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Biological Treatment of Toxic Petroleum Spent Caustic in Fluidized Bed Bioreactor Using Immobilized Cells of *Thiobacillus* RAI01

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Abstract In the present studies, newly isolated *Thiobacillus sp* was used for the treatment of synthetic spent sulfide caustic in a laboratory-scale fluidized bed bioreactor. The sulfide oxidation was tested using Ca-alginate immobilized *Thiobacillus sp.* Initially, response surface methodology was applied for the optimization of four parameters to check the sulfide oxidation efficiency in batch mode. Further, reactor was operated in continuous mode for 51 days at different sulfide loading rates and retention times to test the sulfide oxidation and sulfate and thiosulfate formation. Sulfide conversions in the range of 90–98% were obtained at almost all sulfide loading rates and hydraulic retention times. However, increased loading rates resulted in lower sulfide oxidation capacity. All the experiments were conducted at constant pH of around 6 and temperature of 30±5 °C.

Keywords Autotrophic bacteria · Sulfide oxidation · Response surface methodology · *Thiobacillus sp.* · Fluidized bed reactor · Optimization

Introduction

In a petroleum refinery and petrochemical plant, hydrocarbon feed stocks are cracked to manufacture varied products. The cracked gas from the cracking furnace is scrubbed in a

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caustic wash tower to remove carbon dioxide, hydrogen sulfide, and mercaptans prior to further processing in the plant's cold section. The spent caustic is purged from the wash tower and is laden with sulfidic constituents plus organics such as condensed oils and benzene. This spent caustic scrubbing liquor (spent caustic) is the most common problematic waste stream generated in a petroleum refinery and petrochemical plant. This is primarily due to the sulfide concentrations which can range as high as 6% (expressed as NaHS), depending on the cracking furnace feed stock and wash tower operation. Typical composition of petroleum spent caustic sulfide effluent is as follows; NaHS, 05–6%; Na_2CO_3, 1–5%; NaOH, 1–4%; NaSR, 0–0.2%; soluble oil, 50–200 ppm; benzene, 20–100 ppm; phenols, 20–200 ppm. High levels of sulfides can create odor and safety problems when liberated as a gas. Discharge of spent caustics directly to the plant's wastewater treatment plant can cause operational problems. There is great need to develop processes for sulfide removal from wastewater because of its toxicity, corrosive properties, bad odor, and high oxygen demand [1–5]. On a worldwide basis, most newly constructed grassroots facilities use wet air oxidation for spent caustic treatment [3, 4]. The resulting solution from wet air oxidation process rich in sulfites, sulfates, and thiosulfate will send to plant's wastewater treatment unit. In the anaerobic treatment unit of wastewater treatment plant, the sulfites, sulfates, and thiosulfate will reduce into sulfides by the anaerobic metabolism of sulfate-reducing bacteria. Besides this, the wet air oxidation process operates at a temperature of around 150–190 °C, which results in higher operating costs [4]. Removal of sulfide from the industrial waste streams is presently being done by chemical methods, which are expensive as well as environmentally not benign [3, 4].

The biological sulfide-removing studies employs either photoautotrophic or chemolithotrophic sulfide-oxidizing bacteria (SOB). Bioreactors employing chemotrophic SOB generally achieved higher sulfide loading rate than photoautotrophic systems. The simpler nutritional requirements and higher sulfide tolerance of chemotrophic organisms favored their application in biological sulfide oxidation systems. The bacteria involved in sulfide oxidation belong to a group of colorless sulfur bacteria, of which *Thiobacillus* is the best known. *Thiobacillus* is mostly facultative autotrophic, utilizing reduced inorganic sulfur compounds as electron donors and carbon dioxide as a carbon source. However, some heterotrophic *Thiobacilli* were reported in a sulfide-oxidizing reactor, when the sulfide-laden wastewater contained organic matter [6–9]. Under the present circumstances, sulfide oxidation using biotechnological methods is the best suitable alternative.

Organisms belonging to the group of colorless sulfur bacteria oxidize sulfide to elemental sulfur under circumstances of oxygen-limiting conditions. Based on this feature, many researchers worked for biological oxidation using various types of microorganisms [8, 9].

Immobilization of cells for microbial fermentation for extracellular metabolites offers many advantages over free cell fermentation systems because such systems offer better operational stability, higher efficiency of cells and allow the continuous use of cells, making immobilization a very advantageous procedure. Polysaccharide gel matrices, more particularly Ca-alginate hydrogels, are by far the most frequently used materials for better cell entrapment. Furthermore, this process stabilizes the biocatalysis as well as maintaining the cells uniformly distributed in the reactor, so that each cell is provided with an equal substrate supply [10].

The classical method of experimental optimization involves changing one variable at a time, keeping the others constant. In addition, it is not practical to carry out experiments with every possible factorial combination of the test variables because of the large number of experiments required [11, 12]. This does not consider the effect of interactions of various parameters. Besides this, it is a tedious, cumbersome, and time-consuming process especially when a large number of parameters are taken into account. An alternative and more efficient approach is the use of statistical method. Response surface methodology

(RSM) has been widely used to evaluate and understand the interactions between different process parameters [13]. RSM was applied successfully for optimizing process parameters for various processes in biotechnology [14–16].

In the present study, fluidized bed bioreactor (FBR) parameters were optimized by a 2^4 full-factorial central composite design (CCD) and RSM were used for optimization of reactor conditions. Further synthetic spent sulfide caustic effluent was treated in fluidized bed reactor using calcium alginate immobilized beads.

Materials and Methods

Microorganism and Culture Conditions

Isolation of sulfide-oxidizing bacteria is done from aerobic sludge collected from distillery industry and dairy industry wastewater treatment plant and the cultures were identified as *Thiobacillus* sp. by its phenotypic and genotypic characterization. Further, sulfide oxidation capacities of the isolated cultures were evaluated [10]. The isolated *Thiobacillus sp.* cultures were maintained in liquid broth having composition [10] NH_4Cl, 1.0 g/l; K_2HPO_4, 0.6 g/l; $CaCl_2{\cdot}2H_2O$, 0.2 g/l, $FeCl_3{\cdot}H_2O$, 0.02 g/l, $Na_2S_2O_3{\cdot}5H_2O$, 10 g/l; $CaCO_3$ 3 g/l.

