Chapter 2

Developing Tailor-Made Microbial Consortium for Effluent Remediation

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Additional information is available at the end of the chapter

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Abstract

The work describes a biofilm-based soluble sulphate reduction system, which can treat up to 1600 ppm of soluble sulphate within 3.5 hours of incubation to discharge level under ambient condition using a well-characterized sulphate-reducing bacterial (SRB) consortium. This system ensures the treatment of 1509 litres of sulphate solution in 24 hours using a 220-litre bioreactor. Performance of the system during series operation was compromised, indicating the presence of inhibitor in solution at a toxic level. A single unit bioreactor would be the ideal configuration for this consortium. Modified designs of bioreactors were tested for optimization of the process using response surface methodology (RSM), where the system could function optimally at an initial sulphate concentration of 1250 ppm with a flow rate of 1.8 litre/hour. The time course of sulphate reduction yielded a parabolic profile (with coefficient of determination $r^2 = 0.99$ and p value < 0.05). The rate of sulphate reduction was found to be independent of seasonal variation as well as the specific design characteristic.

Keywords: Sulphate, packed bed reactor, time series analysis, bacterial consortium, Nitrate, radioactive effluent

1. Introduction

Extraction of nuclear materials like Uranium from ores generates effluents containing sulphate. Sulphate mostly comes from the sulphuric acid used for extraction of uranium from its ore. Sulphate is also released as a by-product of different anthropogenic activities such as metal smelting, fuel gas scrubbing, molasses fermentation, tanneries, food processing, coal burning, pulp and paper processing and mining activities [1, 2]. Increase in sulphate concentration in ground water causes various adverse effects such as laxative effect, dehydration, and skin problem, and it also imparts an unpleasant taste to water [3]. It is an eye irritant, causing redness upon exposure. It has also been reported that sulphate pollution results in eutrophication of both surface and ground water. It indirectly enhances phosphate-based eutrophication that can inhibit the growth of different plant species. Na_2SO_4 contamination in the soil can lead to change in freezing temperature by 0.28 °C [4, 5]. The standard level for the presence of sulphate is 250 ppm in drinking water while it is 1000 ppm for waste water. There are different techniques for sulphate demineralization such as reverse osmosis, distillation, ion exchange for drinking water, while methods involving chemical precipitation using chemicals like barium chloride exist for environmental waste disposal. The chemical method of reduction of sulphate using barium chloride also ensures substantial reduction of heavy metals in the form of precipitates. But for the chemical process to function optimally, it is essential that the concentration of the chemical is high and that it is thoroughly mixed with the effluent discharged. The mechanical stirring in case of large volumes may not be a feasible option at the industrial scale. Hence, physicochemical techniques have many drawbacks when their efficiency is compared with the cost of implementation of the technology [6].

Bioremediation happens to be an alternative method of treatment. Biological sulphate reduction is a state-of-the-art technology, which has definite advantages over conventional treatments. Sulphate-reducing bacteria (SRB) play an important role in several biochemical processes. Sulphate is taken up by these microbes as a nutrient and reduced to sulphide, which is then incorporated into sulphur-containing amino acids. Thus, they are significant in sulphur transformation [7]. SRB is heterogeneous, morphologically diverse, physiologically unique anaerobic microorganisms that are widespread in anoxic habitats [8, 9], where they use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide. Both oxidation and reduction reactions for the generation of metabolic energy are important. The sulphide thus produced can be oxidized in the presence of high levels of oxygen by chemolithotrophic sulphur bacteria or under anoxic conditions by phototrophic sulphur bacteria, whereas SRB perform the dissimilatory sulphate reduction [10–12]. In marine sediments, above 50% organic carbon mineralization is carried out by sulphate reduction making the sulphate reducers extremely important for both the sulphur and carbon cycles. However, the use of SRB for bioremediation of waste water has some bottle necks. These include (a) the continuous supply of microbes for sulphate reduction within reasonable time and (b) the survival of the microbes in the environment while maintaining the efficiency of reduction. The literature reported a retention time of 15 days [13], 14 days [14], 10 days [7], 6 days [15], and 1 day [16] while working at laboratory scale with associated problems of clogging, back pressure and need for repeated maintenance. These facts made them non-viable

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for large-scale applications. Hence, the need of the hour was to develop a microbial solution through which rapid removal of soluble sulphate could be carried out in a sustainable manner. To address this issue, the following points had to be considered: (1) appropriate site selection for enrichment of SRB; (2) appropriate medium selection for the same; and (3) consortium optimization and development of packed bed reactor with optimal design for sustained performance of the system.

As an outcome of this study, a consortium was developed using which a packed bed bioreactor–based process has been drawn up, which is by far the fastest and the most stable sulphate removal system. This invention has been filed as an Indian patent and a PCT [17] to protect the intellectual property associated with this invention. It has immense application for industrial effluent treatment. Although biofilm-based bioreactors have been the point of investigation and application for a long period of time [18–21], little progress has been made in terms of real-life industrial application.

