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Effect of Increasing Concentrations of Chloride, Nitrate and Sulphate Anions with Their Counter Cations of Potassium, Sodium and Ammonium on Sulphur Bio-Oxidation by Sulphur Oxidizing Microorganisms

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Abstract

The present study investigates effect of anions such as chloride (Cl^-), nitrate (NO_3^{2-}), and sulfate (SO_4^{2-}) with their counter cations like sodium (Na^+), potassium(K^+), and ammonium (NH_4^+) on sulfur oxidizing microorganisms. Bioprocess parameters such as pH and viable cell count were considered to understand the toxic effect of the anions as well as cations on the sulfur-oxidizing microorganism. The pH and viable cell count data reveals that $Cl^-$and NO_3^{2-} had more inhibitory effects at lower concentrations compared to SO_4^{2-} ions. Effect of the same concentration of Cl^- ion as Na^+, K^+, and NH_4^+ counter cations showed, NaCl as more inhibitory with less viable cell count compared to KCl and NH_4Cl However, all the three salts such as NaCl, KCl and NH_4Cl showed inhibitory effects at 4 g/L Cl^- with an order of toxicity of NaCl> NH_4Cl>KCl. Similar studies conducted on NO_3^{2-} ions with similar counter cations resulted with an order of inhibition for NO_3^{2-} ion as KNO_3>$NaNO_3$>$Ca(NO_3)_2$ indicating a more inhibitory effect of monovalent cations (Na^+ and K^+) then divalent (Ca^{2+}) ion. The viable cell count data for the study supports the pH patterns with time followed by viable cell count for $Ca(NO_3)_2$ supporting the less inhibitory effect of Ca^{2+} ion. While for the higher concentration of $NO_3^{2-}$the Na^+ ion and K^+ ion showed a similar pattern for pH and viable cell count. The higher concentration of SO_4^{2-} was chosen for the study, which reveals that $(NH_4)_2SO_4$ was more deleterious at lower concentrations. The order of inhibition observed was $(NH_4)_2SO_4$>Na_2SO_4>K_2SO_4 for the higher SO_4^{2-} concentrations. Na_2SO_4 and K_2SO_4 showed almost similar effects at their lower concentrations.

Keywords: Ammonium chloride; Bio-Oxidation; Chloride; Nitrate; Potassium; Sulphate; Sulphur Oxidising Microorganism; Sodium

Introduction

The use of micro-organisms to facilitate the extraction and recovery of the base as well as precious metals via bioleaching or bio-oxidation processes from primary ores/concentrates, and secondary resources referred generically as 'biomining', has developed into a successful and expanding area of biotechnology [1]. The microorganisms play an essential role in bioleaching are autochemolithotrophic, and they grow by oxidizing reduced forms of sulfur or ferrous iron (or both) which are found in extreme natural conditions (acid mine drainage and acid rock drainage)

of low pH and high salt and metal ion concentrations [2]. The microbially mediated mineral dissolution processes operate in an aqueous medium, where the process water becomes essential,as the microbial culture has to oxidize the ferrous iron or reduced sulfur species and replicate rapidly to have sufficient microbial population for successful bioleaching. There are many factors including pH and the toxicity of anions (Cl^-, NO_3^{2-}, SO_4^{2-}) and cations (K^+, Na^+, Ca^{2+}) that may affect the growth and hence the bioleaching activity of culture [3]. Many of these ions (KCl, $Ca(NO_3)_2$, and $(NH_4)_2SO_4$ are required as micronutrients for the microbial growth and an essential part of microbial growth medium but their elevated concentrations may consequent deleterious effects [4]. An earlier study on the impact of anions on sulfur oxidation by *A. thiobacillus* have reported the more negative effect of anions at lower pH due to the destruction of positive inside membrane potential was in the series of SCN^-> NO_3^->Cl^->H_2PO_4>HSO_4^- [5]. Iron oxidizers are proved more sensitive in comparison to sulfur oxidizers as they prefer low pH 1-2 and have less tolerance for Cl^- [6]. The activity of S oxidizers gets stimulated in the range of 10-50 mM of the anions, i.e., Cl^-, PO_4^{2-}, K_2SO_4, and Na_2SO_4, above which a complete inhibition of cell growth has been observed **[7].**

The mesophile and thermophile bacteria respond varyingly for saline conditions. In a comparative study on tolerance of different salt concentration reported less viability of acidophilic thermophiles in comparison to mesophile and moderate thermophiles among which the exceeded tolerance limit of mesophiles was 70g/L for NaCl and 350g/L of $MgSO_4 7H_2O$ [8]. There are many studies which suggest that for the same anionic concentrations SO_4^{2-} (Sulfate) is less inhibitory in comparison to Cl^- and NO_2^- which shows low microbial growth, low Fe and S oxidation rates with increased microbial growth lag phase [9-11]. The present study reveals the effect of various anions with their counter cations on the growth of S oxidizing microorganisms. The multiple salts used in the study are based on the fact that these ions are required as a minimal salt medium for microbial growth. The effect of Cl^-, NO_3^-, SO_4^{2-} with their counter cations K^+, Na^+, and NH_4^+ with increasing concentrations was observed on the microbial growth pattern.