Immobilization of *Thiobacillus sp.* Cultures

The isolated *Thiobacillus sp.* cultures grown in 500 ml of thiosulfate media were centrifuged at 7,500 rpm for 10 min. After centrifugation, the bacterial cells were separated by decanting the supernatant aseptically and stored in a vial. The bacterial cell pellet is washed with sterile double-distilled water for three times. The bacterial cell pellet was weighed and 20 mg of cells were added to sodium alginate solution. The 4% sodium alginate solution was prepared by adding 4 g of high viscous sodium alginate in 100 ml of *Thiobacillus sp.* growth media by continuous stirring till the sodium alginate is completely mixed in solution.

The beads are prepared with a peristaltic pump using a pipe of 2-mm diameter in $CaCl_2$ solution. The 4% $CaCl_2$ cross-linking solution was prepared by dissolving 6 g of $CaCl_2$ in 150 ml of double-distilled water. The beads were allowed in cross-linking solution for 3 h. Later, beads were transferred to 150 ml of maintenance medium containing NH_4Cl, 4 g/l; $MgSO_4.7H_2O$, 1 g/l; KH_2PO_4, 2 g/l; and 10 ml of trace element solution overnight. The whole process is carried out in aseptic conditions to prevent any possibility of contamination.

Optimization FBR Operation in Batch Mode for Sulfide Oxidation

Selection of Fluidized Bed Reactor Conditions

In an earlier study, the reactor conditions were optimized by one-factor-at-a-time method, by keeping the other factors at constant level. It was found that sulfide concentration (SC), (VB), $CaCO_3$, and reactor operation time (ROT) had the significant effects on the sulfide oxidation by *Thiobacillus* sp.

Experimental Design and Optimization

The optimum conditions of fluidized bed bioreactor for sulfide oxidation by *Thiobacillus* sp. were determined by means of RSM. The RSM consists of a group of empirical

techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and measured responses according to one or more selected criteria. According to this design, the total number of treatment combinations was 2^k+2k+n0 where k is the number of independent variables and n0 is the number of repetitions of the experiments at the center point.

Based on the best results of one-at-a-time approach, four critical components of production medium were selected and further evaluated for their interactive behaviors by using a statistical approach. The levels of four medium variables viz. SC, 150 mg/l (X1); VB 20 (*v*/*v*, X2); $CaCO_3$ 3% (*w*/*v*, X3), and ROT 36 h (X4) were selected and each of the variable were coded at five levels −2, −1, 0, 1, and 2 by using Eq. 1. For statistical calculations, the variables X_i were coded as x_i according to the following transformation.

The range and levels of the variables in coded units for RSM studies are given in Table 1.

$$x_i = X_i - X_0/\Delta X \tag{1}$$

Where x_i is the dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point, and ΔX the step change.

The behavior of the system was explained by the following quadratic model 2.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_j^2 + \sum \beta_{ij} x_i x_j \tag{2}$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} the interaction effect. The full quadratic equation for four factors is given by model 3.

$$\begin{aligned} Y = {} & \beta_0 + \beta_i x1 + \beta_2 x2 + \beta_3 x3 + \beta_4 x4 + \beta_{11} x1 \times x1 + \beta_{12} x1 \times x2 + \beta_{13} x1 \times x3 + \beta_{14} x1 \\ & \times x4 + \beta_{22} x2 \times x2 + \beta_{23} x2 \times x3 + \beta_{24} x2 \times x4 + \beta_{33} x3 \times x3 + \beta_{34} x3 \times x4 + \beta_{44} x4 \\ & \times x4 \end{aligned} \tag{3}$$

Several experimental designs have been considered for studying such models, and central composite design was selected. For this study, a 2^4 factorial design with eight star points and six replicates at the central points were employed to fit the second-order polynomial model, which indicated that 30 experiments were required for this procedure. STATISTICA 6.0 (Stat Soft, Inc., Tulsa, OK, USA) software was used for regression and graphical analysis of the data obtained.

In order to search for the optimum combination of major components of the production medium, experiments were performed according to the CCD experimental plan (Table 2).

Table 1 Range and levels of the variables in coded units for RSM studies.

Variables	Range and levels					ΔX
	−2	−1	0	1	2	
SC, mg/l, $x1$	50	100	150	200	250	50
VB, % (*v*/*v*), $x2$	10	15	20	25	30	5
$CaCO_3$, %, $x3$	1	2	3	4	5	1
ROT, h, $x4$	12	24	36	48	60	12

Where ΔX is step increment in each variable values

The results of CCD experiments for studying the effect of three independent variables are presented along with the mean predicted and observed responses in Table 2. The regression equations obtained after the analysis of variance (ANOVA) gave the level of sulfide oxidation as a function of the initial values of SC, VB, $CaCO_3$, and ROT.

Construction and Operation of Fluidized Bed Bioreactor

Fluidized bed reactor made up of glass column having dimensions of height, 54 cm; internal diameter 6 cm; effluent inlet and outlet pipe diameter, 8 mm; draft tube height, 52 cm, resulting in a total volume of 1,500 ml. Working volume of FBR was 1,000 ml with packed bed height of 16 cm and fluidized bed height of 48 cm at a minimum fluidization air flow rate of 2.4 ml/min. FBR was equipped with a peristaltic pump (Watson Marlow, UK) for feeding with a feed pipe diameter of 8 mm connected to a glass feed tank having 15-l capacity. Sterile air was supplied through Air filter (PTFE, GELMAN) 0.2-μM size using air pump having capacity of 0.833 to 6.67 ml/min measured using rotameter (Engineers India Ltd.).

Table 2 Design of experiments by central composite design for RSM studies.

