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Bioremediation of sulfate-rich wastewater using lactate-fed sulfidogenic enrichment culture predominantly *Desulfovibrio sp.*: Box-Behnken design optimization

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ABSTRACT

In this study, a sulfidogenic enrichment culture was obtained from lignite mine spoils and utilized for the remediation of sulfate-rich wastewater. By 16S rRNA analysis, the enrichment culture was identified to be predominantly *Desulfovibrio sp.* strain VSV1. Lactate was proved to be the suitable electron donor for the enrichment culture when compared with other volatile fatty acid salts such as formate, acetate, and propionate. By controlling one parameter at a time while others fixed, the parameter such as pH, temperature, and sulfate concentration was optimized as 8, 30°C and 1500 mg/L respectively. This study further investigated on mathematical optimization using Box-Behnken design followed by response surface methodology which yielded the optimum values as pH 7.7, temperature 30°C and sulfate concentration 1392 mg/L. The experimentally optimized multiple process parameters resulted in 95.5% of sulfate reduction comparatively higher than that obtained from single parameter optimization. The results of the present study suggested that the lactate-fed sulfidogenic enrichment culture can be effectively utilized for the remediation of sulfate-rich wastewaters.

Keywords: Sulfidogenic enrichment culture; Mine spoils; *Desulfovibrio sp.*; Sulfate-rich wastewater; Lactate; Box-Behnken design

1. Introduction

Sulfate is one of the major pollutants of water. The occurrence of sulfate ions is common in natural ground and surface waters. Sea water contains an excess amount of sulfate ions through photochemical generation from volcanic SO_2 and H_2S [1]. The pyrites (FeS_2) exist at mine sites which when exposed to oxygen and water, release acidic and sulfate-rich wastewater called acid mine drainage [2]. Apart from mining processes, the other industrial sources of sulfate laden wastewaters are food and pharmaceutical production, fermentation, pulp and paper, tanneries and petrochemical operations [3]. These sulfate-rich wastewaters reduce the availability of potable and usable water by increasing the salinity of receiving water bodies [4]. According to US code of Federal regulations, the sulfate concentration in drinking water should not exceed 250 mg/L [5]. The chemical methods of treating sulfate-rich wastewater such as coagulation, flocculation, floatation, ion exchange and complexation are effective, but their need for separation and appropriate disposal of the solid phase and relatively high cost and energy consumption prefers biological sulfate reduction process [6].

Sulfate-reducing bacteria (SRB) are prominent members of microbial communities with economic, environmental and biotechnological interest. They can exist in various environments such as soils, sediments and domestic, industrial and mining wastewaters. SRB are strictly anaerobic bacteria and included in a group of chemoorganotrophic, which comprises representatives of the genera *Desulfobacter, Desulfomicrobium*, *Desulfovibrio* and *Desulfotomaculum*, among

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others [7]. Biological sulfate reduction process requires sulfate-reducing bacteria (SRB) that uses sulfate as a terminal electron acceptor and obtains energy for their growth and maintenance through coupled oxidation of the organic substrate and reduction of sulfate under anaerobic condition [8]. The biogenic sulfide produced can be utilized for heavy metal removal through insoluble metal sulfide precipitates formed by reacting with dissolved metals. It can also be converted to elemental sulfur via partial sulfide oxidation by sulfide oxidizing bacteria like *Thiobacillus thioparus* [9].

Sulfate-reducing bacteria need special environmental requirements like anaerobic condition, pH, temperature and suitable electron donors for the effective treatment of sulfate-rich wastewaters. SRB can survive in the environment with a pH range of 5–9. The activity of SRB reduced beyond this pH range. Similarly SRB comprises both mesophilic and thermophilic strains with the growth and temperature being significantly affected by temperature. A variety of organic compounds like formate, acetate, propionate, butyrate, lactate and pyruvate could be utilized as electron donors and often simultaneously as carbon source for SRB [10]. Among all, lactate was proved to be a better carbon source and electron donor as it supports the diversity of SRB species which promotes the bioremediation system. On the other hand, sulfate concentration also affects the SRB activity and the kinetics of sulfate reduction [11].

In this present study, a sulfidogenic enrichment culture was utilized for biological sulfate reduction. Using a lactate-fed sulfidogenic enrichment culture, the process parameters such as pH, temperature, and sulfate concentration were optimized to improve the sulfate reduction. A very few works have been reported on statistical optimization of these parameters for effective sulfate reduction. Hence, this study also focused on Box-Behnken design optimization to study the interactions of pH, temperature, and sulfate concentration for maximizing the sulfate reduction (%).

2.Materials and methods

2.1. Microbial source

A microbial culture capable of sulfate-reducing was enriched from mine spoils collected from an opencast mine located in NLC India Ltd., (Neyveli, Tamilnadu, India). The collected samples were stored in sterile poly bags at 4°C for further use.

