

INHIBITION OF BACTERIAL ACTIVITY IN ACID MINE DRAINAGE

by

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ABSTRACT

Acid mine drainage water give rise to rapid growth and activity of an iron- and sulphur- oxidizing bacterium *Thiobacillus ferrooxidans* which greatly accelerate acid producing reactions by oxidation of pyrite material associated with coal and adjoining strata. The role of this bacterium in production of acid mine drainage is described.

This study presents the data which demonstrate the inhibitory effect of certain organic acids, sodium benzoate, sodium lauryl sulphate, quarternary ammonium compounds on the growth of the acidophilic aerobic autotroph *Thiobacillus ferrooxidans*. In each experiment, 10 milli-litres of laboratory developed culture of *Thiobacillus ferrooxidans* was added to 250 milli-litres Erlenmeyer flask containing 90 milli-litres of 9-k media supplemented with $\text{Fe SO}_4 \cdot 7 \text{H}_2\text{O}$ and organic compounds at various concentrations. Control experiments were also carried out. The treated and untreated (control) samples analysed at various time intervals for Ferrous Iron and pH levels.

Results from this investigation showed that some organic acids, sodium benzoate, sodium lauryl sulphate and quarternary ammonium compounds at low concentration (10 -2 M, 10-50 ppm concentration levels) are effective bactericides and able to inhibit and reduce the Ferrous Iron oxidation and acidity formation by inhibiting the growth of *Thiobacillus ferrooxidans* is also discussed and presented

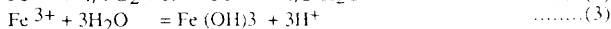
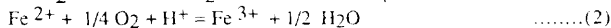
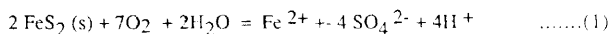
INTRODUCTION

Acid mine drainage (AMD) gives rise to several problems of environmental degradation- especially pollution of aqueous environment. The adverse effects of AMD are many and re potentially serious. Water quality deterioration [1, 2] and corrosion of mining

equipment [3,4] are of much concern to coal mining authorities in the North Eastern Coalfield (NEC) of India where ecology of the surrounding region is badly disrupted. Other undesirable effects are loss of recreational facilities, aquatic life and involvement of additional expenses necessary to prepare the effected water for useful purposes i.e. potable, domestic, industrial, irrigational and recreational.

CHEMISTRY OF ACID MINE DRAINAGE FORMATION AND CONTROL

Prevention and /or control of AMD depends upon an understanding of the chemical, biological and geological factors [5]. Earlier studies [6-8] have described the nature and occurrence of Acid Mine Drainage in North Eastern Coalfield of India. A series of chemical reactions given below describe AMD that results from exposure and weathering of pyritic material (FeS_2) normally present in coal and the associated strata to the combined effects of atmospheric oxygen, water and iron- and sulphur- oxidizing bacteria such as *Thiobacillus ferrooxidans*.



The stoichiometry of equation (1) shows that one mole of FeS_2 produces two moles of acid (H^+). In turn Fe^{2+} generated by reaction (1) can readily oxidize into Fe^{3+} and produces additional three moles of acid (Equation 3). The net result is that for every mole of pyrite oxidized, four equivalent of acid (H^+) are produced. Although this summary is correct at pH above about 4.0, it is only one of three different reactions systems, which vary in significant with pH [9].

At the near neutral pH (stage 1), the rates of oxidation by air and *Thiobacillus ferrooxidans* are comparable. This stage is typical of freshly exposed coal or refuse. Despite the high concentration of pyrite, the rate of oxidation either by oxygen or by *Thiobacillus ferrooxidans* is relatively low and neutral alkalinity of the ground water may effectively neutralized the acid formed at this stage.

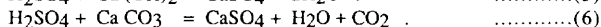
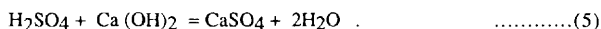
When the neutralizing capacity of the environment is exceeded acid begins to accumulate and then pH decreases (stage 2). As the pH decreases, the rate of iron oxidation by *Thiobacillus ferrooxidans* increases. The action of the bacteria causes increased acid production which serves to further lower pH.