Run number	*x*1	*x*2	*x*3	*x*4	Coefficients assessed by	Sulfide oxidation, %, measured	Sulfide oxidation, %, predicted
1	−1	−1	−1	−1	Full factorial 2^4	72	71.507
2	1	−1	−1	−1	design (16 expts)	68	58.795
3	−1	1	−1	−1		82	77.131
4	1	1	−1	−1		71	70.671
5	−1	−1	1	−1		68	63.129
6	1	−1	1	−1		74	69.169
7	−1	1	1	−1		61	67.005
8	1	1	1	−1		84	79.297
9	−1	−1	−1	1		82	89.123
10	1	−1	−1	1		75	77.663
11	−1	1	−1	1		76	89.495
12	1	1	−1	1		77	84.287
13	−1	−1	1	1		69	77.997
14	1	−1	1	1		78	85.289
15	−1	1	1	1		65	76.621
16	1	1	1	1		81	90.165
17	−2	0	0	0	Star points (8 expts)	92	79.049
18	2	0	0	0		78	79.881
19	0	−2	0	0		65	67.215
20	0	2	0	0		91	77.715
21	0	0	−2	0		82	79.715
22	0	0	2	0		86	77.215
23	0	0	0	−2		45	62.199
24	0	0	0	2		99	90.683
25	0	0	0	0	Central points (6 expts)	65	67.917
26	0	0	0	0		63	67.917
27	0	0	0	0		66	67.917
28	0	0	0	0		67	67.917
29	0	0	0	0		69	67.917
30	0	0	0	0		70	67.917

The reactor was filled with immobilized beads prepared in the previous step along with the maintenance media having composition [10] NH_4Cl, 4 g/l; $MgSO_4 \cdot 7H_2O$, 1 g/l; KH_2PO_4, 2 g/l; and 10 ml/l of trace element solution and 20 g of $NaHCO_3$ as an initial buffer was transferred to the reactor.

The packed bed height of the beads before starting the reactor was 16 cm. The reactor was fed with maintenance medium continuously by the help of peristaltic pump for a period of 4 days at a hydraulic retention time (HRT) of 5 h and during this period the beads were maintained in fluidized state by supplying sterile air at a flow rate of 2.4 ml/min with the help of air pump.

After acclimatizing the immobilized beads with maintenance medium for 4 days, it was gradually replaced with synthetic spent sulfide effluent having initial SC of 500 mg/l along with other nutrients as defined in previous section. Since the typical composition of petroleum spent caustic SC is in the range of 0.5% to 5%, lower concentration was selected for the experimental studies. The total replacement of maintenance medium was done after 20 h of reactor startup. The maintenance medium replacement with synthetic spent caustic effluent was done at every 5-h intervals a step increment of 20%.

Synthetic sulfide spent caustic effluent was prepared in the lab and at different concentrations of sulfide by using sodium sulfide. Synthetic sulfide caustic effluent was mixed with 50 ppm of phenol to test the effect of its presence on the sulfide oxidation activity of the *Thiobacillus* sp. Further, the caustic effluent was treated in a fluidized bed reactor using Ca-alginate immobilized *Thiobacillus* sp. cells for sulfide oxidation under different loading rates.

Varying caustic effluent inlet flow rates without changing initial SC in the effluent varied the sulfide loading rates to the reactor. The reactor was operated at three different inlet flow rates, viz., 0.695, 0.9267, and 1.388 ml/min which resulted in HRTs of 24, 18, and 12 h. This corresponds to the respective sulfide loading rates of 0.020833, 0.027778, and 0.041667 g-S/m^3 per hour. FBR was operated continuously for a period of 51 days under different sulfide loading rates.

Initially, FBR was operated for 9 days at a flow rate of 0.695 ml/min which resulted in 24 h of retention time with 0.020833 g-S/m^3 per hour sulfide loading rate. From 10th to 18th day, the sulfide loading rate was 0.027778 g-S/m^3 per hour with corresponding flow rate of 0.926 ml/min and 18-h retention time. Sulfide loading rate was further increased to 0.041667 g-S/m^3 per hour with a flow rate of 1.388 ml/min on 19th day and operated till 31st day having retention time of 12 h. From 32nd day till the end (51st day), FBR was operated at a flow rate 0.9267 ml/min with 0.027778 g-S/m^3 per hour sulfide loading rate and 18-h retention time.

The samples are drawn at 24-h frequency and analyzed for pH, temperature, DO, sulfate, sulfide, and thiosulfate.

Analytical Methods

Standards methods [17] are used for the analysis of pH, temperature, DO, sulfate, sulfide, sulfur, and thiosulfate. Scanning electron microscope (SEM) was used to study the morphology of immobilized *Thiobacillus* sp. cells in alginate beads. Sample of bead at the end of 51 days of FBR operation was examined. The alginate bead was cut into cross section and fixed with 2% glutaraldehyde and dehydrated in gradient ethanol (10% to 100%). The sample was dried, coated with gold, and observed with an (HITACHI-S3000N, Singapore) electron microscope.

Results and Discussions

Results

Optimization of Batch FBR for Sulfide Oxidation

The lead objective of the RSM was to run rapidly and efficiently along the path of improvement towards the general vicinity of the optimum. It is appropriate when the optimal region for running the process has been identified. The four independent variables, SC, VB, $CaCO_3$ and ROT, in the bioreactor operation were chosen to optimize the sulfide oxidation by *Thiobacillus* sp.

Experiments were performed according to the CCD experimental design given in Table 1 in order to search for the optimum combination of components of the medium. The coefficient of determination (R^2) was calculated as 0.927 for sulfide oxidation (model summary, Table 3), indicating that the statistical model can explain 92.7% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. In this case, the value of the determination coefficient (R^2=0.927) indicates that only 7.3% of the total variations are not explained by the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2=0.859) is also very high to advocate for a high significance of the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. Here, in this case, the adjusted R^2 value is 0.859, which is lesser than the R^2 value of 0.927. At the same time, a relatively lower value of the coefficient of variation (CV=12.9%) indicates a better precision and reliability of the experiments carried out.