2.2. Enrichment of sulfidogenic culture

A mixed sulfidogenic culture was enriched from mine spoils using modified Postgate B medium [12] with composition of (in g/L): K_2HPO_4 0.5, NH_4Cl 1.0, Na_2SO_4 1.0, $FeSO_4{\cdot}7H_2O$ 0.5, sodium lactate (60%) 6 mL, $MgSO_4{\cdot}7H_2O$ 2.0, $CaCl_2{\cdot}6H_2O$ 0.1, yeast extract 1.0. Bromo-ethane-sulphonic-acid was used at a concentration of 3.2 g/L to inhibit the activity of methanogens [13]. Ascorbic acid and Thioglycollic acid were also added at a concentration of 0.1 g/L to promote the sulfate reduction. All reagents used in this study were of analytical grade. The medium was sterilized at a temperature and pressure of 121°C and 15 psi for 20 min. Nitrogen gas was purged into each serum bottles containing sterile medium to remove the oxygen. One gram of mine spoil was inoculated into 100 ml of sterile medium and kept incubated at ambient temperature for seven days till the color of the media changed to blackish grey. After 7 d of anaerobic incubation, about 10 ml of culture obtained was further transferred to 100 ml sterilized medium. The blackening of the medium and H_2S generation was observed as a positive indication for the presence of sulfate-reducing bacteria. This enrichment culture was used as inoculum for batch experiments.

2.2. Isolation and identification of predominant SRB strain

The sulfidogenic enrichment culture was serially diluted up to 10^8 times and the final dilution was used to isolate sulfate-reducing bacteria in sulfate API agar (SIGMA Product Number S 5056) with 4% sodium lactate solution. After incubating the agar plates in an anaerobic jar for 7 d, a black colored colony was observed which was then transferred to a fresh sulfate API broth (SIGMA Product Number S 5181) with 4% sodium lactate solution and incubated. This sulfate API broth containing a pure strain of sulfate reducing bacteria was identified using 16s rRNA analysis.

Genomic DNA was extracted concerning the protocol described for GeneiPureTM Bacterial DNA Purification Kit (Merck, India) and stored at –20°C. The amplification of the extracted DNA was done by the polymerase chain reaction (PCR) using the following thermal cycling conditions: Denaturation (94°C for 3 min), annealing (94°C for 30 s, 50°C for 60 s, and 72°C for 60 s) and extension (72°C for 10 min).

The primers used were 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Cleanup kit (Millipore) and finally sent to Yaazh xenomics (Tamilnadu, India) for 16s rRNA analysis.

The 16s rRNA sequence obtained was searched for nucleotide similarity using online NCBI BLAST tool. The phylogeny analysis of query sequence with the closely related sequence of BLAST results was performed. The software MEGA version 6.0and the method neighbour-joining were used for phylogenetic tree construction.

2.4. Optimization of sulfate reduction

All experiments were carried out in 250 mL serum bottles containing sterile modified Postgate B medium. The preferred electron donor for the sulfidogenic enrichment culture obtained was chosen based on sulfate removal (%), COD removal (%) and biomass yield (mg/L). This study was done by replacing sodium lactate with other electron donors such as sodium propionate, sodium acetate and sodium formate. For the optimization studies, the amount of Na_2SO_4, $FeSO_4{\cdot}7H_2O$ and $MgSO_4{\cdot}7H_2O$ were adjusted for the required sulfate concentration. With the lactate-fed sulfidogenic enrichment culture, the effect of pH (5, 6, 7, 8 and 9) and temperature (20°C, 25°C, 30°C, 35°C and 40°C) on sulfate reduction were analyzed to find the optimum values. Similarly, the initial sulfate concentration (1000 mg/L, 1500 mg/L, 2000 mg/L, 2500 mg/L and 3000 mg/L) was also investigated to find the optimum sulfate concentration

for sulfate reduction. Samples were taken from the bottles at regular intervals and tested for sulfate concentration. All the experiments were done in triplicates and the average values were reported.

2.5. Box-Behnken design optimization

Optimization using *Box-Behnken* design was employed to maximize the sulfate reduction. Based on single parameter optimization, the levels of the independent variables were chosen and coded as shown in Table 1. A three-factor Box-Behnken design consists of a total 15 experiments using independent factors such as pH (A), temperature (B) and sulfate concentration (C) as shown in Table 3. All experiments were conducted in 250 mL serum bottles containing sterile modified Postgate B medium. The statistical software MINITAB 17 was used to generate the experimental design and also for regression and graphical analysis.

The experimental results were fitted using the following second-order equation [Eq. (1)] which correlates between dependent and independent variables. By solving the equation, the optimum values of independent variables can be determined.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (1)$$

where Y: predicted response; β_0: intercept; β_1, β_2, β_3: coefficients of A,B and C; β_{12}, β_{13}, β_{23}: coefficients of cross products; β_{11}, β_{22}, β_{33}: coefficients of quadratic terms.

In addition to this, 3D surface plots and contour plots generated by MINITAB 17 were also analyzed to study the combined effects of independent variables on the response.