2. Selection of inoculum and medium for consortium development

The authors of this chapter have developed a consortium from waste water–fed fish pond at East Kolkata Wetland (EKW), India (22° 27′ N 88° 27′ E) [1] in synthetic medium (DSMZ 641) specific for growing SRB under anaerobic condition, which could reduce soluble sulphate from 1600 ppm to discharge level within three and half hours of incubation at room temperature in a packed bed reactor with stable biofilm for sustained treatment of soluble sulphate. The geographical orientation in terms of slope is such that the entire city's (Kolkata's) run off (which includes contribution of acid rain) drains at EKW. However, there is no toxicity reported in these water bodies. Hence, there is high possibility of these water bodies harbouring efficient SRB. The selection of medium for growth, the inoculum for development of the consortium and the subsequent selection pressures were carefully monitored keeping in mind the need to specifically enrich the SRB with minimal non-SRB so as to ensure insignificant dead mass during bioreactor operation, hence developing a tailor-made consortium for this purpose. Its performance was tested for sulphate reduction from modified synthetic medium (DSMZ 641) prepared using tap water and mining effluent.

Synthetic medium for growing the consortium

For 1 litre of medium **DSMZ 641**, following is the composition:

Solution A

NH_4Cl	1.0 g
Na_2SO_4	2.0 g
$Na_2S_2O_3 \times 5\ H_2O$	1.0 g
$MgSO_4 \times 7\ H_2O$	1.0 g
$CaCl_2 \times 2\ H_2O$	0.1 g

KH_2PO_4	0.5 g
Yeast extract	1.0 g

Then, a pinch of Resazurin is added, which is used as a redox indicator.

Solution B

$Na_2S_2O_4$	0.1 g
$NaHCO_3$	1.68 g
Lactic acid	12 ml
NaOH	4.4 g

Solution C: Trace element solution 1.0 ml (SL10)

HCl (25%; 7.7 M)	10.0 ml
$FeCl_2 \times 4\ H_2O$	1.5 g
$ZnCl_2$	70.0 mg
$MnCl_2 \times 4\ H_2O$	0.1 g
H_3BO_3	6.0 mg
$CoCl_2 \times 6\ H_2O$	190.0 mg
$CuCl_2 \times 2\ H_2O$	2.0 mg
$NiCl_2 \times 6\ H_2O$	24.0 mg
$Na_2MoO_4 \times 2\ H_2O$	36.0 mg
Distilled water	990.0 ml

Solution D: Vitamin solution 10 ml

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine	10.0 mg
Thiamine HCl ×	$2H_2O$ 5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
D-Ca-pantothenate	5.0 mg
Vitamin B_{12}	0.10 mg
P-amino benzoic acid	5.0 mg
Lipoic acid	5.0 mg
Distilled water	1000.0 ml

Sodium azide is also added to prevent the fungal formation.

The following are the modified medium components, which were effective for SRB in terms of nutrient consumption. This medium is far more advantageous in terms of cost and amount of consumption. It is similar to the

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aforementioned media composition with a minor change in components such as ammonium chloride—50% of previously mentioned medium, KH_2PO_4—25% and yeast extract—50%.

Composition of the mining effluent

Sodium (17.5 ppm), potassium (37.3 ppm), manganese (0.03 ppm), nickel (0.026 ppm), magnesium (17.6 ppm), calcium (540 ppm), total carbon (5.893), inorganic carbon (4.477) and total organic carbon (1.419).

Microorganism preferentially gets attached to the surfaces in favourable conditions like moist surface along with the nutrients as a layer called biofilm. Earlier reports indicated the presence of surfaces to stimulate attached bacterial growth under conditions, which are otherwise too dilute to sustain the microbes [22]. The operation was scaled up to 220 litres. The system involved three columns in series of 78, 71 and 71 litres (**Figure 1**). It showed an efficiency of above 50% soluble sulphate (starting from 1600 ppm) reduction within three and half hours under ambient temperature during both batch and continuous modes. The sulphate reduction varied from 65% to 100% within 24 hours. The biofilm could be well sustained in both polypropylene and steel matrix (**Figure 2**) without any maintenance for more than 18 months.

Figure 1. 220-litre packed bed bioreactor for soluble sulphate removal with steel and polypropylene immobilization substrate with defined surface area for bacterial biofilm formation.

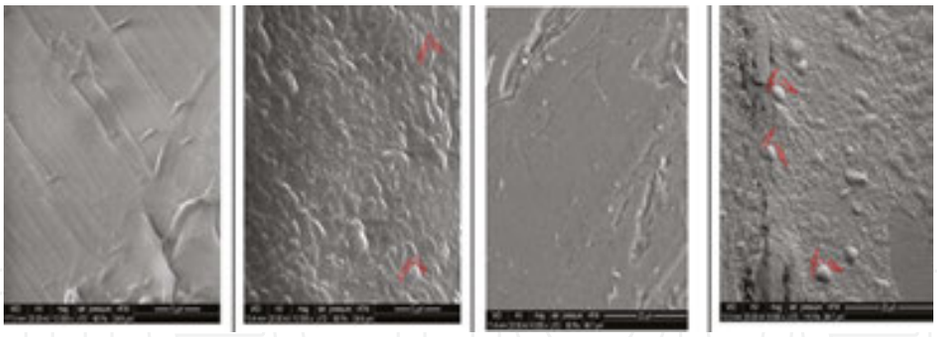

Figure 2. Scanning electron microscopic image of different matrix with and without SRB biofilm. From extreme left, polypropylene matrix (without biofilm), polypropylene matrix (with biofilm), steel matrix (without biofilm), and steel matrix (with biofilm).