By applying multiple regression analysis on the experimental data, the experimental results of the CCD design were fitted with a second-order full polynomial equation. The empirical relationship between sulfide oxidation (Y) and the four test variables in coded units obtained by the application of RSM is given by Eq. 4.

$$\begin{aligned} Y = {} & 67.917 + 0.208 \times x1 + 2.625 \times x2 - 0.625 \times x3 + 7.121 \times x4 + 2.887 \times 1\text{^}2 \\ & + 1.563 \times x1 \times x2 + 4.688 \times x1 \times x3 + 0.313 \times x1 \times x4 + 1.137 \times x2\text{^}2 \\ & - 0.437 \times x2 \times x3 - 1.313 \times x2 \times x4 + 2.637 \times x3 \times x3 - 0.687 \times x3 \times x4 \\ & + 2.131 \times x4\text{^}2 \end{aligned} \tag{4}$$

Where Y is sulfide oxidation in percent and response and $x1$, $x2$, $x3$, and $x4$ are the coded values of the test variables, SC, VB, $CaCO_3$, and ROT, respectively.

Table 3 Model summary and analysis of variance for the quadratic model.

Source of variations	Sum of squares	Degrees of freedom	Mean square	F value	Probability (p)
Regressions	2,251.855	14	160.847	13.619	4.55e−06
Residual	177.159	15	11.811		
Total	2,429.014	29			

R=0.963, R^2 =0.927, Adjusted R^2 =0.859, CV=12.9%

The ANOVA was conducted for the second-order response surface model and the results are given in Tables 3 and 4. The significance of each coefficient was determined by Student's *t* test and *P* values, which are listed in Tables 3 and 4. The larger the magnitude of the *t* value and smaller the *P* value, the more significant is the corresponding coefficient. This implies that the linear effects of VB ($P<0.002$) and ROT ($P<4.10e{-}08$) and interactive effects of SC and $CaCO_3$ ($P<6.62e{-}05$) and quadratic effects of SC ($P<0.001$), $CaCO_3$ ($P<0.001$), and ROT ($P<0.005$) are more significant than the other factors (i.e., $P<0.05$).

The model *F* value of 13.619 and values of prob>*F* (<0.05) indicated that the model terms are significant. The statistically significant model of the optimization studies is given by the following Eq. 5.

$$\text{Sulfide oxidation}(\%) = 67.917 + 2.625 \times x2 + 7.121 \times x4 + 2.887 \times x1\text{^}2 + 4.688 \times x1 \times x3 + 2.637 \times x3 \times x3 + 2.131 \times 4\text{^}2 \quad (5)$$

RSM proved to be a powerful tool in optimizing the reactor conditions for the sulfide oxidation by *Thiobacillus* sp. In the present study, the experimental results clearly showed that the sulfide oxidation was dependent mainly on the SC, $CaCO_3$, and ROT.

The regression model developed can be represented in the form of 2-D and 3-D surface and contour plots. The percent sulfide oxidation for different conditions of FBR can also be predicted from the respective contour plots as shown in Fig. 1a–f. Each contour curve represents an infinite number of combinations of tow test variables with the other two maintained at their respective zero level.

Sulfide Oxidation and Sulfate and Thiosulfate Formation in Continuous Fluidized Bed Bioreactor

A laboratory-scale fluidized bed reactor was operated in continuous mode using Ca-alginate immobilized cells of isolated *Thiobacillus sp.* The results of continuous FBR with immobilized cells of *Thiobacillus* sp. are shown in Figs. 2 and 3.

Table 4 Model coefficients estimated by multiple linear regressions (significance of regression coefficients).

Model term	Parameter estimates	Std. Err.	Computed *t* value	*p* value
Intercept	67.917		48.408	0.000
*x*1	0.208	1.403	0.297	0.771
*x*2	2.625	0.702	3.742	0.002[a]
*x*3	−0.625	0.702	−0.891	0.387
*x*4	7.121	0.702	10.151	4.10e−08[a]
*x*1*x*1	2.887	0.702	4.399	0.001[a]
*x*1*x*2	1.563	0.656	1.819	0.089
*x*1*x*3	4.688	0.859	5.456	6.62e−05[a]
*x*1*x*4	0.313	0.859	0.364	0.721
*x*2*x*2	1.137	0.859	1.732	0.104
*x*2*x*3	−0.437	0.656	−0.509	0.618
*x*2*x*4	−1.313	0.859	−1.528	0.147
*x*3*x*3	2.637	0.859	4.018	0.001[a]
*x*3*x*4	−0.687	0.656	−0.800	0.436
*x*4*x*4	2.131	0.859	3.248	0.005[a]