Material and Methods

Microorganisms and growth culture

The microbial culture for the present study was collected from Lulea University of Technology, Lulea, Sweden. Q-PCR analysis conducted by Bio clear B.V., Netherlands, revealed that the mixed culture of chemolithotrophic, mesophilic acidophilic Fe & S oxidizers, was dominated by *Leptospirillum ferriphilum* (Fe oxidizer) followed by *Acidithiobacillus caldus* (S-oxidiser), and with approximately the same amount of *Acidithiobacillus thiooxidan* (S-oxidiser), *Sulphobacillus sp.* (Fe-oxidiser) and *Ferroplasma* (Archaeal species, Fe-oxidiser). Inoculum for the study was prepared by sub culturing the parental Fe & S mixed microbial culture in a Fe free 0K medium supplemented with 3 g/L elemental Sulphur S^0 to provide selective growth conditions for S oxidizing microorganisms with a working volume of 100 ml (v/v). After several times of sub-culturing microbial culture dominated by *Acidithiobacillus caldus* and *Sulphobacillus sp.* was used as inoculum.

Bio-oxidation experiments with anionic effects

The three different anions selected for the bio-oxidation studies were Nitrate (NO_3^-), Chloride (Cl^-) and Sulphate (SO_4^{2-}) with their counter cations Na^+, NH_4^+ and K^+. The selection was based on their presence in bacterial growth medium (Table 1) and essential role during bacterial bio-oxidation. All bio-oxidation experiments were carried out for three different concentrations of anions such as 0.5 g/L, 1 g/L, and 1.5 g/L and the concentration of cations were calculated accordingly (Table 2,3,4).

Composition	g/L
$(NH_4)_2SO_4$	3
KCl	0.1
K_2HPO_4	0.5
$MgSO_4$	0.5
$Ca(NO_3)_2.4H_2O$	0.01

Table 1: Composition of iron free OK medium.

Nitrate Concentration (g/L)	KNO_3 (g/L)	$NaNO_3$ (g/L)	$Ca(NO_3)_2$ (g/L)
0.5	0.85	0.68	0.67
1	1.16	1.30	1.33
1.5	2.44	2.05	1.99

Table 2: Concentration of nitrate with variable cations in bio oxidation experiments.

Chloride Concentration (g/L)	KCl (g/L)	NaCl (g/L)	NH_4Cl (g/L)
0	0	0	0
2	4.24	3.30	3.01
3	6.31	5.00	4.52
4	8.38	6.60	6.03

Table 3: Concentration of chloride with variable cations in bio oxidation experiments.

Sulphate Concentration (g/L)	Na_2SO_4 (g/L)	K_2SO_4(g/L)	$(NH_4)_2SO_4$
35	51.7	63.5	48.1
40	59.1	72.5	55.0
45	66.5	81.6	61.8

Table 4: Concentration of sulphate ions with variable cations in bio oxidation experiments exclusive of the sulphate ions added via H_2SO_4 addition and OK medium.

The experiments were conducted in 3 parallel sets for each of the anionic salt. It was a bench scale study conducted with a working volume of 50 ml (v/v), 90% (v/v) 0K medium (supplemented with S^0), 10% (v/v) inoculum in a 100ml Erlenmeyer flask. The inoculum pH was 1.8, and 0 K medium pH was 3 when after inoculation the final value of experimental pH was 2.2. The experiments were carried out in an orbital shaker with a rotation speed of 110 rpm, and at a temperature of 30°C supports mesophilic bacterial growth. A positive control contains S oxidizing microorganisms with no anionic salts added in the growth medium and a negative control flask without S oxidizing microorganisms (without MO) to ensure the existence of any other organism during the study were maintained. Two parameters viz., pH and viable cell count were analyzed a day thrice to follow the bio-oxidation trend. Water lost due to evaporation was compensated by regular addition of water based upon flask weight. No efforts were put to maintain sterile conditions. The above experiments were continued until the pH of positive control reached 1 as below pH 1.0 these microbes start to get stressed due to high acidic concentration.

Analytical and instrumentation techniques

A bench top pH meter (Riviera Eutech pH-150) was used for regular pH measurement. Three-point calibration of pH meter was carried out daily by the standard buffers of pH 1.68, 4.0 and 7.0 and the slope obtained ranged from 95 to 100 which ensures good working conditions for pH meter. The Viable cell count was conducted on a bright field compound microscope with 10× eyepiece and 100× objective lenses by loading the microbial culture sample on an improved neubar haemocytometer. The haemocytometer with neubar rulings, where the main divisions separate the grid into 9 large squares with each square having a surface area of one square mm and a depth of the chamber was 0.1 mm. The counting grid lies under a volume of 0.9 mm^3. Microbial culture samples or bioleaching solution samples were diluted enough whenever required to avoid overlapping of the cells with uniformly distribution over the counting grid while sometimes vortex of the solution was done to liberate the attached cells. The magnification varies with different microbial cell sizes and types. A systematic counting of the cells was done in selected squares so that the total count of 100 square cells, while the 4 large squares (1/25 sq. mm) present in the four corners were considered together with one in the middle central square. Precautions were taken not to count the cells lying > 50% outwards the border lines of the square cells. The calculation of the cells per ml of solution was done by adding the total number cells counted in 5 different small squares, which has area of 1/25 mm^2 (i.e., 0.04 mm^2) and depth of 0.1 mm each. So the total volume in each square would be 0.04 multiplied by 0.1 resulting as 0.004 mm^3.

Result and Discussion

Comparative assessment of effect of anions on pH profile

S oxidation is an acid producing process so the pH profile studies shows the bio oxidation kinetics of the bacterial growth medium with time. Present study with cations such as nitrate, chloride and sulphate ions along with their counter cations was carried out to check their various inhibitory effects. The nitrates with its counter ions sodium (Na^+), Pottassium (K^+) and Calcium (Ca^{2+}) indicate the more inhibitory effect of monovalent cations (Na^+ and K^+) then divalent (Ca^{2+}) ion for the same concentration of nitrates. KNO_3 and $NaNO_3$ for 0.85 g/L to 1.16 KNO_3 and 0.68 g/L to 1.30 of $NaNO_3$ having same nitrate content showed almost similar patterns with a slight increase in lag phase and in some extent to pH (1.5-1.8), in respect of control (no salts added) with pH 1.0. The further increase in concentration up to 2.44 g/L for KNO_3 showed an increment of pH>3 which is higher than the pH-3 of negative control (without microorganisms) (Figure 1-3).