3. Optimization of inoculum percentage and immobilization matrix

Inoculum optimization was done based on the extent of sulphate reduction following immobilization of the consortium onto a matrix as per the method of Nasipuri et al. [1]. The inoculum percentage was varied from 2% to 50% (2%, 5%, 10%, 20%, 30%, 40% and 50%) to maximize the sulphate reduction by the system. Optimum sulphate reduction of 70% was obtained with 10% primary inoculum. It implied that with 10% bacterial inoculum maximum metabolic rate was reached, which eventually resulted in a significant sulphate reduction under optimum condition. But further increase in inoculum percentage resulted in no further increase in efficiency in terms of sulphate reduction.

Two types of matrices (polypropylene and steel) with uniform surface areas were tested for the purpose as per the method of Nasipuri et al. [2]. The stainless steel and polypropylene raschig rings showed an overall sulphate reduction of 72.05% and 69.59%, respectively. They were equally efficient as immobilization matrix in terms of sulphate reduction under the same set of conditions. The comparable range of efficiency between stainless steel and polypropylene raschig ring in terms of sulphate reduction might be due to equal lower pressure drop at effective surface areas and same gas velocity. Our data were also supported by the study of Kolev et al. [23]. In this regard, the scanning electron microscopic images were further used to visualize the dense biofilm formation on both types of matrices (**Figure 2**).

4. Biofilm formation and sulphate reduction

Biofilm, a thick layer of microbial cells embedded into secreted extracellular material on inert matrix such as polypropylene or metals, acts as a constant source of inoculum for the system. The method was adopted from Nasipuri et al. [1]. Two millilitre of sample was centrifuged at 10,000 rcf for 10 minutes to pellet down the cells. The supernatant was mixed with 98 ml of distilled water in a 250-ml conical flask. A 5 ml of conditioning mixture containing hydrochloric acid (6%), isopropanol (20%), water (64%) and glycerol (10%) was added to it for proper

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mixing. The mixture was then put onto a magnetic stirrer at the maximum speed for 1 minute with a pinch of barium chloride. The solution was then allowed to stand for 2 minutes for settling the barium sulphate precipitate. The absorbance was taken at 420 nm for soluble sulphate measurement using a dual-beam spectrophotometer by Agilent Technologies.

Similarly, for measurement of biofilm, the method of Martin [24] was adopted. The biofilm-containing matrix was firstly stained for 10 minutes with crystal violet. Vigorous washing was done with distilled water to wash away the loosely bound stain. Ninety-five percent of ethanol was then added to remove the bound stain from the biofilm. The absorbance of the removed stain was measured at 620 nm for biofilm thickness measurements using a dual-beam spectrophotometer by Agilent Technologies.

The effect of biofilm formation by SRB consortium on sulphate reduction was checked for 90 days. The biofilm thickness (left) compared with sulphate reduction (right) performed by the system for the above-mentioned period (**Figure 3a** and **b**) revealed oscillatory nature of both biofilm formation and associated sulphate reduction. It is an inherent nature of a biofilm-based system. The biofilm thickness was not directly correlated with the extent of reduction. This is because biofilm thickness as reflected by the method of Martin et al. [24] consists of active cells, inactive cells and the extracellular polymeric substances, although the reduction is due to the function of just the active cells. Hence, both show an oscillatory pattern but are not directly dependent on one another. The evidence of an oscillatory nature in biofilms was also observed by others [25, 26], which eventually supported the former statement.