[a] Significant at $p<0.05$

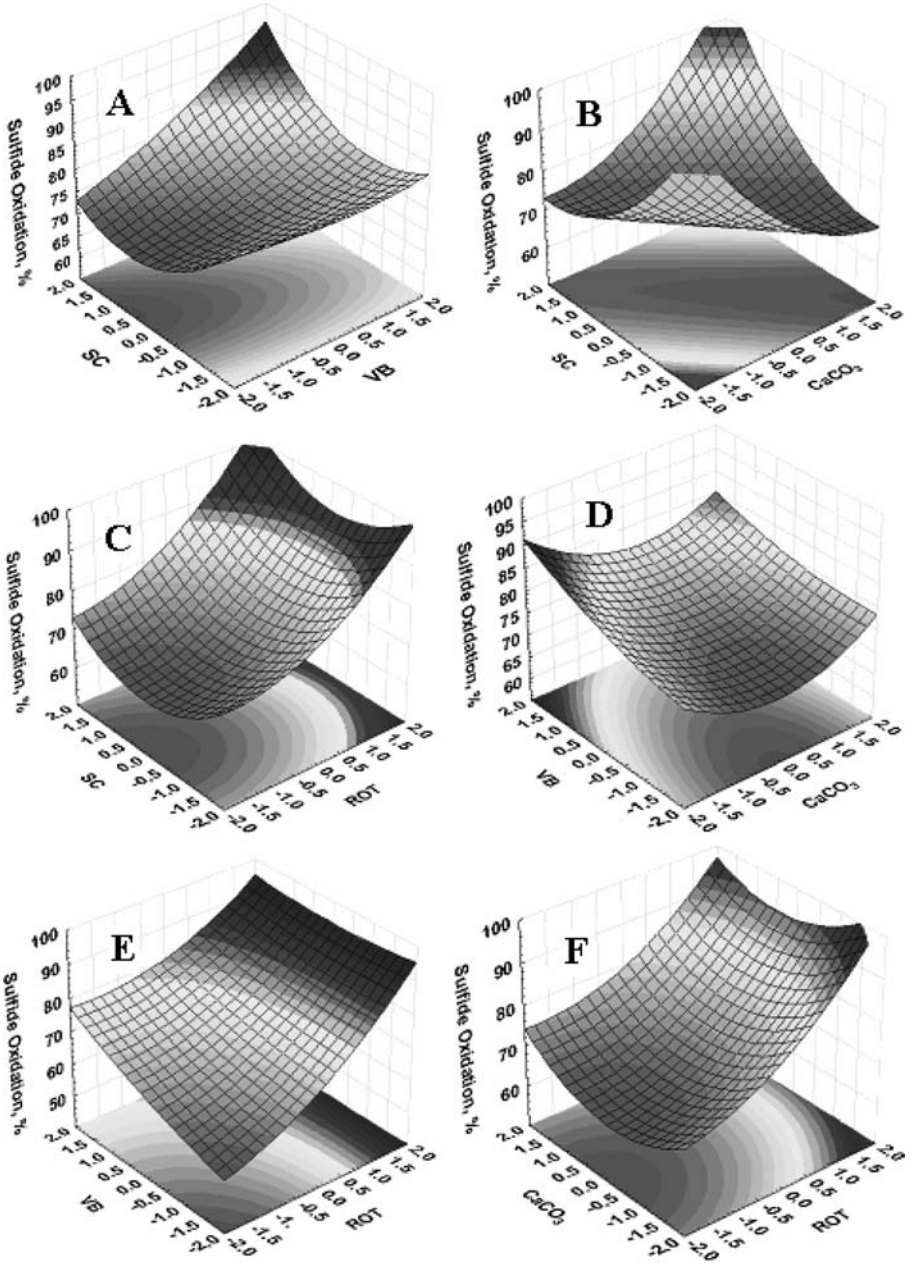

Fig. 1 3-D surface and contour plots of sulfide oxidation by immobilized cells of *Thiobacillus* sp. in FBR: the effect of two variables while the other two are held at 0 levels. **a** 95▪, 90▪, 85▫, 80▫; **b** 100▪, 90▪, 80▫; **c** 100▪, 90▪, 80▫; **d** 90▪, 85▪, 80▫; **e** 90▪, 80▪, 70▫; **f** 100▪, 90▪, 80▫

Figure 2 shows the sulfide oxidation pattern in the FBR during 51 days of operational period. During the entire operational period, fluidization of immobilized beads was done by supplying sterile air at a constant flow rate of 2.4 ml/min and this flow rate was considered as minimum fluidization flow rate.

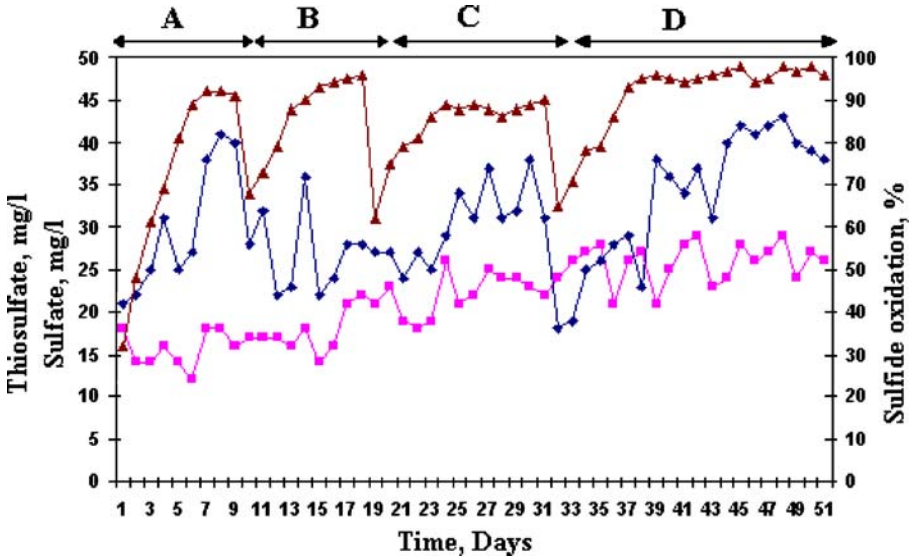

Fig. 2 Variation in percent sulfide oxidation, sulfate and thiosulfate formation in FBR with immobilized cells of *Thiobacillus* sp. at different HRTs. *A* 24-h HRT (1 to 9 days). *B* 18-h HRT (10 to 18 days). *C* 12-h HRT (19 to 31 days). *D* 18-h HRT (32 to 51 day). *triangle*, Sulfide oxidation (%); *diamond*, sulfate formation (mg/l), *square*, thiosulfate formation (mg/l)

Initially, the FBR was operated at 0.695 ml/min effluent flow rate with a sulfide loading rate of 0.020833 g-S/m^3 per hour till the reactor achieved a stable sulfide oxidation efficiency of 92% then the effluent flow rate was increased to 0.926 ml/min with sulfide loading rate of 0.027778 g-S/m^3 per hour where the reactor resulted in a maximum sulfide