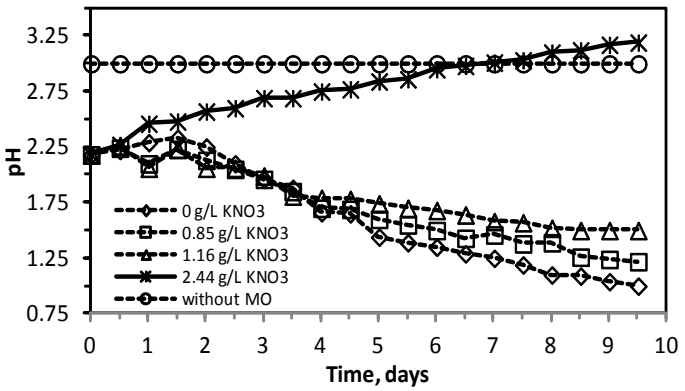

Figure1: Effect of KNO_3^{2-} on pH.

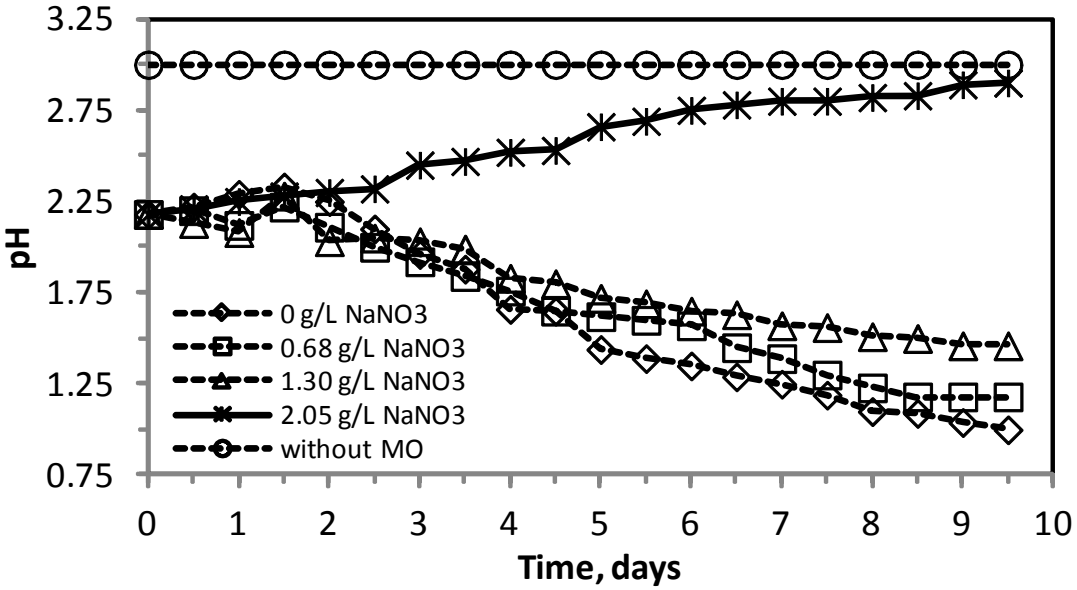

Figure 2: Effect of various concentration of $NaNO_3$.

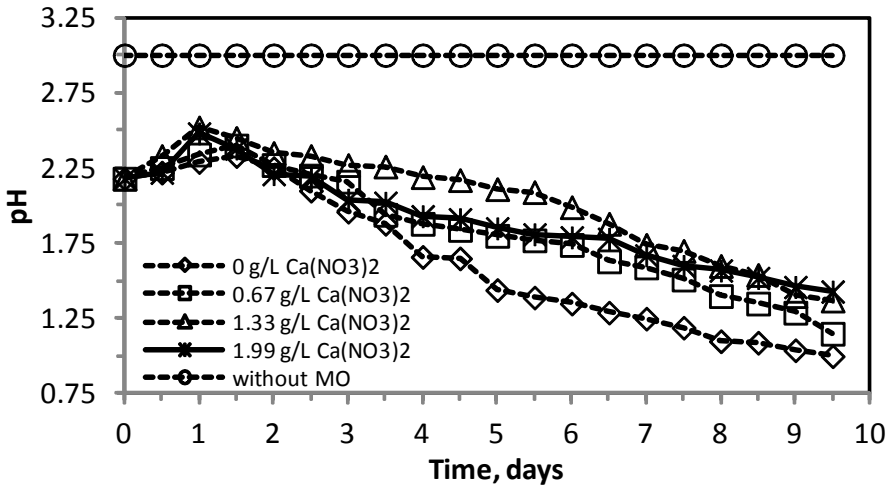

Figure 3: Effect of calcium nitrate on pH.

The higher concentrations also increased the bacterial growth lag phase from 2 days (in case of positive control) to 7 days, while $Ca(NO_3)_2$ showed an utterly different pattern with a slight increase in pH and an almost similar pattern for all increased concentrations (Figure 1-3). The control experiment where no inoculum has added no growth and sulfur oxidation was observed. The pH for the operation was constant to pH-3. The result ensures that the absence of laboratory contamination it also shows lower sulfur oxidation in the absence of sulfur-oxidizing microorganisms. In the positive control experiment, the pH goes below 1 which ensures the complete S oxidation (Figure 1-3).

