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