As the pH in the vicinity of the pyrite falls to than 3, the increased solubility of iron and the decreased rate of $\text{Fe}(\text{OH})_3$ (yellow boy) precipitation affects the overall rate of acid production (stage 3). At this point, ferrous iron is oxidized by *Thiobacillus ferrooxidans* and the ferric -iron in turn oxidizes the pyrite.:

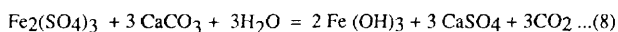
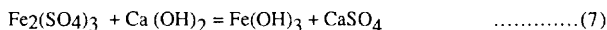


In the third stage, the rate of acid production is high and is limited by the concentration of ferric ions. Fe^{3+} activity becomes significant at a pH of approximately 2.5, a vicious cycle of pyrite oxidation and bacterial oxidation of Fe^{2+} results from the combined effects of reactions 2 and 4. The rate of reaction 2 exerts primary control on the cycle by limiting the availability of Fe^{3+} which is the major oxidant of pyrite [10]. Inhibition of the activity of *Thiobacillus ferrooxidans* would prevent ferric oxidation of pyrite and should therefore reduce acid formation by at-least 75%.

Standard AMD treatment methods involve neutralization of the acid by the addition of a base, oxidation of ferrous iron in an aeration tank or pond, and precipitation of iron compounds in a settling pond. Chemistry of the basic treatment method is relatively straight forward. Neutralization is the reaction of the acid with a base (lime/ limestone).



To remove the ferrous iron, the neutralized water is aerated to produce ferric ions, which react with the base to form insoluble ferric hydroxides:



Although commonly used, Neutralization has several inherent problems. Base can produce water with an unacceptable high pH (greater than 9) and also give rise to sludge disposal/ handling problems apart from high cost. Treatment is not the best solution to AMD problems as it has disadvantage of being necessary for as long as the discharge continues and thus requires manpower, surface facilities and a sludge disposal area indefinitely. Since AMD results from the oxidation of pyrite associated coal and overburden strata, limiting the rate of pyrite oxidation would reduce the amount of acid formed.

acid mine drainage water gives rise to rapid growth and acidity of *Thiobacillus ferrooxidans* (up to 10^8 cells /ml) which greatly accelerate acid producing reactions by oxidation of pyrite through iron oxidation [7 and 11]. Inhibiting bacterial activity, therefore, would be most sensible approach in controlling AMD by using bacterial agents which have been found to control *Thiobacillus ferrooxidans* in an economical and environmentally safe manner[10-16]. This investigation presents an evaluation of the effectiveness (efficiency) of some organic compounds in inhibiting the growth and activity of *Thiobacillus ferrooxidans* in AMD water, which in turn reduces a large part of acid production and hence causing bactericidal control of AMD.

MATERIALS AND METHODS

Media and Culture Condition:-

Thiobacillus ferrooxidans was grown in 9-k media of Silverman and Lundgren [17] which was supplemented with $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1%V/W). The 9-k salts were sterilized by autoclaving at 103.5 kPa for an hour. pH of the media was adjusted to 2.5 prior to autoclaving. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was sterilized by using two micro millipore filter. Solution of 9-k salts and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ were then mixed aseptically and inoculated with pH 2.0 inoculum. Incubation was done at $28 \pm 2^\circ\text{C}$.

The cells of *Thiobacillus ferrooxidans* were harvested at $42000 \times g$ with Sharples super centrifuge and the resulting cell paste was re-suspended in $0.001\text{ N H}_2\text{SO}_4$ and was centrifuged at $4000 \times g$ at 0°C to remove precipitated iron. The centrifugate was collected and again centrifuged at $14000 \times g$ at 0°C and resulting cell paste was washed twice with $0.001\text{ N H}_2\text{SO}_4$ and re-suspended in $0.001\text{ N H}_2\text{SO}_4$ (cell mass obtained from 4 litre of culture was re-suspended in 500 ml of $0.001\text{ N H}_2\text{SO}_4$)

Physio-Chemical Determinations:-

Standard methods were employed for estimation of various constituents in mine water samples [18]. Fe^{3+} iron was determined by colorimetric method based on the absorbance of Fe^{3+} ion- HCl complex at 410 nm. pH measurements were made using Tonishwal Digital pH-meter.