The pH profile for Cl^- ions also followed an interesting pattern. As the Cl^- concentration was kept constant 2, 3 and 4 g/L similar for all cations there was an overall exciting correlation between Na^+ ion and K^+ ion with the same Cl^- concentration. It was found that 4.2 and 6.3 g/L of KCl showed faster kinetics compared to NaCl with 3.3 & 5.0 g/L Cl^- concentration of 2 and 3 g/L respectively. The lag phase in case of NaCl with the concentration of 5.0g/L was increased from 2 to 7 days while it was shorter for

KCl. At Cl^- ion concentration 4g/L, all cations showed complete inhibition as at this level of concentration no growth was observed (Figures 4,5) in case of NH_4Cl there was a slight increase in pH for 4 g/L of Cl^- (Figure 6).

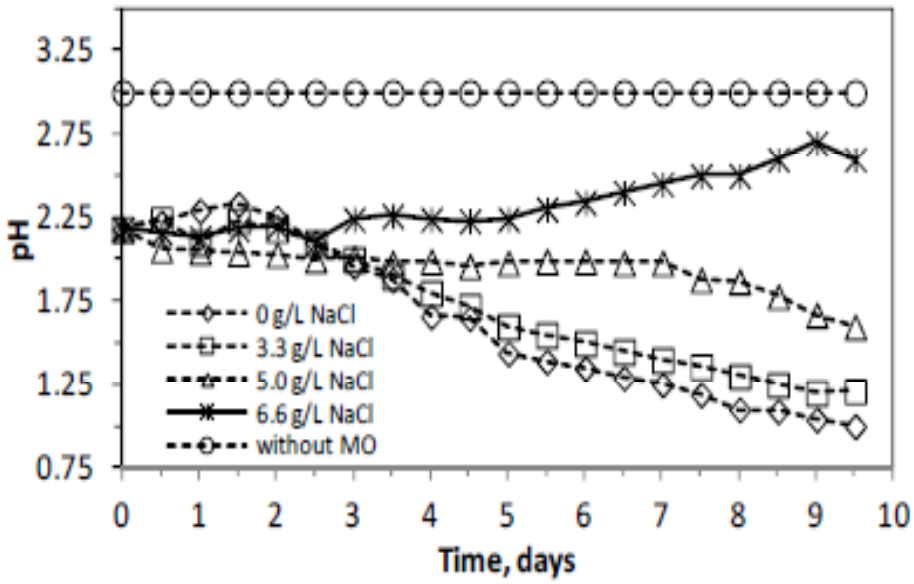

Figure 4: Comparative plot of the change in pH with varying concentration of NaCl.

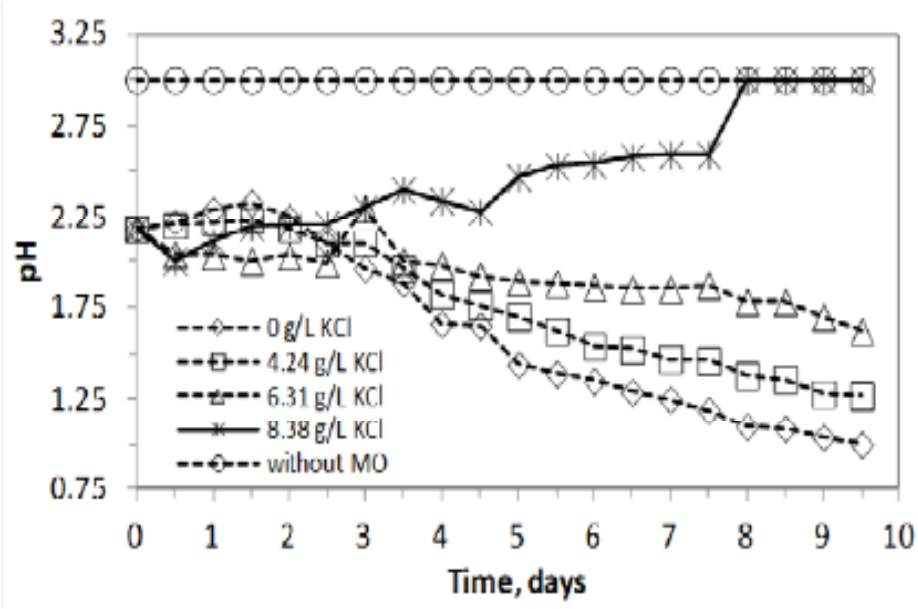

Figure-5: Comparative plot of the change in pH with varying concentration of KCl.

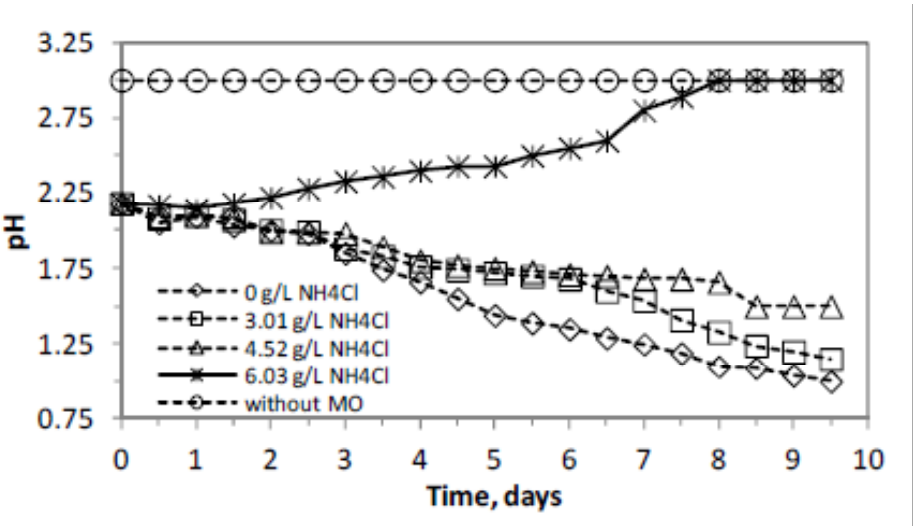

Figure 6: Comparative plot of the change in pH with varying concentration of NH_4Cl.

