STUDIES ON SULFATE REDUCTION TO ELEMENTAL SULFUR UNDER ANAEROBIC/MICROAEROBIC CONDITIONS

A Thesis Submitted

by

Bharati Brahmacharimayum
(Roll No. 08615205)

In Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Centre for the Environment
Indian Institute of Technology Guwahati
Guwahati - 781039, Assam, India

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Dedicated

to

My Family

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Statement

I hereby declare that the matter embodied in this thesis entitled "Studies on Sulfate Reduction to Elemental Sulfur under Anaerobic/Microaerobic Conditions" is the result of investigations carried out by me at Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Dr. Pranab Kumar Ghosh**. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

Dated

Bharati Brahmacharimayum
Roll No. 08615205
Centre for the Environment
IIT Guwahati
Guwahati -781039
Assam, India

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Certificate

It is certified that the work described in this thesis entitled "Studies on Sulfate Reduction to Elemental Sulfur under Anaerobic/Microaerobic Conditions" by **Bharati Brahmacharimayum** (Roll No. 08615205), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at Centre for the Environment, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for any kind of degree.

Dated

Dr. Pranab Kumar Ghosh
Associate Professor
Department of Civil Engineering
IIT Guwahati
Guwahati – 781039
Assam, India

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Abstract

Sulfate rich wastewater with low organic content is being released in increasing quantity due to rising industrial activities. This wastewater can be treated using both physicochemical and biological treatment processes. Though physicochemical treatment process allow faster removal of sulfate but biological treatment through sulfate reduction serves as an effective and economical method as expensive additional units are required when physicochemical methods are employed. In spite of the cost advantage, one of the main drawbacks of biological treatment is the generation of sulfide in the treatment process. In order to make the biological process the best option for treating sulfate rich effluents, the sulfide generated needs to be taken care of. Various sulfide removal options such as gas stripping, precipitation, chemical and biological oxidation are available. The cost effective option would be oxidation of the sulfide to elemental sulfur under microaerobic conditions. The combination of anaerobic sulfate reduction and aerobic sulfide oxidation for treating sulfate rich wastewater has a great potential.

The main objective of this study is to develop an anaerobic/micro-aerobic bioreactor system for sulfate-rich wastewater treatment. The experimental setup comprises of a packed bed reactor for anaerobic sulfate reduction and a sequenced up-flow microaerobic reactor for oxidation of hydrogen sulfide into elemental sulfur. In the microaerobic reactor, dissolved oxygen (DO) was controlled with a mass flow controller for optimizing elemental sulfur formation in the batch as well as with the continuous supply of air.

The bioreactor system was fed with sulfate rich synthetic wastewater and the performance of the reactor system in terms of COD removal efficiency, sulfate reduction and sulfide oxidation potential were observed over a period of 35 months. Regarding the sulfate reduction efficiency, the PBR was able to achieve more than 90% sulfate reduction could be achieved with 2000 mg/L sulfate concentration when lactate was used as the carbon source. The formation of elemental sulfur was observed even in anaerobic condition which might be most probably due to the reaction between the sulfides and sulfites. The utilization of toxic compound such as phenol as

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a co-substrate as well as sole source of carbon for sulfate reduction was investigated. Optimization studies done in the PBR when phenol was added as a co-substrate using response surface methodology showed SO_4^{2-} removal of 86.4 % (from initial 2000 mg/L) with the lactate COD of 1400 mg/L and phenol concentration of 210 mg/L. Phenol was effectively utilized as a sole carbon source for SO_4^{2-} reduction till 550 mg/L but it started to show inhibitory effects at a higher concentration of 750 mg/L. The PBR was able to achieved SO_4^{2-} and phenol removal of 75.5 % and 87% respectively were achieved from an initial concentration of 2000 mg/L of SO_4^{2-} and 512 mg/L of phenol at a HRT of 42 h using phenol was used as a sole carbon source. The performance of the reactor was also observed when subjected to shock loading. Shock loading studies showed that up to 3.5 times increase in the normal input sulfate concentration (2000 mg/L) in the form of sulfate shock loading did not affect the PBR performance irreversibly. Bacterial strains such as *Pseudomonas* species, *Desulfovibrio* species and *Citrobacter* species were isolated and identified from the PBR which have been already reported to have SO_4^{2-} reducing capacity.

With respect to the microaerobic reactor, optimum DO for elemental sulfur formation in the microaerobic reactor was observed to be 70-100 µg/L with a minimum HRT of 17 h when the reactor was operated in continuous mode with continuous air supply. The elemental sulfur which was formed in the microaerobic reactor could be easily separated from the reactor effluents by gravity sedimentation and centrifugation.