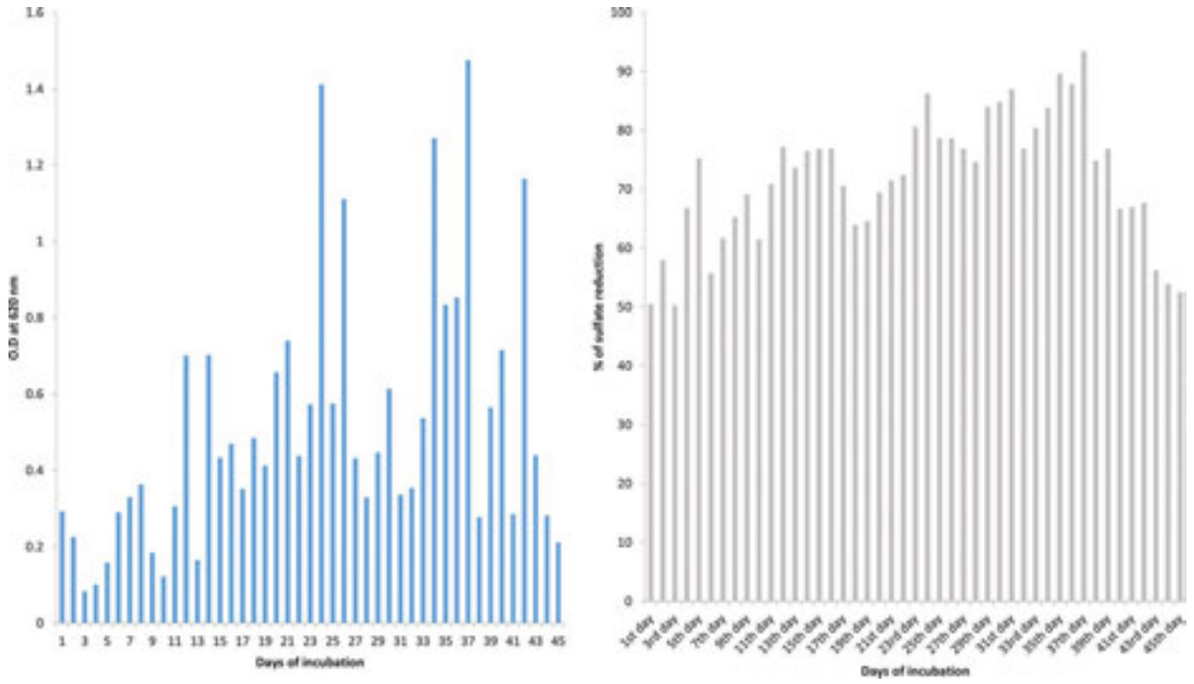

Figure 3. Graphical representation of biofilm formation by the SRB consortium on matrix (left) with associated sulphate reduction (right).

5. Performance of the bioreactor

The performance of the bioreactor in terms of sulphate reduction efficiency of the system starting from an initial concentration of 2000 ppm was measured after 24 hours of incubation according to the method of Nasipuri et al. [1] for a period of 361 days (**Figure 4**). The quarterly data revealed 36.29 ± 16.55, 63.46 ± 15.24, 57.44 ± 17.32 and 91.81 ± 7.97 sulphate reduction, respectively, indicating stabilization of the bioreactor. The data generated from the bioreactor were used for carrying out the time series analysis after detrending (**Figure 5**) the series using Matlab 7.4.0 (R2007a) to study the bioreactor performance in terms of sulphate reduction. The time series plot (**Figure 5**) clearly reflects the inherent oscillatory nature of the system as has been reported earlier by other investigators [25]. This oscillatory nature is an inherent property of the biofilm-based system. Taherzadeh reported such oscillatory property in case of continuous mode operations of reactors due to shearing force, but in this case, the same property is observed even in case of batch mode operation as an outcome of biofilm property, where there exists only minimum of shearing force [27]. However, the impact of seasonal variation may also be important. The effect of seasonal variation on the performance of the system was analysed (**Figure 6**). Its performance appears to be independent of the seasonal variation. This is a positive finding in terms of application of this system on site. Hence, the correlation between ambient temperature and age of the biofilm with that of the performance of the system was determined using Pearson's correlation coefficient. The correlation coefficient among sulphate reduction and biofilm formation, biofilm formation and ambient temperature, sulphate reduction and ambient temperature was observed to be 0.738, 0.538 and 0.284, respectively. It was observed that there was no statistical trend in performance of the system with either ambient temperature or age of the biofilm. It was revealed that the two variables were not related (**Figure 6b**).

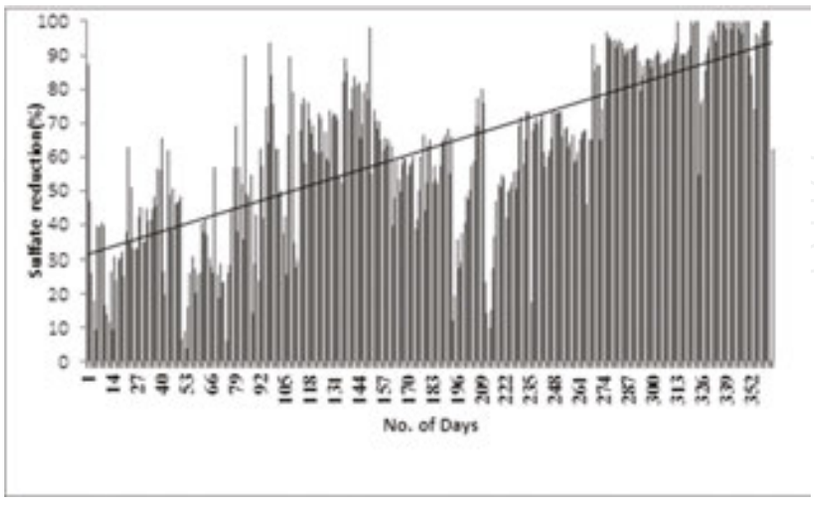

Figure 4. Performance of the SRB consortia in 78-litre packed bed bioreactor for 361 days. The 78-litre bioreactor is constructed of acrylic body and stainless steel for the bottom portion with three nozels for sample collection (distance between first and second nozels is 18 cm, whereas the distance between second and third nozels is 40 cm. The anaerobicity was mentioned by purging nitrogen gas (60 lm/cm^2) into the bioreactor from outside.

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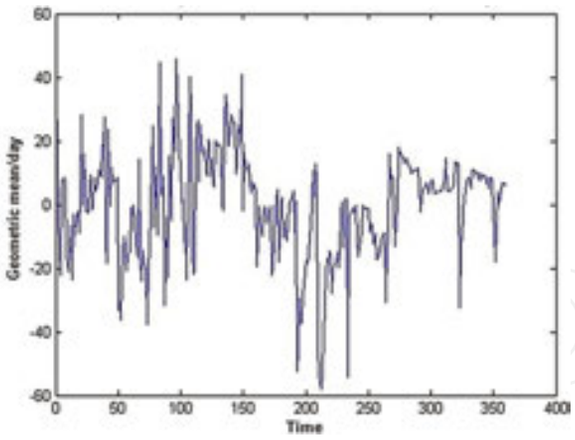

Figure 5. Time series of geometric mean of sulphate dataset after removing trend.

