Optimization of Heavy Metal Removal by Sulfate Reducing Bacteria in a High Concentration Zn-fed Fixed Bed Bioreactor Using Plackett Burman Design Experiments

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Abstract. This study evaluated the combined effect of six processing factors on Zn, Cd removal and sulfate reducing bacteria in a high concentration Zn-fed fixed bed bioreactor. Statistically valid Plackett Burman design of experiments was employed to carry out this study. The results obtained showed a high removal of Zn (99.63%), Cd (99.73%) at a maximum influent Zn concentration of 320 mg/L. Analysis of variance (ANOVA) of Zn and Cd removal revealed that the effect due to glycerol dosage and reflux ratio were highly significant (P value < 0.05). To establish the role of sulfate reducing bacteria (SRB) in the metal removal process, the characteristics of microbial community in fixed bed bioreactor was analyzed by High-throughput Sequencing during the steady operational process. The results obtained shows that the percentage of the dominant sulfate reducing bacteria in the sludge, such as Desulfovibrio, could reach 45.7%. Qualitative EDS analysis of the precipitate was performed. The results revealed that the precipitates as ZnS were confirmed by the EDS spectrum with strong peaks of zinc and sulfur.

1. Introduction

Heavy metal containing wastewater from mining and industrial processing can result in a serious problem toward the environment and living things [1]. Compared to the organic pollutants, which can be biodegraded to harmless chemical species, heavy metals can be classified as toxic and non-biodegradation inorganic pollutants as they could accumulate in the food chain and get absorbed by living organisms, including humans, which result in ion displacement and substitution of essential ions from cellular sites and blocking of functional groups of important molecules and essential nutrient transport systems [2]. Hence, it is very importance to remove heavy metals and sulfate from wastewater prior to its discharge into the environment [3]. Conventional chemical precipitation treatment processes of heavy metal wastewater have been investigated all over the world including addition of lime to raise pH, aeration to oxidize ferrous iron and flocculating and so on. However, those processes produce metal-rich solid products, such as residual sludge, which need disposal in designated landfill sites and long-term management [4-6]. Therefore, an alternative approach of immobilization of heavy metals as sulfides using hydrogen sulfide generated by sulfate reducing bacteria (SRB) is now of considerable research interest [7].

Up to now, bacterial sulfate reduction has been studied to treat heavy metal containing wastewater in laboratory bioreactor including upflow anaerobic sludge blanket (UASB) and in pilot-scale tests [8-10]. This process utilizes SRB to reduce the sulfate to $S^2-$, HS$^-$ and H$_2$S which could precipitate heavy metals in wastewater. SRB belong to a group of morphologically diverse and anaerobic
microorganisms and have an ability to use sulfate as the terminal electron acceptor and organic compounds as electron donors [11]. The process is briefly showed as follows:

\[
\text{Electron donor} + SO_4^{2-} \rightarrow HS^- + HCO_3^- \tag{1}
\]

\[
Me^{2+} + HS^- \rightarrow MeS \downarrow + H^+ \tag{2}
\]

\[
HCO_3^- + H^+ \rightarrow H_2O + CO_2 \tag{3}
\]

The SRB do their activity as oxidation-reduction reactions, in which the carbon is oxidized and the released electron from this reaction is used to reduce sulphate [12]. Heavy metal precipitation by SRB is influenced by a variety of parameters such as carbon resource concentration, pH, sulfate and heavy metal concentration [13]. Several authors have reported the effect of individual parameter on heavy metals treatment. It is important to analyze and characterize the multi-parameters effect on the treatment performance. One way to study the combined effect is by using statistically designed experiments [14]. For example, individual main effect of parameters on a given response can be investigated by employing statistical experimental design technique, such as Plackett-Burman design [15].

In this study, different combinations of high and low levels of six operation parameters were chosen using the Plackett Burman design. ANOVA and Student’s t test were used for statistical analysis of the results to assess the significance and effect of these parameters on Zn, Cd and sulfate reduction in the study.

2. Materials and Methods

2.1. SRB source and culture conditions
Excess sludge obtained from a municipal wastewater treatment plant was used as an inoculum. Element composition measured in the water phase of the sample used as the inoculum in this study comprised of 400 mg/L COD, 40 mg/L TN, 2mg/L TP, 5 mg/L Zn, 1mg/L Cd and 200 mg/L sulfate.