GROWTH INHIBITION EXPERIMENTS

Growth inhibition experiments were carried out in 250 ml Erlenmeyer flasks. In each flask 90 ml of 9-k media (pH 2.5) supplemented with $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ and Organic compound was taken. 9-k salts, $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ solution and solutions of organic compounds were sterilized separately and after cooling to room temperature mixed aseptically 10ml of a standardized cell suspension having iron from cells of *Thiobacillus ferrooxidans* was used as inoculum. Concentration of the organic acids ranged from 10^{-5} M to 10^{-1} M whereas in case of sodium benzoate (SBZ), sodium lauryl sulphate (SLS) and quarternary ammonium compounds concentration ranged from 5 ppm to 50 ppm. Control experiments were also run simultaneously. All flasks were incubated at $30 \pm 2^\circ\text{C}$. Treated and untreated (control) samples were analysed at various time intervals for Fe^{3+} iron, acidity and pH levels. Results are expressed as % inhibition which is calculated as:

$$\% \text{ inhibition} = \left[1 - \left(\frac{\text{absorbance at 410nm, with inhibitor}}{\text{absorbance at 410nm, control}} \right) \right] \times 100$$

Results and Discussions :-

Physio-chemical characteristics of mine water (used as inocula for growth and culture of bacteria) from some NEC mines, are given in Table 1. The mine water is highly acidic with low pH and contains high iron and sulphate contents. Sulphate and iron contents in mine water are primarily released as a result of pyrite oxidation. Presence of Fe^{3+} iron at significant concentrations in these AMD water is ascribed to the bacterial activity since oxidation of Fe^{2+} iron occurs at a considerable rate in solutions having pH greater than 4.5. *Thiobacillus ferrooxidans* exerts a catalytic influence by providing a high concentration of Fe^{3+} iron in acid solutions, with the Fe^{3+} being the oxidizing agent at the pyrite surface (reaction given in equation 4) which becomes significant only when $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio approaches to two or more [5,11]. Further the rate of pyrite oxidation is comparatively very slow by Fe^{3+} iron, when bacteria are not present in the system. *Thiobacillus ferrooxidans* obtains most of the energy it needs to survive by oxidizing Fe^{2+} iron and is protected by an outer membrane which enables it to survive in its acid environments [11].

Inhibition inefficiencies of some organic acids such as oxalic, succinic, formic, propionic, fumaric, acetic and malic acids at various concentrations (range 10^{-5} M to 10^{-2} M) in controlling the oxidation of Fe^{2+} to Fe^{3+} iron by *Thiobacillus ferrooxidans* are depicted in Figures 1 to 3 after 14 to 21 days of incubation - which indicate that all these tested organic acid results in over 70 % inhibition at an optimum concentration of 10^{-2} M except malic acid. Percentage of inhibition was calculated from experimental data based upon ferrous to ferric iron oxidation when compared to the control set (which did not contain organic acids) as shown in figure 4. All these organic acids are found to effectively retard the iron oxidation in the concentration range of 10^{-3} M to 10^{-2} M. The oxidation of Fe^{2+} iron was only $\sim 5\%$ at 10^{-2} M optimum concentration of these organic acids whilst it was 100% in the control (untreated) set; after 21 days of incubation. Oxalic and Fumaric

acids are found to be the most effective and offer almost complete inhibition of the activity *Thiobacillus ferrooxidans*.

