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 ferrooxidians* 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 *ferrooxidians*.. In each experiment, 10 milli-litres of laboratory developed culture of *Thiobacillus ferrooxidians* was added to 250 milli-litres Erlenmeyer flask containing 90 milli-litres of 9-k media supplemented with Fe SO_4 7 H_2O 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 ferrooxidians* is also discussed and presented
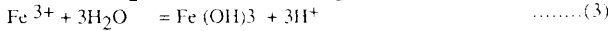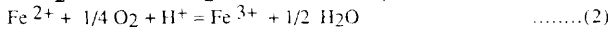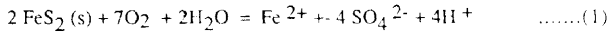
INTRODUCTION

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

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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 *ferrooxidians*.
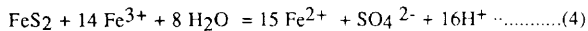
$$2\ FeS_2\ (s) + 7O_2 + 2H_2O = Fe^{2+} + 4\ SO_4^{2-} + 4H^+ \qquad \text{.......(1)}$$
$$Fe^{2+} + 1/4\ O_2 + H^+ = Fe^{3+} + 1/2\ H_2O \qquad \text{........(2)}$$
$$Fe^{3+} + 3H_2O = Fe\ (OH)3 + 3H^+ \qquad \text{........(3)}$$

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 ferrooxidians* 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 *ferrooxidians* is relatively low and neutral alkalinity of the ground water may effectively neutralized the acid formed at this stage.
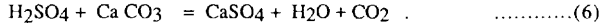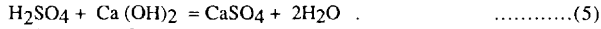
When the neutralizing capacity of the environment is exceeded acid begins to accumulate and them pH decreases (stage 2). As the pH decreases, the rate of iron oxidation by *Thiobacillus ferrooxidians* increases. The action of the bacteria causes increased acid production which serves to further lower pH.

As the pH in the vicinity of the pyrite falls to than 3, the increased solubility of iron and the decreased rate of $Fe\ (OH)_3$ (yellow boy) precipitation affects the overall rate of acid production (stage 3). At this point, ferrous iron is oxidized by *Thiobacillus ferrooxidians* and the ferric -iron in turn oxidizes the pyrite.:
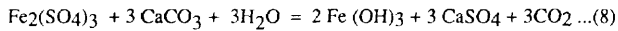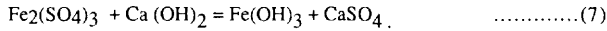
$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O = 15\ Fe^{2+} + SO_4^{2-} + 16H^+ \ \text{............(4)}$$

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 ferrooxidians* would prevent ferric oxidation of pyrite and should therefore reduce acid formation by at-least 75%.

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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).

$$H_2SO_4 + Ca(OH)_2 = CaSO_4 + 2H_2O \quad . \qquad \ldots\ldots\ldots\ldots(5)$$
$$H_2SO_4 + CaCO_3 = CaSO_4 + H_2O + CO_2 \quad . \qquad \ldots\ldots\ldots\ldots(6)$$

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

$$Fe_2(SO_4)_3 + Ca(OH)_2 = Fe(OH)_3 + CaSO_4 \qquad \ldots\ldots\ldots\ldots(7)$$

$$Fe_2(SO_4)_3 + 3CaCO_3 + 3H_2O = 2Fe(OH)_3 + 3CaSO_4 + 3CO_2 \ldots(8)$$

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 ferrooxidians* (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 ferrooxidians* 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 ferrooxidians* 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 ferrooxidians was grown in 9-k media of Silverman and Lundgren [17] which was supplemented with $FeSO_4. 7 H_2O$ (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. $FeSO_4. 7 H_2O$ was sterilized by using two micro millipore filter. Solution of 9-k salts and $FeSO_4. 7 H_2O$ were then \mixed aseptically and inoculated with pH 2.0 inoculum. Incubation was done at $28 +- 2^O$ C.