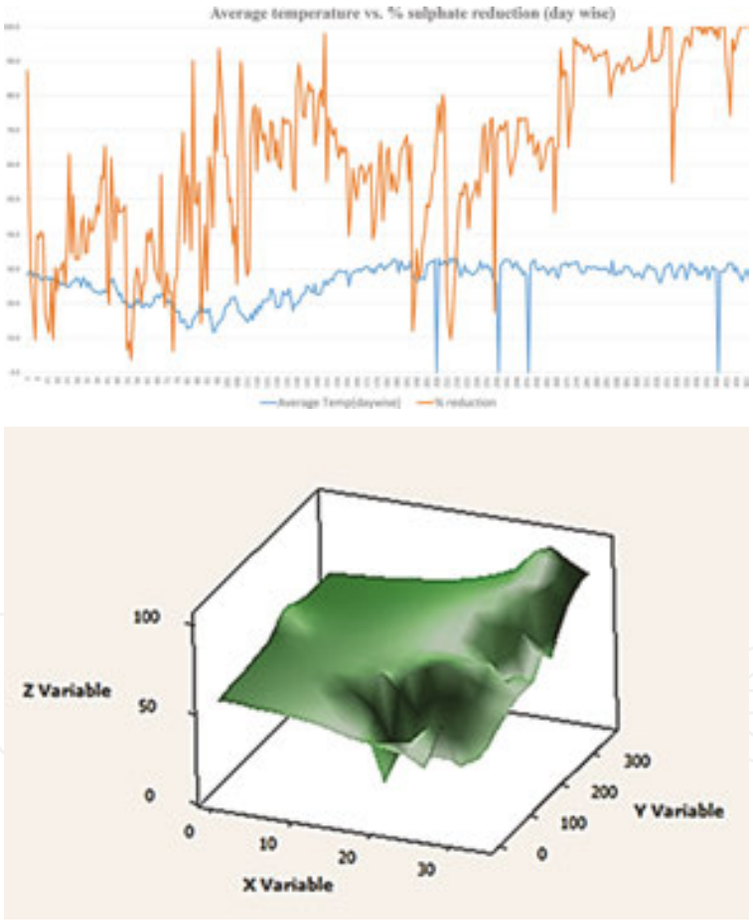

Figure 6. (a) Graph representing biofilm performance in term of sulphate reduction and temperature variation with days. Sulphate reduction indirectly indicates the active biofilm at any point of time. *X* axis represents days that the reactor was functioning, whereas *Y* axis represents performance of the system in terms of sulphate reduction in orange colour and ambient temperature in blue. (b) Surface plot among *X*, *Y* and *Z* variables, where *Z* variable is the percent sulphate reduction, *Y* variable is the age of the biofilm in days and *X* variable is the ambient temperature in degree centigrade.

6. Scalability of the process

The sulphate reduction was monitored under anaerobic condition in a 9-litre bioreactor for 25 days, and the percentage of mean reduction was 56.3 ± 11.03. In case of 78-litre bioreactor, the sulphate reduction was monitored for 361 days, and the percentage of average reduction was 62.36 ± 24.81 with the saturation obtained at around 331 days. The probability distribution of the data was found to be normally distributed. It was evident from the above observations that the efficiency of sulphate reduction was increased by 1.10-fold with scaling up of the bioreactor volume. Our results were quite similar to the study of Sarti et al. [28], where they had also successfully demonstrated sulphate reduction in an anaerobic bioreactor with maximum efficiency of 99%. A similar study of fed batch bioreactor used for sulphate reduction was demonstrated by Silva et al. in the year 2002 with an efficiency of 97% [29]. Although both the studies are similar to ours in terms of reduction efficiency, but unlike those, our system retains the reduction efficiency consistently once it gets stabilized.

7. Optimization of the time of sulphate reduction

The sulphate reduction was further optimized in terms of incubation time in the same bioreactor under identical conditions. The desired level of reduction (for environmental discharge) was reached within three and half hours (**Figure 7**). The data were fitted using Origin8pro (**Figure 8**), which resulted in a polynomial equation of order 5 with $r^2 = 0.97$. The following equation perfectly expresses this desulfurization system:

$$Y = 0.941 + 160.51x - 182.51x^2 + 83.8x^3 - 18.14x^4 + 1.39x^5 \quad (1)$$

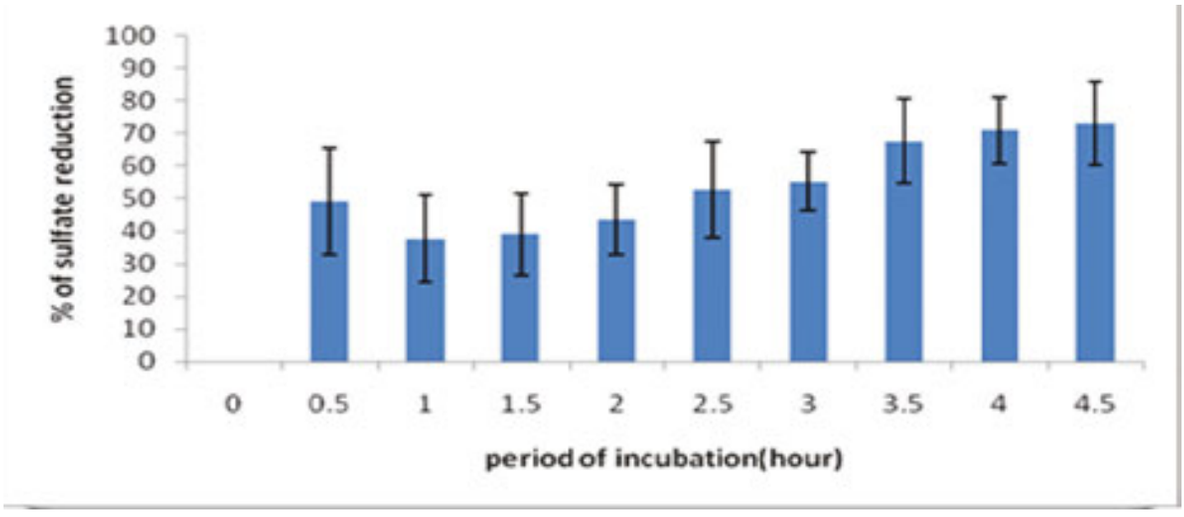

Figure 7. Time course of sulphate reduction by the packed bed bioreactor.

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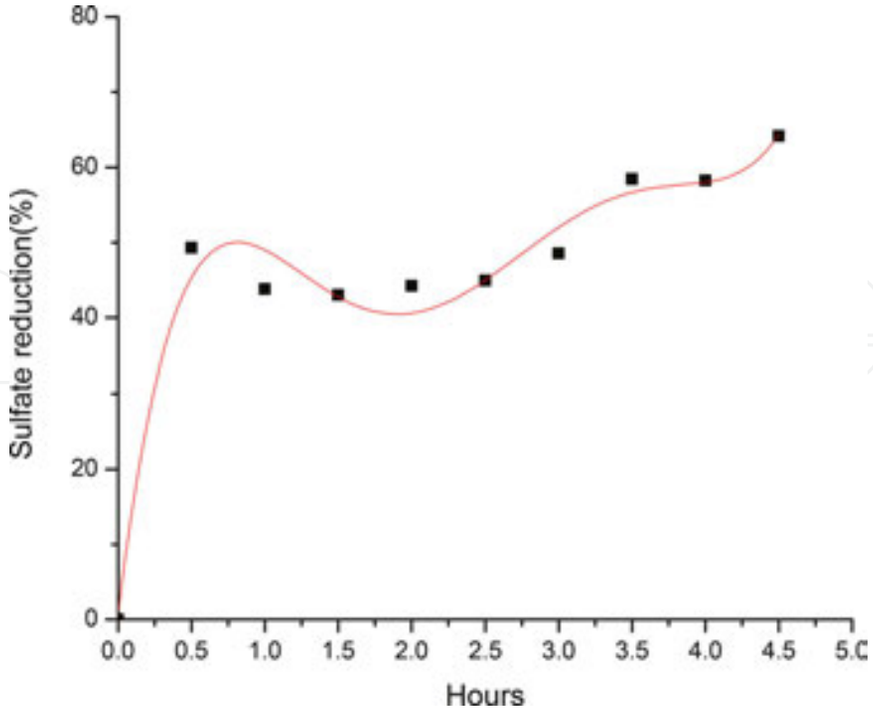

Figure 8. Fifth-order polynomial equation of time optimization of sulphate reduction using Origin8pro.

Here Y denotes the percentage of sulphate reduction, and x signifies the time required for reduction in hours.

8. Effect of height of the bioreactor on performance

The oscillatory nature of the bioreactor performance in terms of sulphate reduction was further analysed by calculating the running mean of the sulphate reduction of the samples taken from each port of the bioreactor as described in **Figure 4**. The result indicated that significant amount of sulphate reduction was observed between the first and second ports (18 cm), in contrast, there was no significant reduction between the second and third ports (40 cm; **Figure 9**). As an explanation to this observation, it can be argued that the compromised performance in the upper layer could be due to the accumulation of hydrogen sulphide gas generated by the system, which has a negative impact on system performance. The phenomenon was also supported by the report of Frank et al. in the year 2013 [30]. To decrease the dead space (where the performance is compromised) and enhance the efficiency of the system, an alternative reactor design was tested (**Figure 10**). Similar designs for one vertical system and one horizontal system were constructed and tested (**Figure 10b**). The performance of the two systems was found to be similar with no significant statistical variation observed using z test for the equality of two means with unknown variances and moderate sample sizes (n = 47 in case of both vertical and horizontal designs). The results are displayed in **Table 1**.