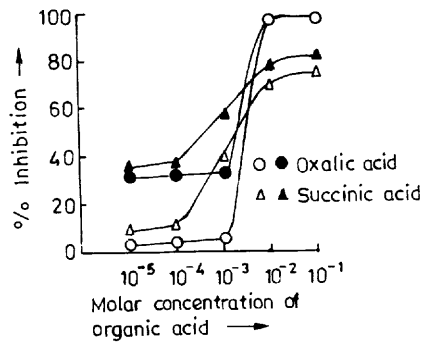


Figure 1 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidans* in Presence of Oxalic and Succinic acids after 14 and 21 days (Solid symbols)

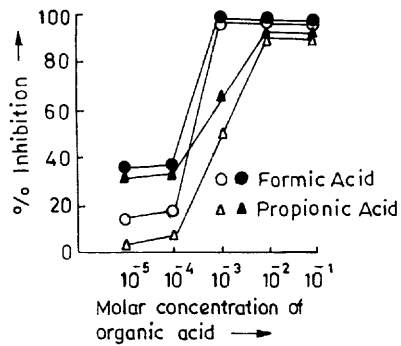


Figure 2 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidans* in presence of formic and propionic acids after 14 and 21 days of incubation

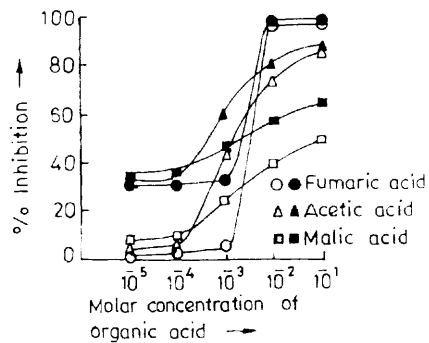


Figure 3 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidans* in Presence of fumaric, acetic and malic acids after 14 and 21 days (Solid symbols) of incubation

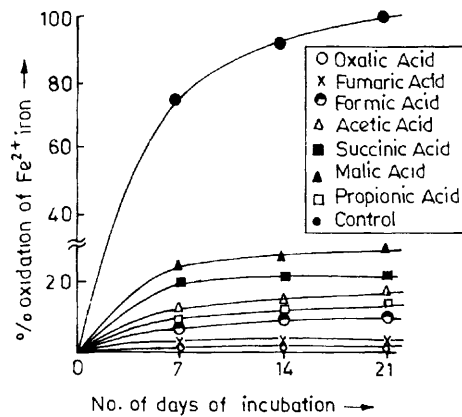


Figure 4 Percentage Oxidation of Ferrous Iron at 10^{-2} M concentration at different organic acids.

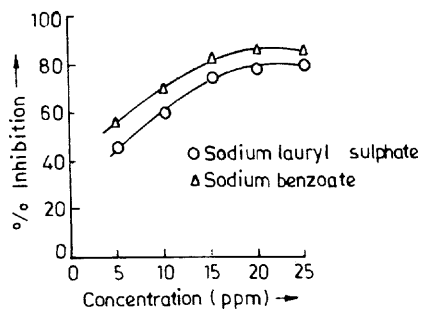


Figure 5 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidans* in Presence Sodium Lauryl Sulphate and Sodium Benzoate after 14 days of incubation

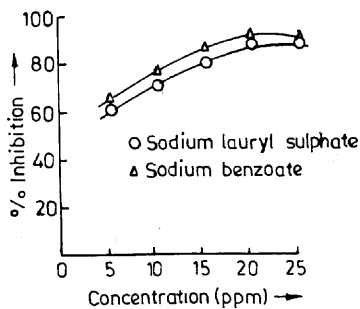


Figure 6 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidans* in presence of Sodium Laurate and Sodium Benzoate after 21 days of incubation

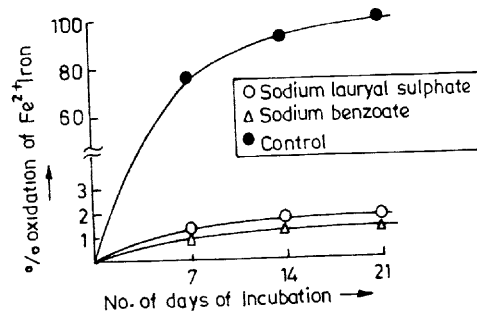


Figure 7 Percentage Oxidation Ferrous Iron at 25ppm concentration of Sodium Lauryl Sulphate and Sodium Benzoate