2.6. Analytical methods

About 10 ml of the sample was taken from the reactor. The cell-free supernatant was used for the analysis of sulfate and soluble COD. Sulfate concentration was estimated by turbidometric method using nephelometer (Systronics Nephelo-Turbidity meter 132). Undissociated H_2S was determined from Henderson-Hasselbalch equation [Eq. (2)] [14].

$$pH = pK' + \log \frac{[HS^-]}{[H_2S]} \quad (2)$$

where K' represents equilibrium constant for the dissociation of H_2S and $pK' = -\log K'$. COD was measured using COD Digester (Lovibond). Biomass yield was calculated by measuring dry weight after removing the iron precipitates through acidification process [15].

Table 1
Level of parameters in Box-Behnken experimental design

Sample	Parameters	Level		
		–1	0	+1
A	pH	7	8	9
B	Temperature (°C)	25	30	35
C	Sulfate concentration (mg/L)	1000	1500	2000

Table 2
Effect of electron donors on various parameters

Parameters	Sodium lactate	Sodium propionate	Sodium formate	Sodium acetate
% Sulfate removal	81.6	56.5	72.9	33.5
% COD removal	28.3	18.6	48.5	12.5
Biomass yield (g/L)	0.142	0.109	0.095	0.058

3. Results and discussion

3.1. Characterization of the sulfidogenic enrichment culture

A black colored culture as shown in Fig. 1a was seen after seven days of anaerobic incubation period. The black color formation was due to the reduction of sulfate to sulfide by the action of sulfate-reducing bacteria. Through the microscopic observation of the enriched culture, it was clear that the cells were Gram-negative. A red fluorescence was obtained as a result of desulfoviridin test as shown in Fig. 1b. It was reported that all *Desulfovibrio* species were gram negative, capable of producing H_2S and desulfoviridin positive [16]. According to Bergey's manual of systematic bacteriology [17], all the species of the genus *Desulfovibrio* produce sirohydrochlorin chromophore of desulfoviridin pigment and the characteristic red fluorescence. Hence the dominant sulfate reducing bacteria present in the sulfidogenic enrichment culture belongs to the genus *Desulfovibrio*.

3.2. Isolation and identification of the predominant SRB strain

A black colored colony observed in sulfate API agar plate was noted as the predominant sulfate reducing bacterial strain from the sulfidogenic enrichment culture and designated as strain VSV1. The partial 16s rRNA analysis of the strain yielded a sequence with 994 base pairs (bp) which was then submitted to NCBI GenBank database with the accession number KY510920. The gene sequence analysis was done using online BLAST tool which indicated that the

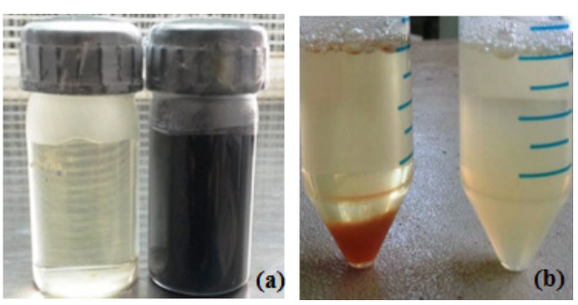

Fig. 1. Microbial culture (a) Sulfidogenic enrichment culture (b) Desulfoviridin test.

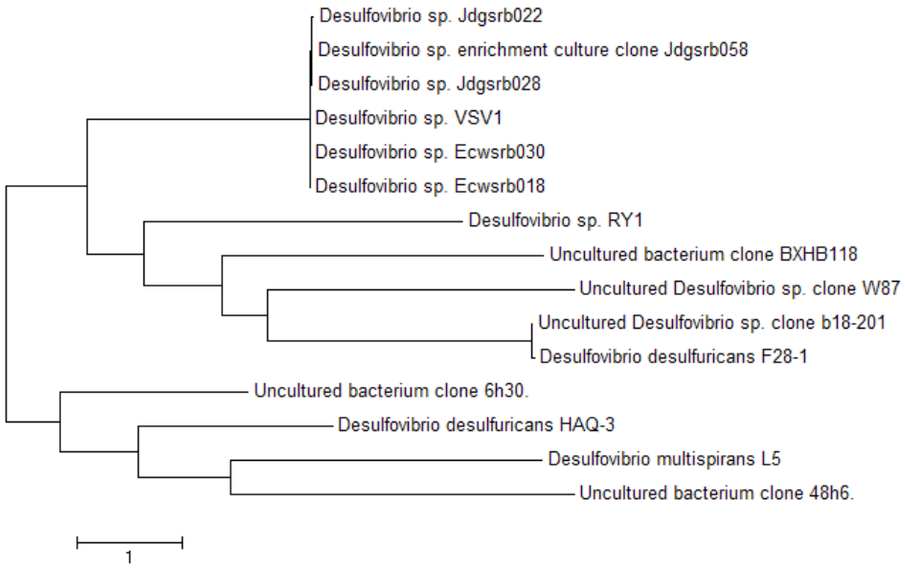

Fig. 2. Phylogenetic relationship between the isolated strain VSV1 and other known sulfate reducing bacterial strains.

isolated strain contains a sequence that is unique to the phylum Proteobacteria and more particularly to the member of the genus *Desulfovibrio sp*. Based on the 16S rRNA sequence of the isolated strain VSV1 and sequences of other sulfate reducing bacterial strains obtained from GenBank database, a phylogenetic tree was constructed as shown in Fig. 2 using the neighbour-joining method. The phylogentic relationship between the isolated strain VSV1 and other known sulfate reducing bacterial strains was clearly shown in Fig. 2.