The comparative assessment of bacterial bio oxidation resulted that for the same concentration of SO_4^{2-} (45 g/L) the NH_4^+ ion showed maximum inhibition effect at a concentration of 64.8 g/L $(NH_4)_2SO_4$ compared to 66.5 g/L of Na_2SO_4 and 81.6 g/L of K_2SO_4. The NH_4^+ ion was inhibitory at lower concentrations then Na^+ and K^+ ions for the same SO_4^{2-} concentration. The pH for the negative control without microorganisms remained constant at pH 3 during the whole experiment. The pH for elevated concentrations of Na_2SO_4, K_2SO_4 and $(NH_4)_2SO_4$ was increased up to 3.03 to 3.49 respectively (Figure 7,8,9). Concentration of 51.7 g/L and 59.1 g/L of Na_2SO_4, 63.5 g/L of K_2SO_4 and 51.1g/L and 58.0g/L was adapted by the bacterial culture after a 2 days lag phase after which a gradual decrease in pH to the value of 1.5-1.8 was observed (Figure 7,8,9). These observations indicate that although bacteria can tolerate the salt concentration up to some extent a higher salt concentration exerts a negative effect on bacterial growth and viability.

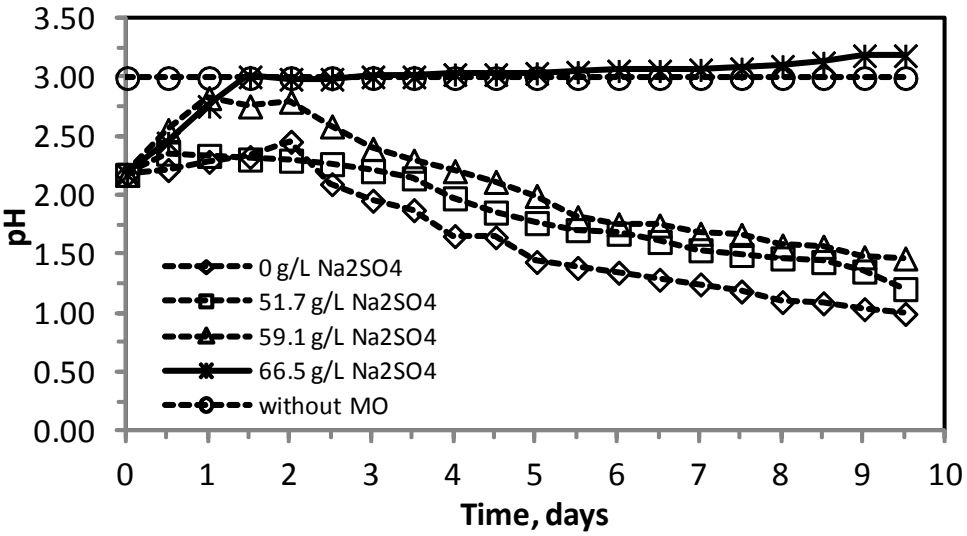

Figure 7: Comparative assessment of pH for various concentration of Na_2SO_4.

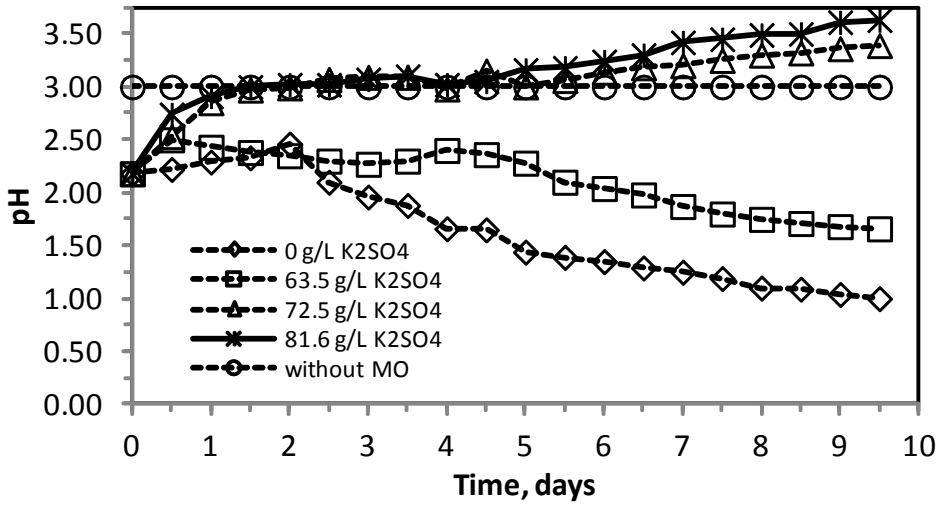

Figure 8: Comparative assessment of pH for various concentration of K_2SO_4.