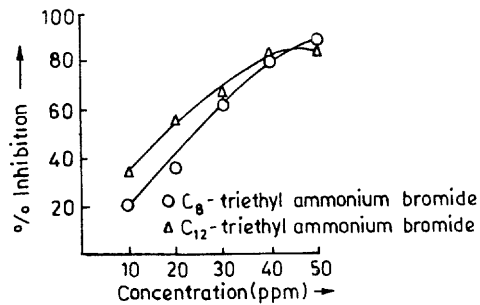


Figure 8 Percentage Inhibition of Iron Oxidation by *Thiobacillus ferrooxidans* in presence of Quaternary Ammonium Compounds after 14 days of incubation

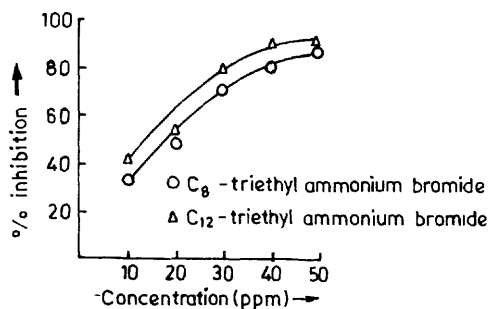


Figure 9. Percentage Inhibition of Iron Oxidation by *Thiobacillus ferrooxidans* in presence of Quaternary Ammonium Compounds after 21 days of incubation

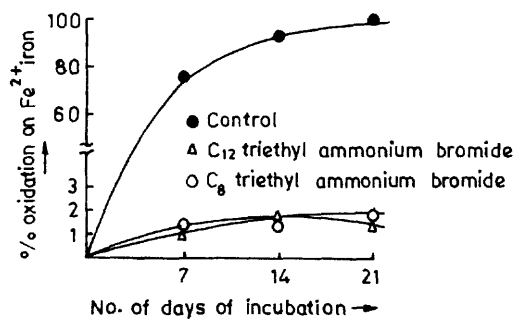


Figure 10 Percentage Oxidation of Ferrous Iron at 50 ppm concentration of Quaternary Ammonium Compounds

Table 1 Physio-Chemical Characteristics of Acid Mine Water Samples

Mine Water Sample	Physical Appearance with Suspension	pH	Acidity ppm	Sulphate ppm	Ferric Iron ppm	Ferrous -iron ppm
Ledo (Tirap)						
Sample 1	Yellow	2.6	2410	4325	546	83
Sample 2	Yellow-reddish	2.5	980	3210	236	60
Sample 3	Yellow	2.7	2040	3050	160	190
Jeypore						
Sample 1	Yellow-reddish	2.45	2350	3110	588	320
Sample 2	Light Yellow	2.84	2040	4116	427	402
Sample 3	Clear	2.90	1090	2420	210	335
Baragolai						
Sample 1	Light Yellow	3.98	190	984	33	27
Tipong						
Sample 1	Yellow	3.87	210	611	22	17

The inability of iron-oxidizing bacteria to utilize organically complexed iron suggested the possibility of using Fe^{2+} complexing organic acids to limit iron oxidation by *Thiobacillus ferrooxidans*. Exceptional extraordinary efficiency of oxalic acid as inhibitor for iron oxidation by *Thiobacillus ferrooxidans* in acid mine water, is due to its strong reducing action in addition to complexation. Other organic acids are very good complexing agents for Fe^{2+} iron.

Organic acids are used for this study mainly because some chemoautotrophs are unable to grow on some organic media [21]. The mode by which organic acids inhibit growth of certain chemoautotrophs depends upon the nature of organic acid as well as species of the organisms [13,14]. The toxicity of some low molecular weight organic acids inhibiting the growth and metabolism, depends upon the relative electronegativity and relative lipid solubility of the acid in cell envelope. Inhibitory action of organic acids may be either due to their direct influence on iron oxidizing enzyme system or they may non-selectively disrupt cell envelope or membrane [14], blocking the functions associated with cell envelope or membrane [11]. These organic acids retard iron oxidation and disrupt cell integrity. Regarding the mechanism of disruption, organic acids may react with cations, contributing to the structural integrity of the components of the cell envelope of bacteria.