3.3. Selection of suitable electron donor for the sulfidogenic enrichment culture

Electron donors are very crucial for the biological treatment of sulfate-rich wastewater. The choice of electron donors has much impact on the growth of SRB and thereby reduction of sulfate also. Low molecular weight compounds like volatile fatty acids (VFA), alcohol and organic acids can be used as the electron donors for SRB [12]. Batch experiments were done using different volatile fatty acid salts to compare sulfate reduction, soluble COD removal, and biomass yield and shown in Table 1. The overall metabolic reactions carried out by SRB when low-molecular-weight volatile fatty acids act as energy sources may be summarized as follows [18].

$$4HCOO^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^- (\Delta G = -146.7kJ\ /\ reaction) \quad (3)$$

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- (\Delta G = -47.3kJ\ /\ reaction) \quad (4)$$

$$2CH_3CH_2COO^- + 1.5SO_4^{2-} \rightarrow 1.5HS^- + 0.5H^+ + 2HCO_3^- + 2CH_3COO^- \quad (\Delta G = -88.8kJ\ /\ reaction) \quad (5)$$

$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+ \quad (\Delta G = -159.6kJ\ /\ reaction) \quad (6)$$

From Table 1, we can infer that when sodium lactate was utilized as a carbon source, the maximum reduction in sulfate was about 81.6% followed by sodium formate 72.9% and least 33.5% in the sodium acetate. The most effective sulfate reduction was observed with lactate, due to its total consumption [19]. Hence, a biomass yield of 0.142 g/L was obtained in the case of lactate and found to be maximum when compared to others. Furthermore, the genus *Desulfovibrio* belongs to non-acetate oxidizers, and hence the enrichment culture oxidized lactate more efficiently [10]. Acetate production during lactate utilization by SRB was witnessed from Eq. (6). Since SRB cannot completely oxidize acetate even with excess sulfate levels, the acetate production during lactate oxidation was considered a major drawback. The acetate remaining in the effluent contributes largely to the residual COD [12] of 71.7%. Similarly, in the case of acetate used as an electron donor, only 12.5 % of COD was utilized and resulted in 33.5% of sulfate removal. But, when formate was used as an electron donor, a maximum COD removal of 48.5% was achieved with the sulfate removal of 72.9%. From Eq. (3), it is understood that four moles of formate are required for every mole of sulfate to be reduced. Hence higher % COD removal was achieved in the case of formate.

3.4. Optimization of sulfate reduction

The impact of process parameters such as pH, temperature and sulfate concentration on sulfate reduction (%) was examined by controlling one parameter at a time while others fixed.

3.4.1. Influence of pH

SRB was found to survive in the environment within the pH range of 5–9. Hence the effect of pH in the range of 5–9 was studied on sulfate reduction efficiency and shown in Fig. 3a. At pH 8, a maximum sulfate removal efficiency of 84% was achieved. At pH 7, the decrease in sulfate removal rate was lesser and found to be 75%. But, when the pH increased to 9, the sulfate removal rate dropped to 48%. This situation was in accordance with the report that optimum pH for sulfate reducers was in the range of 6.9–8.5 [10]. Similarly, when pH was lowered to 6 and 5, the sulfate reduction efficiency drastically decreased to 54% and 36% respectively. These results indicated that the acidic pH of 5 and 6 significantly affect the SRB activity and thereby sulfate removal efficiency. Hence, in this study, a pH value of 8 was found to be optimum for better sulfate removal efficiency.

The drop in sulfate removal efficiency at low pH could be well explained concerning the inhibitory effect of sulfide. The state of sulfide produced solely depends on pH and may exist in different forms such as undissociated hydrogen sulfide (H_2S), hydrosulfide (HS^-) and free sulfide (S^{2-}). At pH below 6, undissociated H_2Sdominates whereas in the pH range 6–8 H_2S dissociates into HS^-. Near pH 12, HS^- further dissociates into S^{2-} [20]. At low pH, undissociated H_2S pass the cell membrane and impede their metabolic activity by combining with iron in cytochromes or any-other metal containing compounds and thus negatively influenced sulfate reduction. Another factor contributing to this negative impact on sulfate reduction would be proton concentration. Because, at low pH increased proton concentration results in higher energy consumption for proton pumping across the cell membrane which might have caused SRB growth inhibition [21]. A similar case of low sulfate reduction at pH below 6 was reported in a study remediating industrial acid mine water. On the other hand the drop in sulfate reduction above pH 8 could be attributed to a report that at high pH, low concentration of undissociated H_2S might be toxic to SRB [22]. In the present study, the undissociated H_2S at pH 9 was measured as 28.67 mg/L. At pH 7 and 8, the high sulfate reduction efficiency obtained in this study was in agreement to a general trend that SRB were able to tolerate higher sulfide concentrations with an increase in pH from 6.8 to 8.5. However, the increase in sulfate reduction efficiency with increase in pH from 7 to 8 could be related to a study reported that the tolerable level of sulfide increased when the pH increased from 7.1 to 8.1 [23].