2.2. Experimental set-up
The schematic diagram of SRB system is presented in Figure 1.

![Figure 1. Schematic diagram of SRB system](image)

The synthetic wastewater was pumped from influent tank to precipitation tank. Part of precipitation tank effluent was pumped to fixed bed SRB bioreactor. The fixed bed SRB bioreactor effluent which comprised of $S^{2-}$, $HS^-$ and $H_2S$ was refluxed to precipitation tank. The precipitation tank was equipped with a stirrer to promote the precipitation reaction. The heavy metals precipitation was carried on in the tank and $R_1$ represent the reflux ratio between precipitation tank and SRB bioreactor. For satisfactory fluidization a reflux system was designed in SRB bioreactor and reflux ratio was represented as $R_2$.

The SRB system were first inoculated with 10% (v/v) of the enrich SRB consortium and fed continuously with synthetic wastewater according to the experiment design plan.

2.3. Analytical methods
The concentrations of Zn and Cd were measured by an atomic absorption spectrophotometer after acidifying with concentrated nitric acid to prevent metal precipitation. Sulfate and pH were measured according to standard methods. The microstructure and qualitative analysis of precipitates were realized by scanning electron microscope(SEM) and energy dispersive spectrometry(EDS). The precipitates were obtained after filtering the sample from the bottom of precipitation tank through 0.45 μm membrane filters. The filter paper was dried in an oven for 2 h at 105 °C and then thin coated by Au for SEM and EDS analysis.

2.4. Microbial community analysis
To study the microbial structure community in the SRB bioreactor, high-throughput sequencing technology was used. The total DNA of sample was extracted by PowerWater DNA Isolation Kit. After qualitative detection with agarose gel electrophoresis and determination by Nanodrop2000, the V4 hypervariable region of 16S rRNA gene was amplified with the primers of 515F (5′-GTGCCAGCMGC CGCGGTAA-3′) and 909R (5′-CCCCGYCAATTCTTMTTTR A GT-3′). Gene sequencing was performed on an Illumina MiSeq platform by Sangon Corp method (Shanghai, China).

3. Results and Discussion

3.1. Heavy metal precipitation form SRB system
Table 1 presents the complete matrix of designed experiments and the results. The Minitab 17 was used to designed the experiments and analyze the data. The designed 12 experimental runs were carried out and the experimental data achieved was input to the software to calculate the predicted values. The results obtained reveal the the removal efficiency of each of Zn, Cd and sulfate varied depending upon the selected operation parameters. The results show a maximum removal for Zn(99.82%), followed by Cd(99.73%), and sulfate(40.00%). An overall removal efficiency of more than 80% for each of Zn and Cd was achieved. The results also show that experimental run 9 resulted in maximum sulfate reduction rate (40.00%), followed by that in in runs 4 and 10. The sulfate reduction efficiencies were the minimum in the experimental run 6.

Table 1. Plackett-Burman experimental design matrix.

| Exp run | A (mg/L) | B (mg/L) | C (mg/L) | D (%) | E (%) | F | Removal rate (%) |
|---------|----------|----------|----------|-------|-------|---|-----------------|
|         | Zn       | SO₄²⁻    | Glycerol | R1    | R2    | pH | Zn  | Cd  | SO₄²⁻ |
| 1       | 320      | 4000     | 250      | 200   | 200   | 5  | 93.13 | 91.33 | 11.00 |
| 2       | 220      | 2000     | 250      | 100   | 100   | 5  | 84.09 | 84.00 | 16.50 |
| 3       | 220      | 4000     | 250      | 100   | 100   | 6  | 87.50 | 85.60 | 20.00 |
| 4       | 320      | 4000     | 500      | 100   | 200   | 6  | 99.63 | 99.73 | 38.75 |
| 5       | 320      | 2000     | 250      | 100   | 200   | 6  | 99.28 | 94.67 | 20.00 |
| 6       | 320      | 4000     | 250      | 200   | 100   | 5  | 86.25 | 84.00 | 5.75  |
| 7       | 320      | 2000     | 500      | 100   | 100   | 5  | 94.56 | 94.13 | 34.00 |
| 8       | 220      | 2000     | 250      | 200   | 200   | 6  | 99.82 | 99.7  | 22.50 |
| 9       | 220      | 2000     | 500      | 200   | 200   | 5  | 98.64 | 96.60 | 40.00 |
| 10      | 220      | 4000     | 500      | 100   | 200   | 5  | 99.50 | 99.50 | 36.00 |
| 11      | 320      | 2000     | 500      | 200   | 100   | 6  | 98.94 | 98.6  | 33.00 |
| 12      | 220      | 4000     | 500      | 200   | 100   | 6  | 99.55 | 93.3  | 27.75 |