The efficacy of SBZ, SLS and quaternary ammonium compounds (C8-triethylammonium bromide and C12-triethylammonium bromide), is also evaluated. All of these are found to be effectively inhibit the iron oxidation by *Thiobacillus ferrooxidans* and demonstrate more than 70 % inhibition efficiencies at each of the tested concentrations of > 10 ppm as shown in Figures 5 to 10, after 14 and 21 days of incubation. It appears that these organic inhibitors behave as bacteriostats at the tested concentrations up to 20 ppm since inhibition efficiency goes on increasing with the increase in concentration of these compounds. Further it is expected that the concentration higher than 20 ppm, these may behave as bactericides. SBZ, SLS and quaternary ammonium compounds at higher concentration thus can effectively be used for bactericidal control of acid mine drainage by killing the bacteria and slowing down the oxidation of acid-forming pyrite.

It is suggested that SLS (including other ionic surfactants) act by disorganizing the lipid fraction of catoplasmic membrane, thereby allowing the seepage of H^+ into the cell [22]. Alteration of the semi-permeable properties of the catoplasmic membrane, is the most typical mode of inhibitory action by SLS [23] and if the membrane is sufficiently damaged, the loss of the intracellular material into the suspending medium would occur. Disorganization of some bacterial cell membrane components might be caused by the action of SLS [24]; resulting in the seepage of H^+ ions from the surrounding acidic environment into the cell. The bacteria are thus attacked by the acid which they help to produce. The metabolism and thereby Fe^{2+} oxidation is slowed down as a result of seepage of H^+ into cellular cytoplasm. Quaternary ammonium compounds by virtue of their strong surface activity may destroy the membrane and exhibit similar action of SLS and other anionic surfactants.

SBZ addition to AMD water can result in the formation of organic precipitate during neutralization of acid water. SBZ has been previously studied as a fungicidal additive for preservation of SLS solutions used in experiments on the control of bacterially catalysed acid formation in refuse material, although no bactericidal action was ascribed directly to the benzoates [25,27]. If the precipitated organic compound consists of ferric salt of benzoate, the species liberated from this precipitate during redissolution in acidified water may include benzoic acid which is inhibitory to bacteria.

By abating acid mine drainage, selective inhibition of *Thiobacillus ferrooxidans* has potential value. These tested organic compounds on their application improved dramatically water quality of AMD. Acidity was reduced by more than 85% but pH was found to remain

in the acidic range i.e 3.1 to 4.7. Organic acids were very good inhibitors for the growth and activity of *Thiobacillus ferrooxidans*. However, the importance of organic acids as inhibitors depends on their lack of toxicity to other organisms (particularly acid tolerant heterotrophs) in the concentration range employed, which should be evaluated.

SBZ, SLS and quaternary ammonium compounds are effective inhibitors to growth of *Thiobacillus ferrooxidans* and are environmentally safe at effective inhibitory concentration range. SBZ is a safe chemical and suitable for use in human food materials (used as food preservative) at comparatively higher concentration than SLS. Therefore the use of SBZ as an alternative chemical must be favoured.

Work is in progress for evaluation of these tested organic compound in controlling acidification by *Thiobacillus ferrooxidans* from pyrite and coal refuse material. The objective of further studies are to identify environmentally acceptable compounds that can : (i) be added to active acid producing sites to effect neutralization of existing acidity, (ii) precipitate as insoluble compounds from these water as a means of storage of the reserve compound after neutralisation is accomplished, and (iii) re-dissolve and exhibit bacterial action upon reappearance of acid mine water condition, which otherwise would enable *Thiobacillus ferrooxidans* to colonise and subsequently accelerate the acid generation process.

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