3.4.2. Influence of temperature

Temperature proved to have a significant effect on SRB growth and sulfate reduction kinetics. SRB comprises both mesophilic and thermophilic species. In this study, the sulfate reduction (%) was estimated for different temperatures in the range of 20–40°C, and the results were shown in Fig. 3b. The optimum temperature for the sulfate reduction was found to be 30°C which is the optimum temperature reported for *Desulfovibrio* species [10]. When the temperature was reduced to 25°C and increased to 35°C, the sulfate removal rates were 68% and 72% respectively which is found to be closer to the maximum removal rate of 84% at

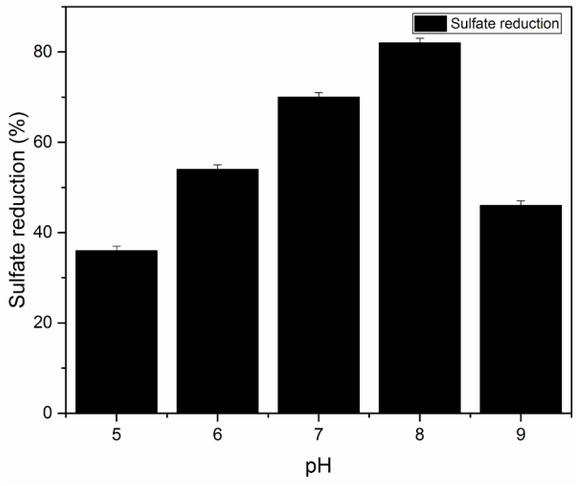

Fig. 3. Optimization of sulfate reduction (a) pH.

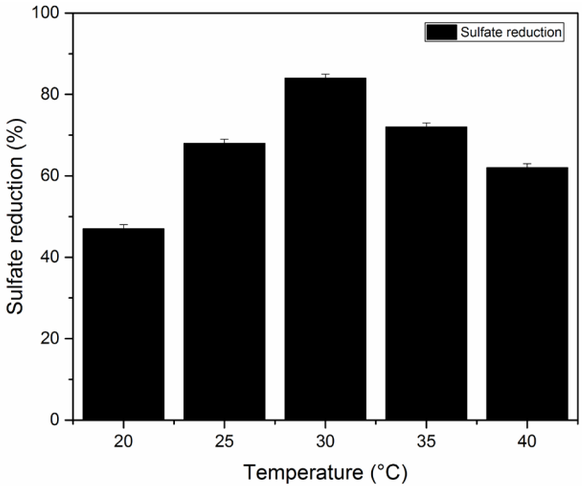

Fig. 3. Optimization of sulfate reduction (b) Temperature.

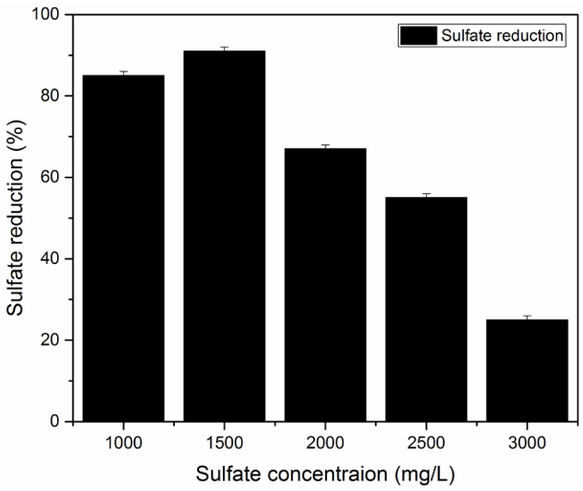

Fig. 3. Optimization of sulfate reduction (c) Sulfate concentration.

30°C. Further, increase or decrease of temperature beyond this range, dropped the sulfate removal rates drastically to 62% and 47% at 40°C and 20°C respectively. An optimum temperature of 35°C was reported for the sulfate reducing bacteria which belongs to *Desulfovibrio* genus [24]. This contradiction was in agreement with the statement that a temperature range of 25–35°C was suitable for *Desulfovibrio* species [10]. In the same way, another study also reported the effect of temperature on the kinetics of sulfate reduction in continuous reactors in which the temperature increased from 20°C to 35°C increases the volumetric reduction rate of sulfate [25].