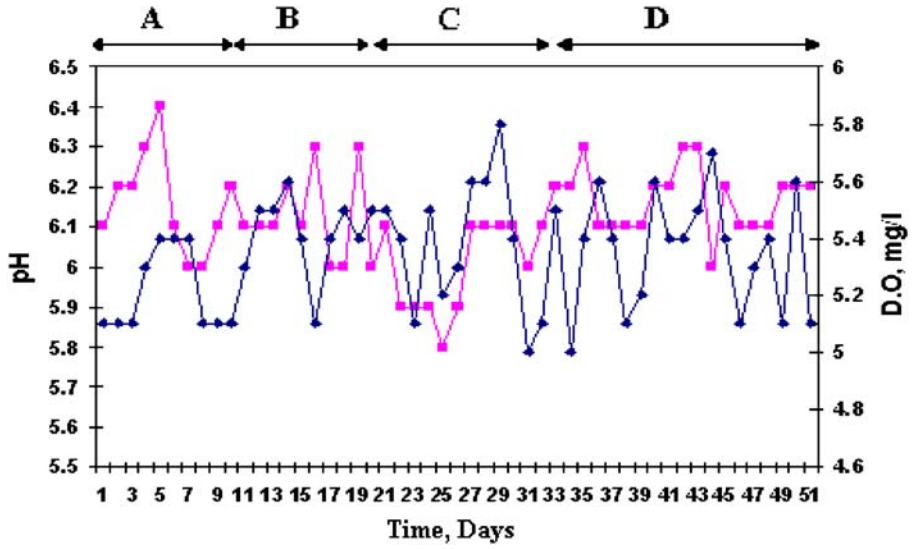

Fig. 3 Variation in pH and DO in FBR with immobilized cells of *Thiobacillus* sp. during entire period of operation. *A* 24-h HRT (1 to 9 days). *B* 18-h HRT (10 to 18 days). **C.** 12-h HRT (19 to 31 days). *D* 18-h HRT (32 to 51 days). *square*, pH, *diamond*, DO (mg/l)

oxidation of 96%. Further, loading rate was increased to 0.041667 g-S/m^3 per hour with a corresponding flow rate of 1.388 ml/min where the sulfide oxidation was dropped to 90%. At this stage, the flow rate was again changed to 0.926 ml/min with a retention time of 18 h and a maximum sulfide oxidation of 96–98% was recorded till the end of reactor operation.

Sulfide oxidation in the system has shown almost a similar pattern throughout the operational period and could attain a maximum of 98% at a sulfide loading rate of 0.027778 g-S/m^3 per hour and then dropped to 90% at 0.41667 g-S/m^3 per hour.

Figure 2 shows the sulfate and thiosulfate formation in the FBR during entire period of operation. The sulfate and thiosulfate pattern almost followed a uniform trend for the entire 51 days of operation at all sulfide loading rates and hydraulic retention times. Formation of sulfate in a biological sulfide oxidation process all depends on the amount of oxygen supplied and it follows the reaction explained in the previous sections.

The formation of thiosulfate will not occur in biological sulfide-oxidizing system [6, 7]. However, it can be excluded that the formation of the thiosulfate resulted from the sulfide auto oxidation process [9]. The sulfate and thiosulfate formation in the reactor was in the range of 18–43 and 12–29 mg/l, respectively.

Since all the experiments were conducted at constant O_2 supply rate, the sulfate and thiosulfate formation can best studied under varied dissolved oxygen conditions. Further, thiosulfate formation may increase at higher sulfide loading rates and lower hydraulic retention time because of chemical oxidation of sulfide. Presence of phenol compounds in the effluent did not affect the sulfide oxidation activity of the *Thiobacillus* sp.

Variation in pH and DO in the Reactor

Figure 3 shows variation pH of the system and change DO in the reactor. Since the air supply was done at a constant flow rate, the DO in the system was maintained almost constant. Uniform pH pattern in the reactor shows the least possibilities of formation of sulfide into sulfuric acid by aerobic oxidation.

Scanning Electron Microscopic Analysis of Immobilized Ca-alginate Beads

Sample of immobilized beads were taken from the reactor column in order to verify, by means of scanning microscopy analyses, whether the microbial immobilization had occurred in the Ca-Alginate matrix. Analyses were performed on cut section of the immobilized beads using an SEM at 6 kV and 5,500 magnifications. SEM of cross section through bead stocked with *Thiobacillus sp*. shows growth throughout particle not just at periphery.

Sulfur Mass Balance During FBR Operation

In the present studies, a sulfur mass balance was made for the entire 51 days of FBR operation. Since the effluent used was of synthetic nature, it was assumed that inlet source of sulfur to the FBR was only in the form of sulfides. Exit stream of the FBR was analyzed for sulfide, sulfate, and thiosulfate. At the end of each stable operational period and before varying the sulfide loading rate and changing the retention time to the reactor, elemental sulfur at the bottom of FBR was quantified. Table 5 shows the sulfur mass balance during 51 days FBR operation. FBR was operated under three different flow rates resulting in different loading rates. The sulfur mass balance was done for different flow rates. First

Table 5 Sulfur mass balance in FBR during 51 days of biological sulfide oxidation process using *Thiobacillus* sp.

Sl no	FBR Operation, days	Flow rate, ml/min	S in inlet stream	S in outlet stream					Unaccounted sulfur, mg
			Sulfide, mg	Sulfate, mg	Thiosulfate, mg	Sulfide, mg	Sulfur, mg	Total S, mg	
1	9	0.695	4,500	270	140	1,225	886	2,521	1,979
2	9	0.926	6,000	324	210	827	1,762	3,123	2,877
3	13	1.388	12,999	786	574	2,100	2,756	6,216	6,783
4	20	0.926	13,334	892	688	2,267	3,268	7,115	6,219
Total	51		36,833	2,272	1,612	6,419	9,172	18,975	17,858

9 days sulfide feed flow rate was 0.695 ml/min with total sulfur mass (S) of 4,500 mg. The exit stream contained 270, 140, 1,225, and 886 mg of S in the form of sulfate, thiosulfate, sulfide, and sulfur, respectively. At 0.926 ml/min, inlet feed flow with 6,000 mg of S was fed to FBR and the FBR was operated for 9 days. Under these conditions, exit stream S resulted in 324 mg of sulfate, 210 mg of thiosulfate, 827 mg of sulfide, and 3,123 mg of sulfur. Similarly, the FBR was operated for 13 days at 1.388 ml/min of feed flow rate with 12,999 mg of inlet S and exit stream had 786 mg of sulfate, 574 mg of thiosulfate, 2,100 mg of sulfide and 2,756 mg of sulfur as S. During last 20 days of FBR operation exit stream had 892, 688, 2,267, and 3,268 mg of S in the form of sulfate, thiosulfate, sulfide, and sulfur, respectively, and at this stage the inlet S was 13,334 mg. The table also shows the amount of unaccounted S during the entire 51 days of FBR operation. Total S inlet was 36,833 mg and the measured S in the exit stream was 18,975 with an unaccounted S of 17,858.