For a better interpretation and assessment of the significance of the selected operation parameters on Zn removal as well as on the Cd and sulfate reduction by SRB, the results obtained were analyzed statistically in terms of Student’s test and analysis of variance(ANOVA). The ANOVA of Zn, Cd and sulfate removal are presented in Table 2, 3 and 4 respectively. In these ANOVA tables, the high Fisher’s F value and low probability P value demonstrate the precision of the
regression model in explaining the variations in the results. The value of Fisher’s F and P illustrate whether the level means significantly different from each other or not. Student’s t test was used to illustrate the effect of the parameters on Zn, Cd and sulfate reduction (Table 5), which is often used to verify the significance of the coefficients of the regression model parameter on the given response. The estimated coefficients of individual effect of these parameters and the associated T and P values were also showed in Table 5.

From Table 5, an increase in glycerol dosage showed positive effect with P values of 0.006, 0.007 and 0.000 on Zn, Cd and sulfate reduction, respectively. The parameter F (pH) showed a positive effect on Zn removal with a P value of 0.026. It was report that SRB could show better activity in basic Ph range than in acidic Ph range. The influence due to parameters B( sulfate concentration), D(R1) on sulfate removal were negative with P value of 0.049 and 0.058, whereas parameter E(R2) showed a positive effect with P value of 0.029. The increase in sulfate concentration resulted in a negative effect on bacterial activity and also sulfate removal. Higher concentrations of sulfate reverse in the activity of the sulfate reducing bacteria, which could be due to inhibition fo metabolism and as a result inhibition of SRB growth at higher sulfate concentration. In addition, in the condition of high sulfate sulfate concentration the amount of dissolved sulfide increase, therefore, the redox potential increases which causes inhibitory effect on SRB.

All these effects of parameters on Zn and Cd removal are better illustrated in the form of Pareto charts which is showed in Figure 2. The effect due to the individual parameter is represented by horizontal bars in the charts. And the those extending past the vertical line on the chart represent the significant ones (α=0.05). The results indicated that an increase in glycerol dosage and R2 showed positive effect on Zn and Cd removal. Glycerol dosage is a very important factor, because biological sulfate reduction involves two steps. In the first step, SRB oxidize glycerol by utilizing sulfate as an electron acceptor and generating hydrogen sulfide. In the second step, the biologically produced hydrogen sulfide reacts with heavy metals to form metal sulfide precipitates.

| Table 2. Analysis of variance of Zn removal |
|--------------------------------------------|
| Source          | DF | Seq SS | Adj MS | F     | P    |
| Main effects     | 6  | 353.719 | 58.953 | 8.58  | 0.016 |
| A               | 1  | 0.601  | 0.601  | 0.09  | 0.779 |
| B               | 1  | 7.965  | 7.965  | 1.16  | 0.331 |
| C               | 1  | 138.353 | 138.353 | 20.13 | 0.006 |
| D               | 1  | 11.520 | 11.520 | 1.68  | 0.252 |
| E               | 1  | 127.368 | 127.368 | 18.53 | 0.008 |
| F               | 1  | 67.912 | 67.912 | 9.88  | 0.026 |
| Residual error  | 5  | 34.359 | 6.872  |       |      |
| Total           | 11 | 388.078 |        |       |      |

| Table 3. Analysis of variance of Cd removal |
|--------------------------------------------|
| Source          | DF | Seq SS | Adj MS | F     | P    |
| Main effects     | 6  | 358.715 | 59.786 | 7.56  | 0.021 |
| A               | 1  | 1.182  | 1.182  | 0.15  | 0.715 |
| B               | 1  | 16.882 | 16.882 | 2.13  | 0.204 |
| C               | 1  | 150.993 | 150.993 | 19.09 | 0.007 |
| D               | 1  | 2.901  | 2.901  | 0.37  | 0.571 |
| E               | 1  | 146.301 | 146.301 | 18.50 | 0.008 |
| F               | 1  | 40.456 | 40.456 | 5.11  | 0.073 |
| Residual error  | 5  | 39.548 | 7.910  |       |      |
| Total           | 11 | 398.263 |        |       |      |
Table 4. Analysis of variance of Sulfate removal