3.4.3. Influence of sulfate concentration

A sulfate concentration of 1000 mg/L was used for single parameter optimization studies of pH and temperature. Sulfate concentration has a significant impact on SRB growth and sulfate reduction [13]. In this study, to analyze the influence of sulfate concentration on bioremediation system, its concentration was varied from 1000 mg/L up to 3000 mg/L with a constant lactate concentration which resulted in different COD/sulfate ratios. From Fig. 3c, it was identified that when sulfate concentration was increased from 1000 mg/L to 1500 mg/L, a slight increase in sulfate reduction was observed and found to be 91%. For the sulfidogenic enrichment culture used in this study, the optimal sulfate concentration was noted as 1500 mg/L. A similar study was proclaimed with an optimal sulfate concentration of 1500 mg/L for the sulfate-reducing system utilizing organic marine wastes as a nitrogen source [26]. But further increase in sulfate concentration from 1500 mg/L to 3000 mg/L influenced sulfate reduction negatively. This could be attributed to the fact that increase in sulfate concentration increased the redox potential and reduced the COD/sulfate ratio to a great extent. Higher redox potential was reported to inhibit the growth of SRB [11]. Similarly, a very low COD/sulfate ratio was also known to inhibit sulfate reduction [27]. Thus, at a higher sulfate concentration of 3000 mg/L, sulfate reduction reached a minimum of 25%.

Another factor responsible for a decline in sulfate reduction was sulfide concentration. Biological sulfate reduction results in the production of sulfide. This sulfide at higher concentration affects the metabolic activity of SRB and cause SRB growth inhibition. The minimum sulfide concentration that inhibits SRB growth by 50% was noted as 900 mg/L [28]. Thus, the high dissolved sulfide produced at high sulfate concentration (above 1500 mg/L) might have inhibited SRB growth and thereby sulfate reduction also. Lactate has not been reported to inhibit SRB growth [11]. However, acetate in its undissociated form was reported to inhibit SRB growth at lower pH values (≤6) [29]. Since the present system was operated at an optimum pH of 8, it could be hypothesised that either lactate or acetate was not responsible for a decline in sulfate reduction.

3.5. Box-Behnken design optimization

Response surface methodology deals with response optimization based on interactions among the variables. The interactions of three factors pH (A), temperature (B) and sulfate concentration (C) were assessed for different experimental combinations, and the corresponding response values are shown in Table 3. The results revealed that all the three factors have a considerable effect on sulfate reduction.

Table 3
Box-Behnken design matrix and results

Run	A: pH	B: Temperature (°C)	C: Sulfate concentration (mg/L)	Sulfate reduction (%)
1	8	25	1000	56
2	9	35	1500	32
3	9	30	1000	71
4	8	25	2000	12
5	7	35	1500	76
6	8	30	1500	42
7	7	30	1000	58
8	8	35	1000	25
9	7	30	2000	65
10	8	35	2000	68
11	8	30	1500	48
12	7	25	1500	55
13	9	30	2000	91
14	8	30	1500	92
15	9	25	1500	92

3.5.1. Statistical analysis and model development

Using MINITAB 17 software, the following quadratic model was developed by multiple regression analysis on the experimental data.

$$Y = -2815 + 494.3A + 61.72B + 0.1188C - 28.83A^2 - 0.8033B^2 - 0.000050C^2 - 1.750AB + 0.00050AC + 0.000400BC \quad (7)$$

where A, B and C represent pH, temperature and sulfate concentration respectively; Y accounts for the predicted sulfate reduction (%). The coefficients with a positive sign in the regression equation suggested a synergistic effect, while those with negative sign denoted an antagonistic effect. Hence it was evident from Eq. (7) that the linear terms A, B, C had a positive influence on the response while second-order terms A^2, B^2 and C^2 impacted negatively. The interaction terms AC and BC with positive sign indicated that there would be an increase in response value with an increase in these parameters. On the other hand, the results were also analyzed using analysis of variance (ANOVA). The Linear coefficient of quadratic, interaction effects and p-values were presented in ANOVA Table (Table 4). Fischer variance ratio (F) of 71.43 indicates that the model is significant. The values of "Probability > F < 0.05 indicated that the model terms viz. A, C, A^2, B^2, C^2, AB were statistically significant. The smaller p-values indicate the greater consequence of the corresponding coefficients.

Table 4
ANOVA table

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value	Inference
Model	8217.82	9	913.09	71.43	0.000	significant
A	2812.5	1	2812.50	220.01	0.000	significant
B	3.13	1	3.13	0.24	0.642	
C	528.12	1	528.12	41.31	0.001	significant
AB	306.25	1	306.25	23.96	0.004	significant
AC	0.25	1	0.25	0.02	0.894	
BC	4.00	1	4.00	0.31	0.600	
A^2	3069.64	1	3069.64	240.13	0.000	significant
B^2	1489.26	1	1489.26	116.50	0.000	significant
C^2	584.64	1	584.64	45.73	0.001	significant
Error	63.92	5	12.78			
Lack-of-fit	63.25	3	21.08	63.25		
Pure error	0.67	2	0.33			
Total	8281.73	14				

The coefficient of determination (R^2) of the model was noted as 0.9923 which indicated that the model could not explain only about 0.0077 of the total variation. It should be pointed out that the difference between predicted R^2 (0.8776) and adjusted R^2 (0.9784) was found to be within 0.2. Hence the predicted R^2 was in good agreement with the adjusted R^2. This further confirmed a good predictability of the model.