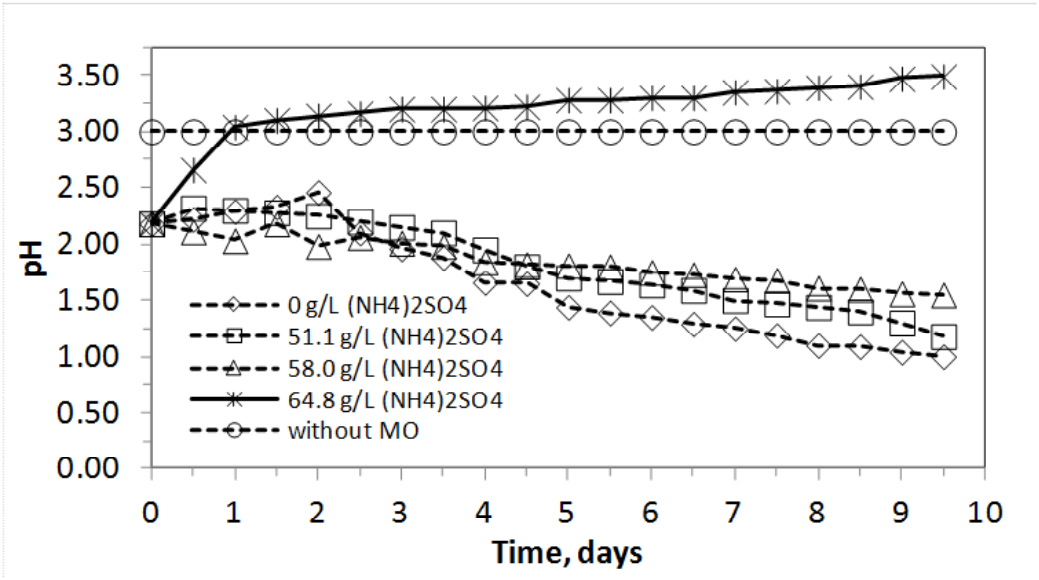

Figure 9: Comparative assessment of pH for various concentration of $(NH_4)_2SO_4$.

Microbial cell viability analysis

The control flask having S Oxidizing microorganisms without any salt inserts showed the complete S Oxidation with a viable cell count 2.8×10^5 viable cells/ml. Increase in concentration of KNO_3 and $NaNO_3$ showed direct effect on cell viability (Figure 10, 11). A steep decrease had observed at 2.44 g/L of KNO_3 and 2.05 g/L of $NaNO_3$. Due to less inhibitory effect of calcium nitrate as compare to other anions, it doesn't't seem to be any toxic effect on cells the decrease in number was possibly due to its inhibitory effect for cell growth and replication rate (Figure 12). The cell count observed was 2.54×10^5 cells/ ml for 0.67 g/L, 2.07×10^5 cells/ ml and 1.97×10^5 cells/ml for 1.33 g/L and 1.99 g/L of $Ca(NO_3)_2$ which was very close to the cell count of control flask 2.85×10^5 cells/ml.

Considering the effect of Cl^- ion on viable cells dynamics it was observed that the viable population increase was relatively faster in case of KCl compared to NaCl and it was also supported by the trend obtained in the change in pH (Figure 13,14,15). The

interesting thing observed was the viable cell count for NH_4Cl at concentration of 3 g/L chloride had a highest increase in the population compared to NaCl and KCl. The reason behind such happening is also clearly stated in the oxidation potential derived from the plot showing the pH change with time.

The K_d (cell death) value was higher for Na^+ and K^+ in comparison to NH_4^+ ion. The inhibitory concentration for microbial growth was 66.5 g/L, 81.6 g/L and 64.8 g/L of Na_2SO_4, K_2SO_4 and $(NH_4)_2SO_4$ respectively. The viable cell count data strengthen the pH profile data for bio-oxidation which states that ammonium sulphate has more inhibitory effects at lower concentrations of NH_4^+ in comparison to Na^+ and K^+ for the same concentration of SO_4^{2-} ion (Figure 16,17,18).

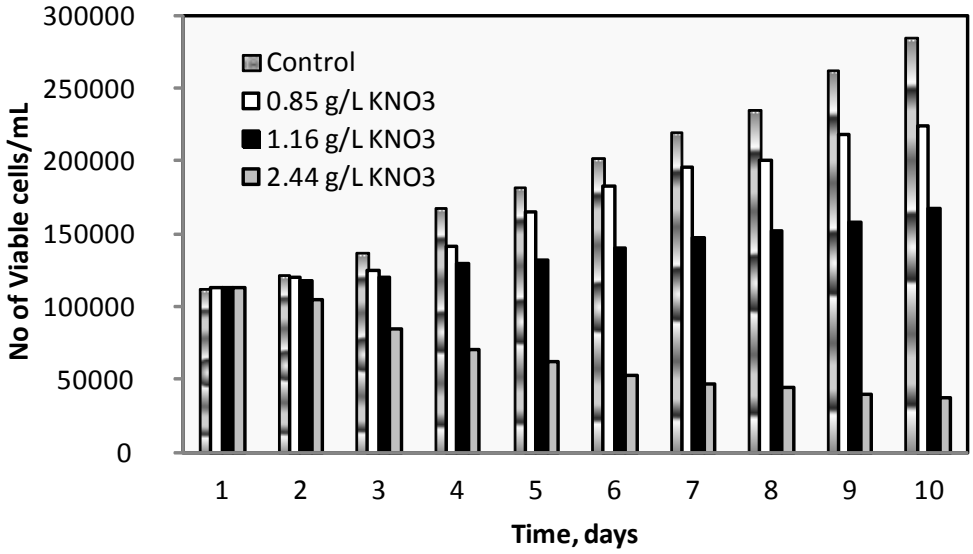

Figure 10: Comparative plot of the change in viable cell count with varying concentration of KNO_3.