Discussions

The RSM is an effective sequential and stepwise procedure and defines the effect of the independent variables, alone or in combination, on the process. The lead objective of the RSM was to run rapidly and efficiently along the path of improvement towards the general vicinity of the optimum. It is appropriate when the optimal region for running the process has been identified [13].

Biological treatment of sulfidic spent caustic could be an inexpensive alternative for the physicochemical process. The sulfide is biologically oxidized to elemental sulfur under oxygen-limiting conditions whereas sulfate is formed at excess amounts of oxygen. It is known that a certain fraction of the HS^- is converted to sulphuric acid (H_2SO_4) instead of the desired elemental sulfur. A major disadvantage of sulfate formation is that less insoluble and reusable elemental sulfur is formed and the sulfate ions have to be removed by the addition of makeup water. The products of sulfide oxidation, other than sulfur, such as sulfate and thiosulfate are highly water soluble and difficult to separate. Therefore, the sulfide removal studies focused mainly on the partial oxidation of sulfide to sulfur that could be efficiently separated from the waste stream. Elemental sulfur is a desired end product of sulfide oxidation because of its nontoxic, settlability nature, less oxygen requirement, and possibility of its reclamation and reuse as a valuable by-product in industrial applications and metal bioleaching processes. The overall biochemical reactions occur during sulfide oxidation under different sulfide to oxygen ratio and it indicates that

reactions producing sulfate is thermodynamically more favored and energy yielding and hence preferred by the SOB [18–21].

The advantage of this biological sulfide oxidation system is no chemicals are required except oxygen. Certain strains of the sulfur-oxidizing bacteria belonging to the genus *Thiobacillus* can oxidize free sulfide to elemental sulfur. During the process, they derive energy for growth from the oxidation of reduced sulfur compounds but they are rather sensitive to concentration of sulfide and only survive if the sulfide concentration is low [19–21].

Various researchers have studied the oxidation of sulfide in reactors like continuous stirred tank reactor and packed bed reactor using isolated strains. In biological sulfide oxidation based on the oxygen concentration, end product will be produced. Under oxygen-limiting conditions, that is at O_2 concentration below 0.1 mg/l, sulfur is the end product of the sulfide oxidation, while sulfate is formed under circumstances of sulfide limitation [20–23]. Few reports are available for the petroleum spent caustic removal by biological methods [24–26] and it was found economical when compared with physicochemical methods [25].

The formation of sulfur is preferred because it is insoluble, nontoxic, settlability nature, less oxygen requirement, possibility of its reclamation and reuse as a valuable by-product in industrial applications, and metal bioleaching processes and can be easily recovered from the water stream. The formation of end product is not only depending on the sulfide concentration but also on the amount of oxygen supply to the reactor.

So far, little attempt was made to study the biological oxidation of sulfide in a fluidized bed reactor and there are almost no reports about the use of Ca-alginate immobilized cells of *Thiobacillus sp.* for sulfide oxidation in a fluidized bed reactor. An important reason for not using free cell reactor in sulfide oxidation process is to prevent the formation of sulfate by oxidation of elemental sulfur by free cells of *Thiobacillus sp.*

Research work has been done for the isolation of autotrophic *Thiobacillus* sp. from different habitats, identification, and sulfide oxidation at different concentrations in different reactors like continuous stirred tank reactor, fluidized bed reactor, etc. using free and immobilized cells. Immobilization of *Thiobacillus* sp. gave good results in terms of elemental sulfur formation rather than forming sulfates and thiosulfates. Also, use of fluidized bed reactor with immobilized cells resulted in rapid sulfide oxidation because of good mixing and agitation for better oxygen mass transfer [26–29].

The fate of unaccounted S needs to be addressed in the exit stream in order to better understand the biological sulfide oxidation system. Sulfur is stored in externally excreted sulfur globules of sulfur-oxidizing bacteria. As long as dissolved sulfide is available, sulfur is stored in globules. When sulfide is depleted, the stored sulfur is oxidized [30]. Sulfur production by the sulfur-compound-oxidizing bacteria is a complex phenomena. Elemental sulfur produced by sulfur-compound-oxidizing bacteria (“biosulfur”) has properties distinctly different from those of crystalline elemental sulfur. The hydrophilic properties of “biosulfur” are the most striking of these differences. As a result of this, biologically produced sulfur can be dispersed in aqueous solutions, whereas crystalline inorganic sulfur is hydrophobic and will not be wetted by an aqueous solution. Biological sulfide oxidation is complex process with not only sulfate and thiosulfate as end products but also many polysulfides as possible products. The *Thiobacillus* has the ability to produce both hydrophobic crystalline sulfur which settles at the bottom during sulfide oxidation and hydrophilic (water soluble) sulfur [30–32]. However, identification of the nature of elemental sulfur in globules produced by sulfur-compound-oxidizing bacteria is not straightforward. Several studies have been performed with several different techniques but unfortunately the interpretation of the results sometimes seems to be contradictory [31–33].

Conclusions

RSM for the optimization of FBR conditions for the better sulfide oxidation was tested. The CCD was a good design for the optimization of variables in the present work. The model developed for CCD had R^2 values of 0.9723. Continuous operation fluidized bed reactor with immobilized cells of *Thiobacillus sp.* reduces the possibilities of sulfate formation during the sulfide oxidation process. Sulfide is the primary nutrient source for the *Thiobacillus sp*. in sulfide oxidation process and elemental sulfur is the immediate oxidative product. If sulfide concentration depletes in the system, the *Thiobacillus sp*. start feeding on elemental sulfur for its food source which results in high sulfate formation. Since elemental sulfur is the immediate product of biological sulfide oxidation under oxygen limitation conditions, in a free cell biological sulfide oxidation system, produced sulfur particles will support for the attachment of cultures and results on oxidation of sulfur particles into sulfates. Hence, it is necessary to avoid contact between sulfur particles and free cells. Under these circumstances, cell immobilization technology is of much use to avoid possible contact between cells and sulfur particles and facilitating the system to convert sulfide into elemental sulfur. Also, sulfate formation is more in the case of excess oxygen concentration during biological sulfide oxidation rather than producing sulfur as end product. Therefore, cell immobilization method is of much use to prevent more sulfate formation and control excess oxygen concentration during biological sulfide oxidation using autotrophic *Thiobacillus* sp. Although a complete sulfur balance was not made on the reactor system, the results of sulfide oxidation and sulfate and thiosulfate formation shows that most of the sulfur (65–75%) in the system is converted into elemental sulfur.