Fig. 4 shows the predicted versus actual plot for the response (sulfate reduction). It was noticed from the plot that the divergence of the predicted values (from regression model) and actual values (from experimental runs) from the line showing the ideal result was very little. This suggested that the predicted and actual values of responses were related excellently. Thus, a quadratic model developed here demonstrated to be successful in correlating process variables and responses.

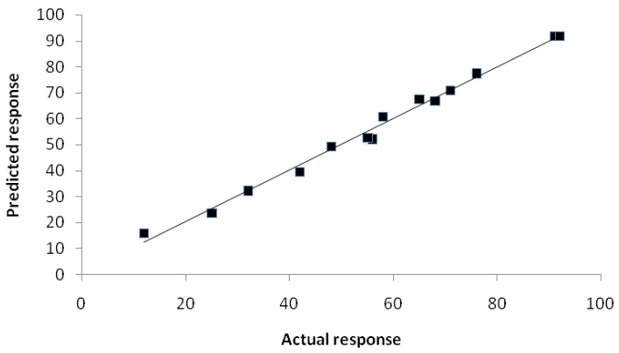

Fig. 4. Plot of actual response versus predicted response for sulfate reduction (%).

3.5.2. Interaction effect of process variables on responses

In response surface methodology, to analyze the nature of responses to different combinations of process variables concerning the regression model, the two-dimensional contour and surface plots need to be considered. Fig. 5a and Fig. 5b represent the interaction effects of pH and temperature on sulfate reduction (%). The third parameter sulfate concentration was held at a constant value of 1500 mg/L. It was observed that both the factors were found to influence the sulfate reduction. The results showed an increase in pH up to a value of 8.4 resulted in higher sulfate reduction (%). Likewise, an increase in temperature from 27°C but not above 34°C also resulted in higher sulfate reduction (%). It was reported that the mutual interactions between the process parameters would be significant when the contour plot is elliptical [30]. Hence, the elliptical contour plot obtained as shown in Fig. 3a proved the notable interactions of pH and temperature on sulfate reduction.

Fig. 6a and Fig. 6b show the combination effects of temperature and sulfate concentration on sulfate reduction by sulfidogenic mixed culture. pH was kept constant at 8 for this study. It was revealed that the sulfate reduction could be achieved above 90% only when the sulfate concentration and temperature were in the range of 1100–1580 mg/L and 28–32°C respectively. To the best of our knowledge, this is the first study to report the interaction effect on sulfate reduction involving temperature as one of the process variables.

The contour and surface plots for the interaction effect of pH and sulfate concentration on sulfate reduction are given in Fig. 7a and Fig. 7b respectively. The result indicated that the sulfate reduction was found to be above 90% with an increase in sulfate concentration from 1000 mg/L to 1650 mg/L. On the other hand, an increase in sulfate concentration above 1650 mg/L showed negative influence on sulfate reduction suggesting it inhibitory to SRB growth. A pH range of 7.25 to 8.20 was observed to have a substantial impact on sulfate reduction. A reverse effect was noted when the pH maintained beyond this range. Of course, a similar kind of trend was recognized in studying the effect of sulfate reduction while using organic marine wastes as

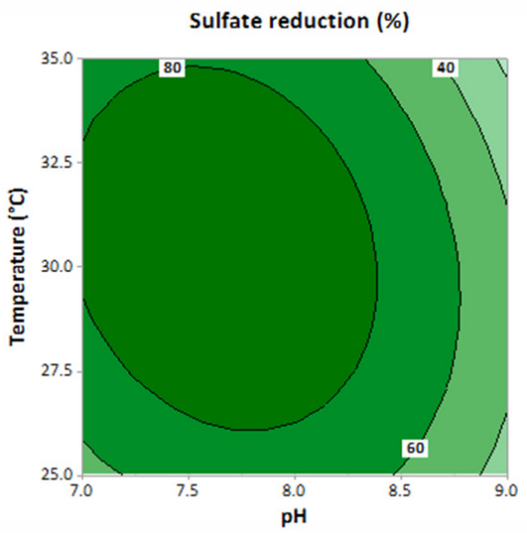

Fig. 5. Interaction effects of pH and temperature (a) Contour plot.

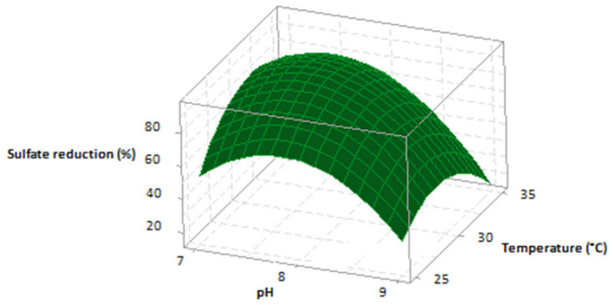

Fig. 5. Interaction effects of pH and temperature (b) Surface plot.