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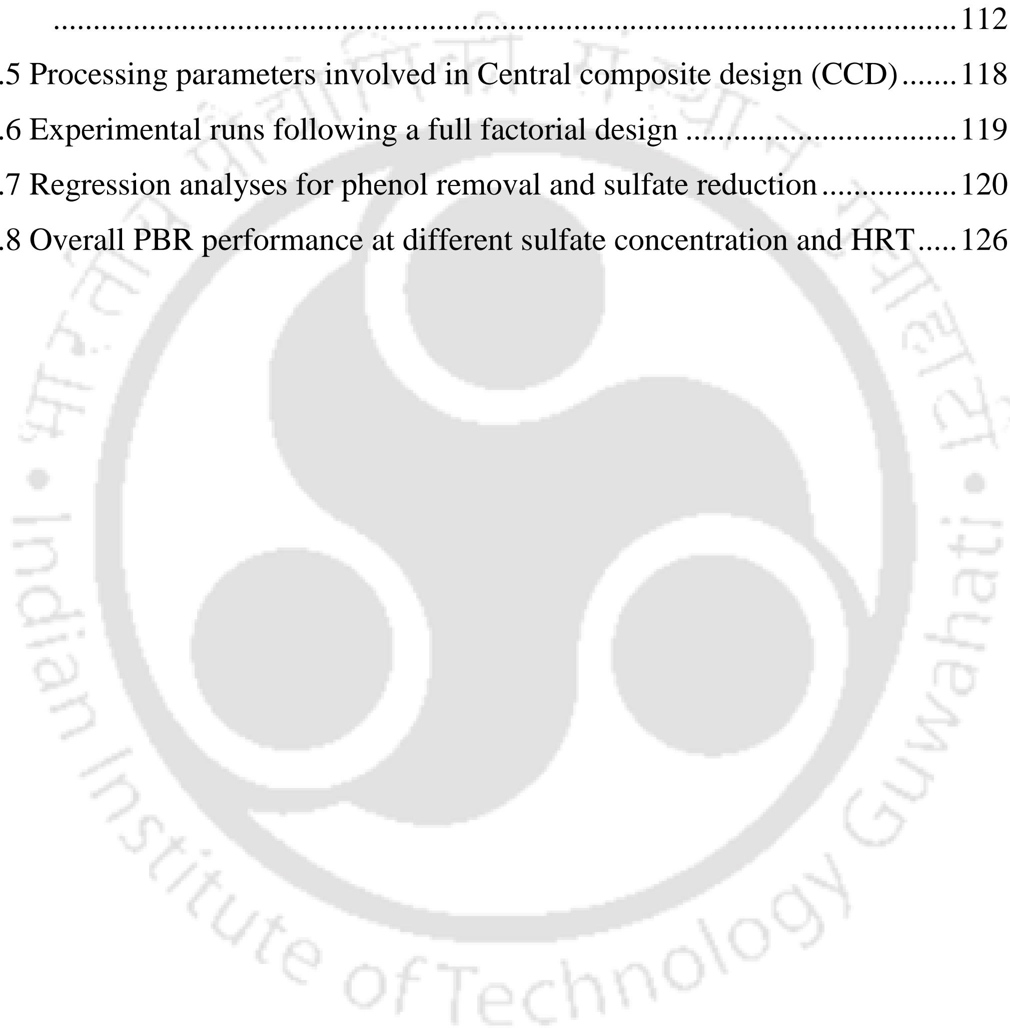

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Abbreviations

H_2S, HS^- and S^{2-}	Sulfides
HSO_3^-	Sulfites
S^0	Elemental sulfur
$S_2O_3^{2-}$	Thiosulfate
SO_4^{2-}	Sulfate
COD	Chemical oxygen demand
h	Hour
HRT	Hydraulic retention time
k	Maximum substrate utilization rate (day^{-1})
K_s	Half velocity constant ($mg\ l^{-1}$)
PBR	Packed bed reactor
R_{OS}	Molar ratio of oxygen to sulfide
SRB	Sulfate reducing bacteria
VFA	Volatile fatty acid
%	Percentage
MLVSS	Mixed liquor volatile suspended solids
MLSS	Mixed liquor suspended solids
PUF	Polyurethane foam

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RSM	Response surface methodology
PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
T-RFLP	Terminal Restriction Fragment Length Polymorphism
DO	Dissolved oxygen
L	Litre
CO_2	Carbondioxide
H_2	Hydrogen
ΔG	Gibbs' free energy
H^+	Hydrogen ion
SLR	Sulfate loading rate
SRR	Sulfate removal rate
PUB	Phenol utilizing bacteria
MB	Methanogenic bacteria
OLR	Organic loading rate
ORP	Oxidation-reduction potential
ATP	Adenosine triphosphate
APS	Adenosine 5' phosphosulfate
LC-MS	Liquid Chromatography-Mass Spectrometry
FESEM	Field Emission Scanning Electron Microscopy