The cells of *Thiobacillus ferrooxidians* were harvested at 42000 x g with Sharples super centrifuge and the resulting cell pasted was re-suspended\in 0.001 N H_2SO_4 and was centrifuged at 4000 x g at 0° C to remove precipitated iron. The centrifugate was collected and again centrifuged ar 14000 x g at 0^OC and resulting cell paste was washed twice with 0.001 N $H2SO4$ and re-suspended in 0.001 N $H2SO4$ (cell mass obtained from 4 litre of culture was re-suspended in 500 ml of 0.001 N $H2SO4$)

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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 Erlemeyer fl ks. In each flask 90 ml of 9-k media (pH 2.5) supplemented with $FeSO_4$. 7 H_2O and Organic compound was taken. 9-k salts, $FeSO_4$. 7 H_2O solution and solutions of organic compounds were sterlized separately and after cooling to room temperature mixed aseptically 10ml of a standardized cell suspension having iron frown cells of *Thiobacillus ferrooxidians* 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 $30 +- 2^{o}C$. Treated and untreated (control) samples were analysed at various time intervals for Fe3+ iron, acidity and pH levels. Results are expressed as % inhibition which is calculated as:

% inhibition = [1 -(absorbance at 410nm, with inhibitor / absorbance at 410nm, control)] x 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 ware 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 ferrooxidians* exert 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 Fe^{3+}/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 ferrooxidians* 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 oxiudation of Fe^{2+} to Fe^{3+} iron by *Thiobacillus ferrooxidians* are depicted in Figures 1 to 3 after 14 tro 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 excedpt 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 10-3M to 10-2M. The oxidation of Fe2+ iron was only , -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 Fumeric

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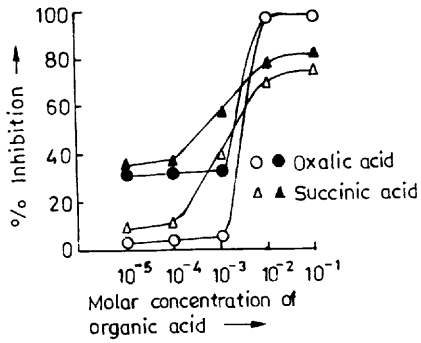

Figure 1 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidians* in Presence of Oxalic and Succinic acids after 14 and 21 days (Solid symbols)
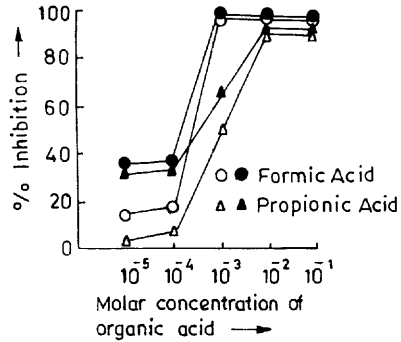

Figure 2 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidians* in presence of formic and propionic acids after 14 and 21 days of incubation
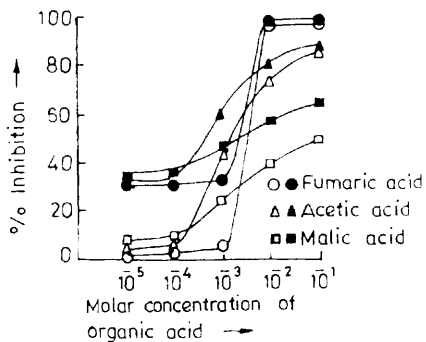
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Figure 3 Percentage Inhibition of Iron Oxidation by *Thiobacillus Ferrooxidians* in Presence of fumaric, acetic and malic acids after 14 and 21 days (Solid symbols) of incubation
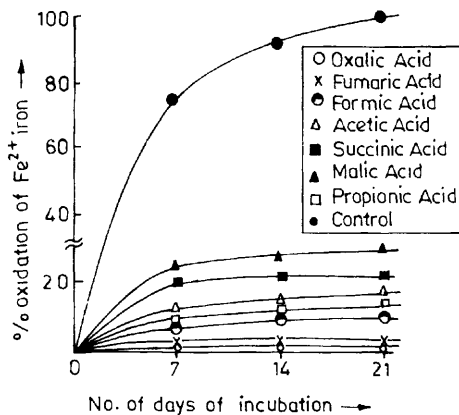

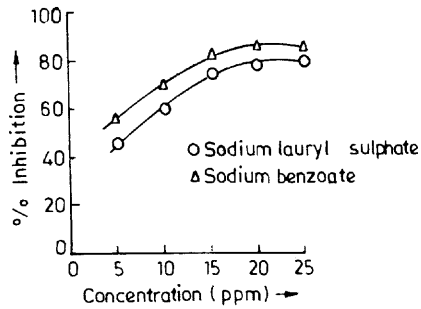Figure 4 Percentage Oxidation of Ferrous Iron at 10^{-2} M concentration at different organic acids.