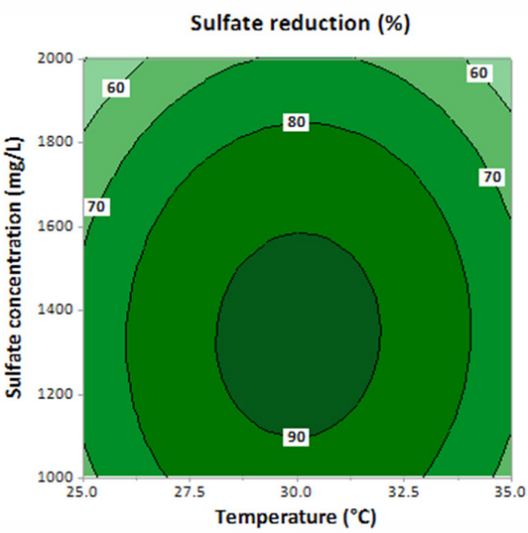

Fig. 6. Interaction effects of temperature and sulfate concentration (a) Contour plot.

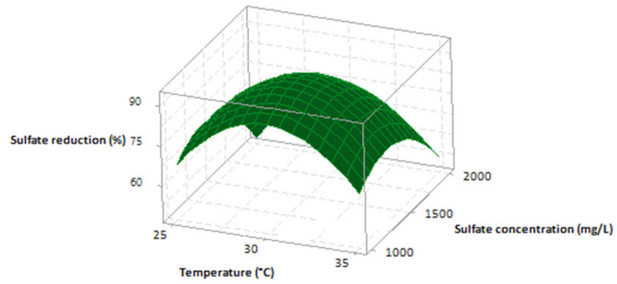

Fig. 6. Interaction effects of temperature and sulfate concentration (b) Surface plot.

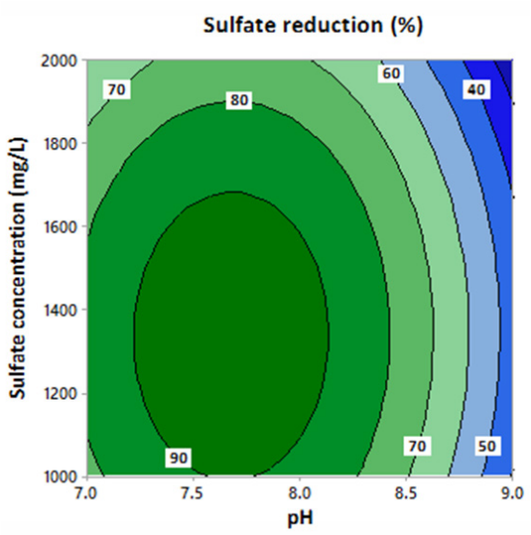

Fig. 7. Interaction effects of pH and sulfate concentration (a) Contour plot.

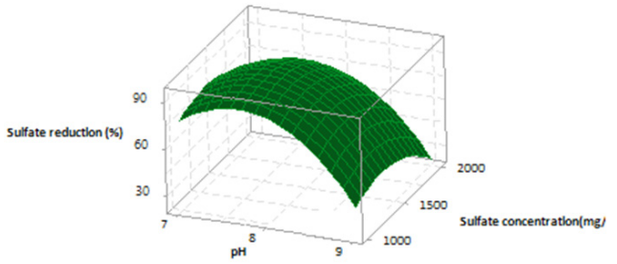

Fig. 7. Interaction effects of pH and sulfate concentration (b) Surface plot.

Table 5
Model validation

pH	Temperature (°C)	Sulfate concentration (mg/L)	Sulfate reduction (%)	
			Predicted	Actual
7.7	30	1392	96.0059	95.5421

the nitrogen source. It was also reported a sulfate concentration of 1500 mg/L and pH of 7.5 above which it causes adverse to SRB growth [26].

3.5.3. Process validation

The optimum conditions of the parameters to achieve maximum sulfate reduction was computed from the theoretical model based on Eq. (6) which corresponds to sulfate concentration of 1392 mg/L, pH of 7.7 and temperature of 30°C. The theoretically optimized points were validated with an experiment which yielded a sulfate reduction of 95.5 % closer to the theoretical value. It was very apparent from Table 5 that the experimental value was found to be in close agreement with the theoretical value. It should also be highlighted that the sulfate reduction achieved from Box-Behnken optimization was comparatively higher than that obtained from single parameter optimization. Hence statistical optimization using Box-Behnken design and response surface methodology proved to maximize the sulfate reduction capacity by the lactate-fed sulfidogenic enrichment culture predominantly *Desulfovibrio sp.*

4. Conclusion

In the present investigation, a lactate-fed sulfidogenic enrichment culture was engaged for the remediation of sulfate-rich wastewaters. The predominant sulfate reducing bacterial strain was identified to be *Desulfovibrio sp.* strain VSV1. This study mainly focused on the optimization of pH, temperature and sulfate concentration by controlling one parameter at a time keeping others fixed as well as mathematical optimization. The Box-Behnken design optimization yielded an optimum value of pH of 7.7, temperature 30°C and sulfate concentration 1392 mg/L which resulted in sulfate reduction of about 95.5%. The Box-Behnken design optimization proved to maximize the sulfate reduction better than single parameter optimization. Therefore, the lactate-fed sulfidogenic enrichment culture predominantly *Desulfovibrio sp.* can be effectively utilized for the remediation of sulfate-rich wastewaters.

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