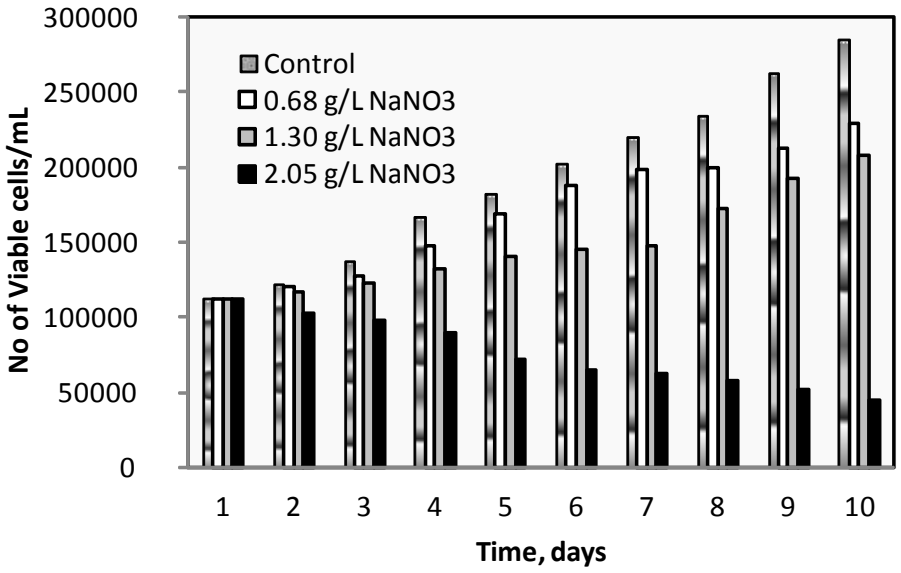

Figure 11: Comparative plot of change in viable cell count with varying concentration of $NaNO_3$.

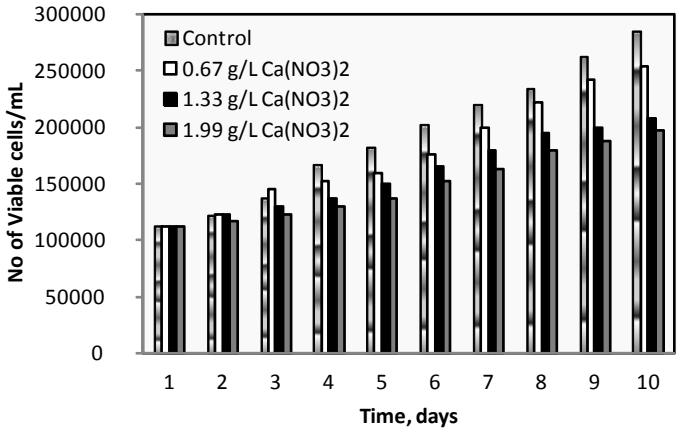

Figure 12: Comparative plot of change in viable cell count with varying concentration of $(Ca)_2NO_3$.

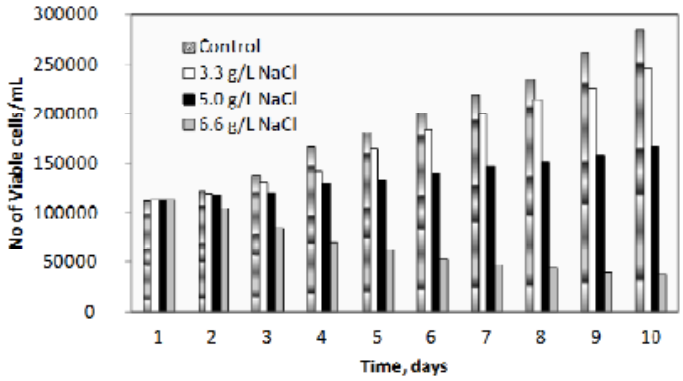

Figure 13: Comparative plot of change in viable cell count with varying concentration of NaCl.

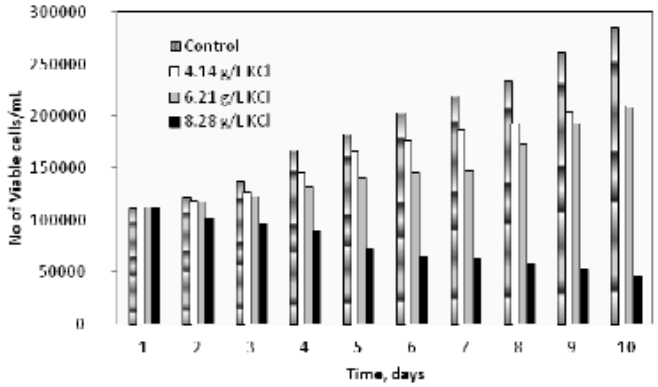

Figure 14: Comparative plot of change in viable cell count with varying concentration of KCl.

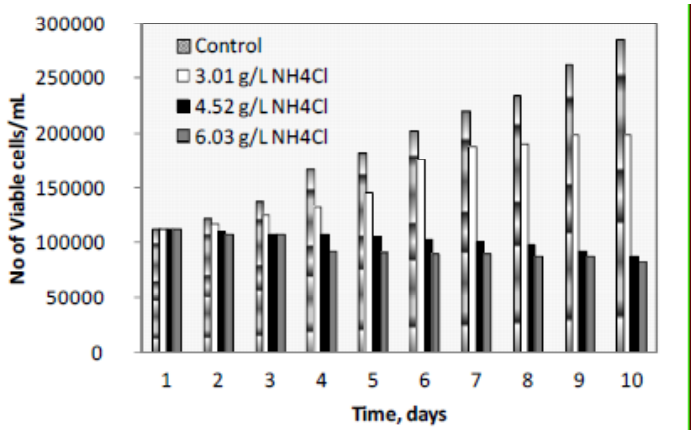

Figure 15: Comparative plot of change in viable cell count with varying concentration of NH_4Cl.