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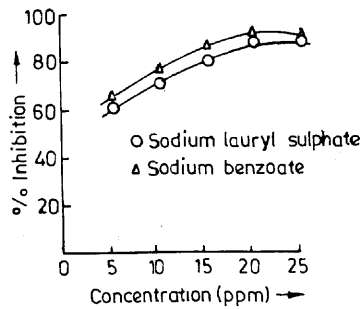Benzoate after 14 days of incubation

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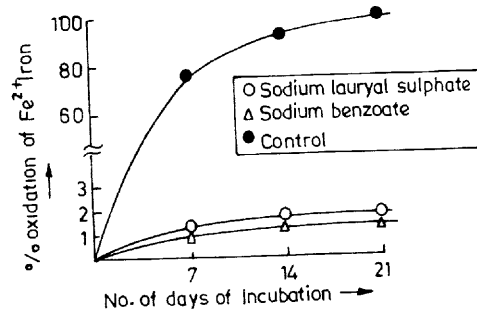Benzoate after 21 days of incubation

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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
Ferrooxidians* in presence of Quaternary Ammonium
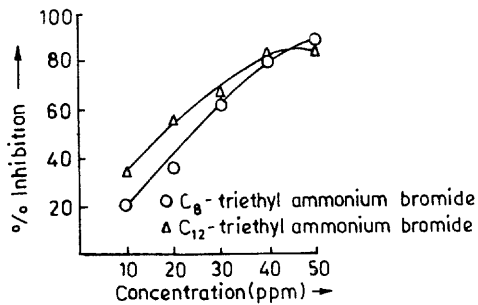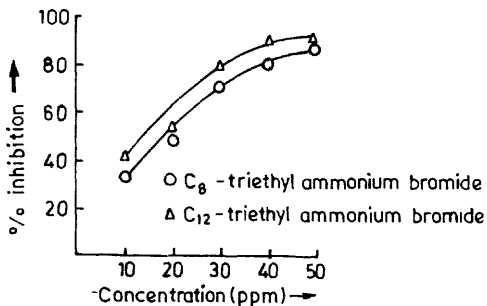Compounds after 14 days of incubation

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Figure 9.   Percentage Inhibition of Iron Oxidation by*Thiobacillus Ferrooxidians* in presence of Quarternary Ammonium Compoundsafter 21 days of incubation

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

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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

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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 ferrooxidians* . Exceptional extraordinary efficiency of oxalic acid as inhibitor for iron oxidation by *Thiobacillus ferrooxidians* in acid mine water, is due to its strong reducing action in addition to complexation. Other organic acids are very good complexing agents fot 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 molucular 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 oxidising enzyme system or yhey may non-selectivity 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 quarternary ammonium compounds (C8-triethylammonium bromide abd C 12- triethylammonim bromide), is also evaluated. All of these are found to be effectively inhibit the iron oxidation by *Thiobacillus ferrooxidians* 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 uo to 20 ppm since inhibition efficiency goes on incresing with the increase in concentration of these compounds. Further it is expected that the concentration higher than 20 `ppm, these may behave as bacterialcides. 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, therby 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 demaged, the loss of the intracellular material into the suspending medium would occur. Disorganization of some bacterial cell membrane conponents might be caused by the action of SLS [24]; resultingin the seepage of H+ ions from the surrounding acidic environment into the cell. The bacteria are thus attacked by the acid which they help ro produce. The metabolism and thereby Fe 2+ oxidation is slowed down as a result of seepage of H+ into cellular cyptoplasm. Quaternary ammonium compounds by virtue of their strong surfacew activity mat 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 contol of bacterially catalysed acid formation in refuse material, although no bacterialcidal action was ascvribed directly to the benzoates [25,27]. If the precipitated organic compound consisits 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 ferrooxidians* 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

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in the acidic range i.e 3.1 to 4.7. Organic acids were very good inhibitors for the growth and activity of *Thiobacillus ferrooxidians* . 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 ferrooxidians* 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 ferrooxidians* from pyrite and coal refeuse 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 od acid mine water condition, which otherwise would enable *Thiobacillus ferrooxidians* to colonoise and subsequently accelerate the acid generation process.

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