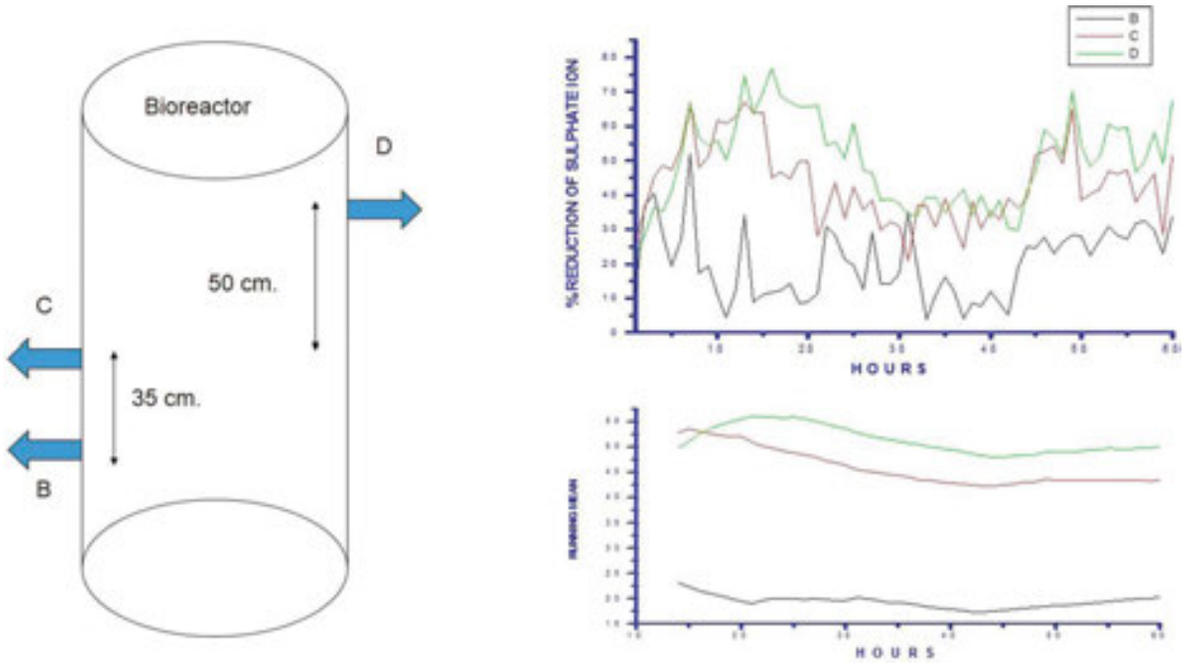

Figure 9. Left panel shows the position of the ports on the bioreactor column. The right top panel shows actual sulphate reduction at the different ports, whereas the right bottom panel shows running mean of sulphate reduction from three different ports of the bioreactor for 60 hours. Bottom curve for the first port, middle one for the second port and the upper curve was for the third port.

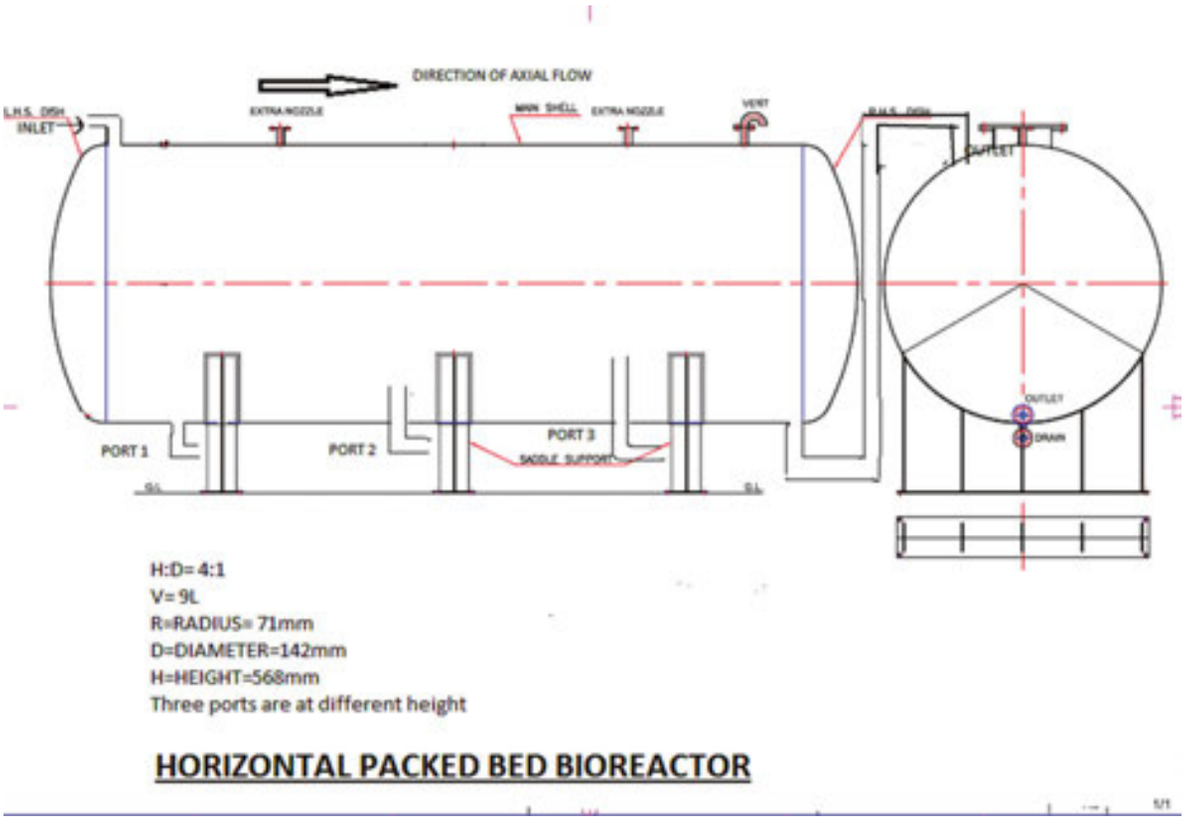

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Figure 10. (a) Modified design of packed bed bioreactor. (b) Actual bioreactors constructed using plastic material.