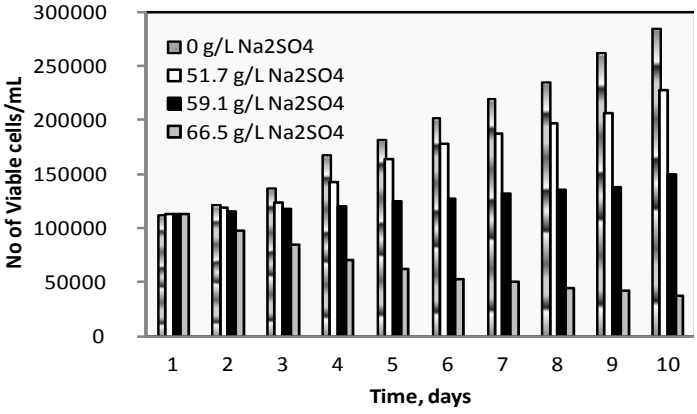

Figure 16: Comparative plot of change in viable cell count with varying concentration of Na_2SO_4.

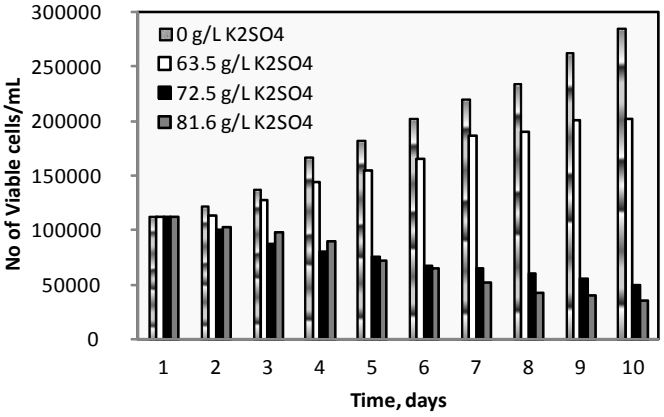

Figure 17: Comparative plot of change in viable cell count with varying concentration of K_2SO_4.

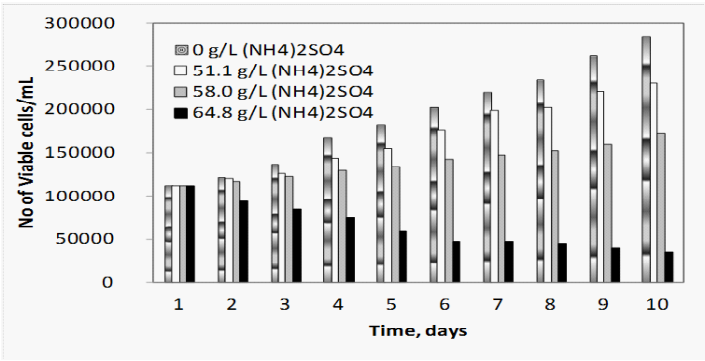

Figure 18: Comparative plot of change in viable cell count with varying concentration of $(NH_4)_2SO_4$.

The viable cell count for 51.7 g/L, 59.1 g/L of Na_2SO_4 63.5 g/L of K_2SO_4 regained after a short period of log phase. The present data also reveals the fact that SO_4^{2-} ion has less lethal effect than other two foresaid anions. Sulphate has negative effects at comparatively high concentrations while the other two ions (Cl^- and NO_3^{2-}) are deleterious effects even at lower concentrations. The possible reason behind this differential effect can be explained through cell membrane potential and ion transport into the cell. High concentration of anions in the growth medium leads to the entry of anions into the cell cytoplasm which disturbs the cell homeostasis by disrupting the inside positive potential. The cell membrane permeability for the different anions determines the extent of inhibition. On the other hand, the more negative potential inside attracts the anions which again moves into the cytoplasm based on their permeability. The order of cell permeability (H^+> K^+>Na^+> NH_4^+) of anions gives a possible reason that why the same anion with varying counter ions showed varying results. The divalent ions have less permeability for cell membrane that can be observed in our study as Ca^{2+} which showed almost negligible effects and SO_4^{2-} ion which was only inhibitory at its higher concentration. The other possible reasons for the inhibitory effects are disturbance in osmotic gradient and destruction of bacterial cell membrane.

Conclusion

The present study reveals the effect of various concentrations of ions on S Bio Oxidation. The effect of various counter ions with the same anion showed complete inhibition at higher concentrations. The pH and viable cell count data reveals that Cl^- and NO_3^{2-} had more inhibitory effects at lower concentrations than SO_4^{2-}. Effect of various anions for the same concentration of Cl^-, was NaCl> NH_4Cl>KCl, the Na^+ ion was more inhibitory with less viable cell count and higher pH then K^+ and NH_4^+. K^+ ion was less inhibitory then Na^+ and NH_4^+ while all three cations showed similar toxicity at the concentration i.e. 4 g/L of Cl^-. The order of inhibition for nitrate ion was as follows Ca $(NO_3)_2$<< $NaNO_3$<KNO_3 indicates the more inhibitory effect of monovalent cations (Na^+ and K^+) then divalent (Ca^{2+}) ion for the same concentration of nitrates. The viable cell count data for the study supports the pH patterns with time. The viable cell count for Ca $(NO_3)_2$ was close to the control experiment's reading which supports the less inhibitory effect of Ca^{2+} ion. While for the higher concentration of nitrate the Na^+ ion and K^+ ion showed the similar pattern for pH and viable cell count. The higher concentration of sulphate was chosen for the study which reveals that $(NH_4)_2SO_4$ was more deleterious at lower concentrations. The order of inhibition observed was $(NH_4)_2SO_4$> Na_2SO_4> K_2SO_4 for the higher SO_4^{2-} concentrations. Na_2SO_4 and K_2SO_4 showed almost similar effects at their lower concentrations.

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