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References

1. World Bank Group (1998). *Petrochemicals manufacturing pollution prevention and abatement handbook*. Washington: The International Bank for Reconstruction and Development.
2. Roger, C. H. (1994). *Industry, technology, and the environment: competitive challenges and business opportunities*. Washington, D.C.: US Congress.
3. Tania Mara, S. C., & Clayton, B. M. (2000). Wet air oxidation of refinery spent caustic: A refinery case Study. In: NPRA Conference, San Antonio, Texas, USA.
4. Claude, E. E. Robert, J. L., & Bruce, L. B. (1994). Wet air oxidation of ethylene plant spent caustic. In: American Institute of Chemical Engineers Sixth Annual Ethylene Producers Conference Annual Meeting Atlanta, Georgia USA.
5. WHO (1981). *H_2S, Environmental health criteria, 19*. Geneva: WHO.
6. Buchanan, R. E., & Gibbons, N. E. (1974). *The Thiobacilli, Bergey's manual of determinative bacteriology* (pp. 78–89, 8th ed.). Baltimore: Williams and Wilkins.
7. Vishniac, W., & Santer, M. (1957). The *Thiobacilli*. *Bacteriological Reviews*, *21*, 185–213.
8. Kuenen, J. G., & Roberson, L. A. (1992). *Biodegradation*, *3*, 239–254.
9. Manahan, S. E. (2000). *Aquatic microbial biochemistry: Environmental chemistry.* Boca Raton: CRC Press.
10. Ravichandra, P., Gopal, M., Gangagni, R. A., Ramakrishna, M., & Annapurna, J. (2007). *Journal of Environmental Biology*, *28*(4), 819–823.
11. Akhnazarova, S., & Kafarov, V. (1982). *Experiment optimization in chemistry and chemical engineering.* Moscow: Mir Publications.
12. Myers, R. H., & Montgomery, D. C. (1995). *Response surface methodology: Process and product optimization using designed experiments* (1st ed.). New York: Wiley-Interscience.
13. Khuri, A. I., & Cornell, J. A. (1987). *Response surfaces: Design and analysis*. New York: Marcel Dekker.
14. Ravichandra, P., Subhakar, Ch., Pavani, A., & Annapurna, J. (2008). *Bioresource Technology*, *99*, 1776–1786.

15. Himabindu, M., Ravichandra, P., Vishalakshi, K., & Annapurna, J. (2006). *Applied Biochemistry and Biotechnology*, *134*, 143–154.
16. Radhika, T., Kiran, K. D., Ravichandra, P., & Lakshmi, N. M. (2007). *Applied Biochemistry and Biotechnology*, *141*, 187–201.
17. Clescerl, L. S., Greenberg, A. E., & Eaton, A. D. (1998). *Standard methods for the examination of water and wastewater* (20th ed.). Washington DC: American Public Health Association.
18. Janssen, J. H., Ma, S. C., Lens, P., & Lettinga, G. (1997). *Biotechnology and Bioengineering*, *53*, 32–40.
19. Kethum, P. A. (1995). *Microbiology: Concepts and applications*. Oakland: Wiley.
20. Cees, J. N., Buisman, B., Geraali, G., Peter, I., & Lettinga, G. (1990). *Biotechnology and Bioengineering*, *35*, 50–56.
21. Cees, J. N., Buisman, B., Peter, I., Anne, H., Janssen, A. J. H., Robert, T. H., et al. (1991). *Biotechnology and Bioengineering*, *38*, 813–826.
22. Suzuki, I. (1999). *Canadian Journal of Microbiology*, *45*, 97–105.
23. Paul, F. H., & Wei, Z. (2001). *Water Research*, *35*(15), 3605–3610.
24. Jan, S., Svitelskaya, A., Mark, B. V., Look, W., Hulshoff, P., Lettinga, G., et al. (2004). *Water Research*, *38*, 4331–4340.
25. Sublette, K. L. (1997). *Applied Biochemistry and Biotechnology*, *65*(1–3), 695–706.
26. Julie, A. C., Robert, R. B., Kathleen, D., Ravi, K., & Sublette, K. L. (2000). *Applied Biochemistry and Biotechnology*, *84–86*, 707–720.
27. Ravichandra, P., Gopal, M., Gangagni, R. A., Ramakrishna, M., & Annapurna, J. (2007). *Journal of Applied Sciences*, *7*, 2188–2193.
28. Ravichandra, P., Ramakrishna, M., Rao, A. G., & Annapurna, J. (2006). *Journal of Engineering Science and Technology*, *1*, 21–30.
29. Krishnakumar, B., Majumdar, S., Manilal, V. B., & Ajit, H. (2005). *Water Research*, *39*, 639–647.
30. Vetter, R. D. (1985). *Marine Biology*, *88*, 33–42.
31. Steudel, R. (1989). In H. G. Schlegel, & B. Bowien (Eds.), Autotrophic bacteria (pp. 289–303). Madison: Science Technology Publishers.
32. Pickering, I. J., George, G. N., Yu, E. Y., Brune, D. C., Tuschak, C., Overmann, J., et al. (2001). *Biochemistry*, *40*(8), 138–145.
33. Prange, A., Chauvistre, R., Modrow, H., Hormes, J., Truper, H. G., & Dahl, C. (2002). *Microbiology*, *148*, 267–276.