| Source          | DF | Seq SS  | Adj MS | F   | P   |
|-----------------|----|---------|--------|-----|-----|
| Main effects    | 6  | 1335.86 | 222.64 | 25.00 | 0.001 |
| A               | 1  | 34.17   | 34.17  | 3.84 | 0.107 |
| B               | 1  | 59.63   | 59.63  | 6.70 | 0.049 |
| C               | 1  | 1078.26 | 1078.26| 121.08 | 0.000 |
| D               | 1  | 53.13   | 53.13  | 5.97 | 0.058 |
| E               | 1  | 81.38   | 81.38  | 9.14 | 0.029 |
| F               | 1  | 29.30   | 29.30  | 3.29 | 0.129 |
| Residual error  | 5  | 44.53   | 8.91   |      |      |
| Total           | 11 | 1380.39 |        |      |      |

Table 5. Significance of different parameters on contaminants removal in terms of Student’s t-test

| Term          | A        | B        | C       | D       | E       | F       |
|---------------|----------|----------|---------|---------|---------|---------|
| For Zn removal| Effect   | 0.448    | -1.629  | 6.791   | 1.960   | 6.516   | 4.758   |
|               | Coef     | 0.224    | -0.815  | 3.396   | 0.980   | 3.258   | 2.379   |
|               | T        | 0.30     | -1.08   | 4.49    | 1.29    | 4.31    | 3.14    |
|               | P        | 0.779    | 0.331   | 0.006   | 0.252   | 0.008   | 0.026   |
| For Cd removal| Effect  | 0.628    | -2.372  | 7.094   | 0.983   | 6.983   | 3.672   |
|               | Coef    | 0.314    | -1.186  | 3.547   | 0.492   | 3.492   | 1.836   |
|               | T       | 0.39     | -1.46   | 4.37    | 0.61    | 4.30    | 2.26    |
|               | P       | 0.715    | 0.204   | 0.007   | 0.571   | 0.008   | 0.073   |
| For sulfate reduction | Effect | -3.375 | -4.458 | 18.958 | -4.208 | 5.208 | 3.125 |
|               | Coef    | -1.688   | -2.229  | 9.479   | -2.104  | 2.604   | 1.563   |
|               | T       | -1.96    | -2.59   | 11.00   | -2.44   | 3.02    | 1.81    |
|               | P       | 0.107    | 0.049   | 0.000   | 0.058   | 0.029   | 0.129   |

Individual parameter effect, such as pH, heavy metal concentration and so on, on sulfate reduction by SRB is well reported in the literature. However, there is a very less understanding on the combined effect of more than one parameter on treatment performance. It can be observed from results that Zn and Cd precipitation by SRB through sulfate reduction. The obtained high Zn and Cd removal rates were due to its low solubility product with sulfide. It was reported that the order of removal of heavy metals as sulfide salts is attributed to their respective solubility product constant values. For example, the solubility product constant of Zn sulfide is $2 \times 10^{-25}$. The substantial percentage of Zn removal can be explained due to its low solubility product with sulfide. The other reason for high percentage of Zn removal is due to the feature of Zn for be bounded by extracellular polymeric substances (EPS) extracted from SRB. Charge density, attractive interaction and conformation types of the polymer are the parameters which determin the affinity of different metal ions. The results further suggest that Zn at a high concentration of 320 mg/L did not inhibit the growth of SRB. At a high influent Zn concentration, the sulfate reduction efficiency was slightly lower. An increase in Zn concentration showed negative effect with Student’s t-test value of 1.96 on sulfate reduction. Possible explanation may be the presence of bacteria cells facilitated metal precipitation and the high level of sulfide precipitates could act as a barrier between the cells and their essential growth nutrients. Therefore, the high influent heavy metal concentration could result in the drop of sulfate reduction. In addition, in others biological sulfate reduction process, the heavy metal containing wastewater was directly feeding into bioreactor, and in the reactor both sulfate reduction and metal precipitation occurs. In such process, high concentration of heavy metals will perform toxic effect on microbial activity, and result in the failure in treatment. In this studied novel process, by
using water recycle (R2), the produced water soluble H$_2$S was pumped to precipitation tank and precipitated with metal to form metal sulfides, thus the heavy metals concentration and H$_2$S will maintain in low concentration. Thus only low concentration of heavy metals was fed into SRB bioreactor. Such process will decrease the toxicity of heavy metal to the microbial community. The high tolerance of SRB in fixed bed bioractor was due to the biofilm structurion. The metal resistance of SRB bilfilm varies with the species by developing a variety of specific resistance emechanism such as metal exclusion by permeability barrier and reduction in metal sensitivity of biofilm. It was reported that the toxic effects of binary mixtures of heavy metal were obviously higher than the one of individual heavy metal. However, in the present study, 99~100% of Zn and Cd removal efficiencies were obtained during the experiments. The results might also be due to the use of an indigenous SRB consortium cultured from the municipal wastewater treatment plant which accepted the industrial heavy metal containing wastewater. The advantage of using indigenous consortia may be that indigenous consortia contained multi-species which had adapted to a heavy metal polluted enviroment by developing a variety of resistance mechanisms.