***z* Test: two sample test for equality of means with unknown population variances and large sample sizes**	**H_0: $\mu_1 = \mu_2$**	**H_1: $\mu_1 \neq \mu_2$**
	Variable 1	**Variable 2**
Mean	50.27085106	48.98255319
Known variance	267.57	302.71
Observations	47	47
Hypothesized mean difference	0	
Z	0.369846189	
$P(Z \leq z)$ one tail	0.355748549	
z Critical one tail	1.644853627	
$P(Z \leq z)$ two tail	0.711497098	
z Critical two tail	1.959963985	

Table 1. Statistical validation of sulphate reduction using different designs of the bioreactor.

From the above observation, it was clear that the diminished performance in the upper layer was not due to accumulated hydrogen sulphide gas. There might be other factors responsible for this performance variation. As the bioreactor design was proper for the current system under investigation, the optimization of process was done using response surface methodology (RSM) under ambient condition and implemented using design expert 9 software as displayed in **Table 2**. Experimental and predicted responses were found to be broadly similar.

		Factor 1	Factor 2	Response 1	Predicted
Standard	Run	A: sulphate concentration (ppm)	B: flow rate (litre/hour)	Sulphate reduction (%)	Sulphate reduction (%)
3	1	719.67	2.82	59.66	55.62
4	2	1780.33	2.82	66.05	60.3
8	3	1250.00	3.00	46.54	56.57
1	4	719.67	1.98	60.72	58.46
9	5	1250.00	2.40	49.07	50.63
13	6	1250.00	2.40	49.07	50.63
2	7	1780.33	1.98	68.21	60.3
11	8	1250.00	2.40	49.07	50.63
10	9	1250.00	2.40	49.07	50.63
12	10	1250.00	2.40	49.07	50.63
5	11	500.00	2.40	58.63	54.05
7	12	1250.00	1.80	53.85	52.93
6	13	2000.00	2.40	63.6	58.79

Table 2. Table representing the experimental design for system optimization using response surface methodology.

From the above analysis, the optimum sulphate reduction condition was determined at an initial sulphate concentration of 1250 ppm and at a flow rate of 1.8 litre/hour (**Figure 11**). The mathematical equation derived from the model is given below. The values of each term are given in the coefficient table (**Table 3**).

$$\text{Equation for sulphate reduction} = 49.07 + 2.61 \times A - 1.69 \times B - 0.28 \times AB + 8.02 \times A^2 + 2.56 \times B^2 \quad (2)$$

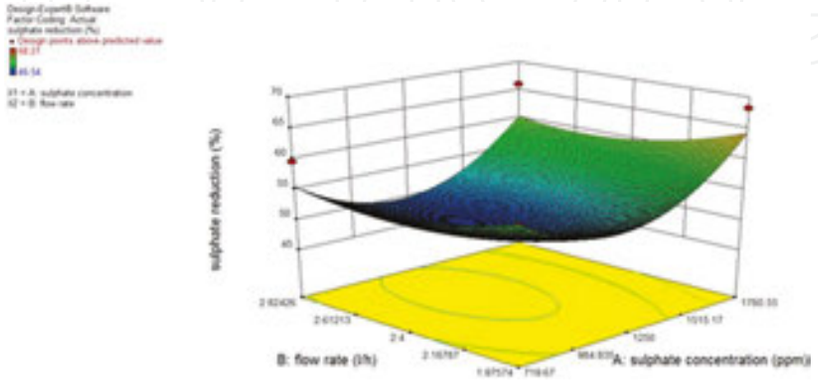

Figure 11. The model graph for sulphate reduction in response to sulphate concentration and flow rate.

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Response	Intercept	*A*	*B*	*AB*	A^2	B^2
Sulphate reduction	49.07	2.61358	−1.69474	−0.275	8.02375	2.56375
p		0.1428	0.3199	0.9057	0.0021	0.1748
Legend		$p < 0.01$	$0.01 \leq p < 0.05$	$0.05 \leq p < 0.10$	$p \geq 0.10$	

Table 3. Statistical validation of the optimization study.

where *A* is the sulphate concentration, and *B* is the flow rate.

9. Conclusion

The work contained in this chapter describes a biofilm-based soluble sulphate reduction system operating within 3.5 hours using a well-characterized SRB consortium from 1600 ppm to discharge level under ambient condition. This ensures the treatment of 1509 litres of sulphate solution in 24 hours using a 220-litre bioreactor. A single-unit bioreactor would be the ideal configuration for this consortium. Time kinetics of sulphate reduction yielded a parabolic form significantly ($r^2 = 0.99$; $p < 0.05$). Rate of sulphate reduction was found to be independent of seasonal variation. The bioreactor designs tested during this study had practically no effect on the performance of the system. This system was the fastest sulphate-reducing system at pilot scale, which could run without maintenance for a long time with the ability to withstand an initial sulphate concentration of 1250 ppm at a flow rate of 1.8 litre/hour optimally under ambient condition. Hence, the process has been filed as an Indian patent and a PCT to protect the intellectual property associated with this invention. It has immense application for industrial effluent treatment in future.

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