![Figure 2](image1.png)

**Figure 2.** Pareto chart showing the effect of different parameters on Zn (a) and Cd (b) removal

### 3.2. Qualitative EDS analysis

In order to confirm the heavy metal removal mechanism by the SRB through sulfate reduction in this study, qualitative EDS analysis of the precipitates obtained from precipitation tank at the end of the experiments was performed.

![Figure 3](image2.png)

**Figure 3.** The SEM image (a) and EDS spectrum (b) of the precipitates

Metal precipitates are visible from Figure3(a). The precipitates as ZnS was confirmed by the EDS spectrum with strong peak of zinc and sulfur shown in Figure 3(b). The present of sulfur peak in the spectra in attributed to the metal sulfide precipitaion as a result of biological sulfate reduction reaction.
Sulfate reduction along with metal sulfide formation confirmed that sulfidogenesis is the predominant mechanism for heavy metal removal by SRB. Metal sulfide have been attributed to the major precipitation of Zn by biological activity of SRB in the present study, which is confirmed by a simultaneous decrease of sulfate and Zn concentration in the effluent and EDS spectrum with strong peaks of zinc and sulfur. Among the peaks due to the different elements, only the peak due to sulfide is significant indicating that metals were presented as sulfides in the precipitate. All other forms such as M(OH)₅, MCO₃ were not significant. The metal sulfide precipitates could be recovered by smelting process. The results indicated that Zn removal was possible only in the presence of sulfate and sulfide produced from sulfate reduction by SRB was responsible for Zn removal as ZnS. Although heavy metals may also have been removed through sorption to the biomass or by hydroxide and carbonate precipitation, sulfide precipitation is the dominant mechanism while other mechanisms play only a minor role for heavy metal removal in SRB system.

3.3. Analysis of SRB community
The microbial community structure in SRB bioreactor on genus level was showed in Figure 4. *Desulfovibrio* was the dominant microbe in the sample which was obtained from SRB bioreactor at experimental run 9. The detected potential SRB included *Desulfovibrio* (45.7%), *Sulfurospirillum* (1.6%), *Clostridium* (0.24%).

![Figure 4. Microbial community structure on genus level in SRB bioreactor](image)

It was reported that SRB was sensitive to acidity. When the pH drop from 3.25 to 3.0, the sulfate reduction efficiency decreased from 38.3% to 14.4%. Thus most of reported papers were operated at neutral pH. The decrease of pH value could result in the drop of sulfate reduction. In our study, *Desulfovibrio* became the dominant microbe under the condition of pH of 5.0~6.0. It was reported that the *Desulfovibrio* could survive at acid pH of 2.5. The works about isolating the acid-tolerant SRB will be carried on. The SRB technique operated under acid pH is very important, which will border the application area of this process.

4. Summary
This study demonstrated the precipitation of Zn and Cd at the condition of high influent Zn concentration of 320 mg/L. A very high removal of Zn and Cd was achieved at both low and high initial concentrations. Statistically valid Plackett Burman design of experiments was employed to carry out this study. Analysis of variance (ANOVA) of Zn and Cd removal revealed that the effect due to glycerol dosage and reflux ratio were highly significant (P value < 0.05). High-throughput sequencing technology was used to study the microbial community structure. The results indicated that SRB was the dominant microbe in SRB bioreactor. The precipitates as ZnS was confirmed by the EDS spectrum with strong peak of zinc and sulfur. Overall, this study proved very good potential of SRB in the anaerobic biomass for metal precipitation with a high tolerance.
5. References

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