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Chapter 1

INTRODUCTION

Sulfate (SO_4^{2-}) is one of the most abundant anions found in the environment. It is a common constituent of many natural water and wastewater, and present, sometimes, in high concentrations. Various industrial activities such as pulp and paper industries, mining and mineral processing, production of explosives, scrubbing of flue gases, petrochemical industries, galvanic processes, battery, paint and chemical manufacturing, food processing (molasses, seafood, and edible oil), and pharmaceutical industries release around 200 to 50,000 mg/L of SO_4^{2-} containing wastewater (Carrondo, 1983; Dijkman, 1995; Habets and de Vegt A.L, 1991; Mendez et al., 1995; Svardal K., 1993). In the present scenario, very less attention has been given to the mitigation of sulfate rich wastewater even though large quantities of sulfate are being released into the environment anthropologically owing its relatively low direct environmental impact compared with other pollutants. Sulfate becomes a pollutant if it is released in excess leading to various environmental hazards and impacts upon its discharge into the natural environment. Presence of sulfate can cause a pungent odor and taste in drinking water and may have a laxative effect. Release of sulfate in the aquatic environment and subsequent sulfide formed from sulfate reduction alter the natural sulfur cycle and may cause damage to the natural ecosystem. Excessive quantities of released sulfate can affect public water supplies and pose health threat to life forms. The upper concentration limit of sulfate in water intended for human consumption is recommended at 250 mg/L (Sawyer CN et al., 2009). In the absence of dissolved oxygen and nitrate, sulfate is converted to sulfide (H_2S) by acting as a source of oxygen or electron acceptor. After discharge to the sewage system, the sulfide might cause toxicity and malodor problems and may also lead to corrosion problems of the sewer pipes in the long run, thus hampering the proper functioning of the sewage systems. In addition, H_2S is fatally toxic to humans, causing death within 30 min at gaseous concentrations of only 800–1000 mg/L, and instant death at higher concentrations (Speece, 1983). Therefore, sulfate rich wastewater require appropriate treatment before being released into the environment.

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The options available for treatment of sulfate rich wastewater include physicochemical and biological treatment methods. Physicochemical methods are very effective but suffer from challenges relating to separation, appropriate disposal of the solid phase, relatively high cost, and energy consumption (Silva et al., 2002). In case of biological treatment SO_4^{2-} bearing wastewater is generally treated using anaerobic processes (Dries et al., 1998; Omil et al., 1996; Percheron et al., 1997; Visser et al., 1993). The biological SO_4^{2-} reduction process is mediated by a group of microorganisms known as SO_4^{2-} reducing bacteria (SRB) which form a specialized group of microbes that use sulfate as terminal electron acceptor for their respiration. Under anaerobic conditions, heterotrophic SRB uses SO_4^{2-} as terminal electron acceptor for the degradation of organic compounds (Elferink et al., 1994). However, the main demerit of anaerobic treatment of SO_4^{2-} rich wastewater is the production of sulfides. Various physico-chemical and biological techniques such as stripping, precipitation, chemical oxidation or biological oxidation were adopted for treating sulfide from effluent (Buisman and Lettinga, 1990; De Smul and Verstraete, 1999; Jensen and Webb, 1995). Amongst these methods, biological oxidation of sulfide into elemental sulfur is reported as a cost effective alternative when compared with physico-chemical techniques (Lens et al., 2000). Reduction of sulfide into elemental sulfur by direct introduction of air into 'anaerobic' bioreactor systems for sulfide removal during treatment of sulfate-rich wastewater has been investigated previously. By regulating the oxygen dosing, micro-aerobic conditions can be generated in anaerobic reactors to maintain an acceptable reducing environment for anaerobic microorganisms to degrade the organic matter (Fox and Venkatasubbiah, 1996; Khanal and Huang, 2003; Zitomer and Shrout, 2000). The process of sulfate-to-sulfide reduction by SRB and sulfide-to-S^0oxidation by sulfate oxidizing bacteria (SOB) occurring in the same reactor is of practical interest (van der Zee et al., 2007). The SRB and SOB activities can be effectively regulated by controlling the level of dissolved oxygen (Okabe et al., 1995). Various studies have concluded that combination of anaerobic sulfate reduction and aerobic sulfide oxidation system had potential for treating sulfate rich wastewater (Chuang et al., 2005; Fox and Venkatasubbiah, 1996; Khanal and Huang, 2003). However, integrating SRB and

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SOB in a single reactor is difficult due to low S^0 conversion (Xu et al., 2013). Under oxygen-limited conditions, S^0 is the major end product of the sulfide oxidation, whereas under fully oxygenated condition, sulfide will be completely oxidized to SO_4^{2-} (Cirne et al., 2008). Elemental sulfur production is favorable because it is neither inhibitory nor highly soluble, forming a solid precipitate that may produce dense sludge that settles well (Guiot et al., 1997). The produced sulfur can be separated from the liquid stream and reuse as fertilizer or as raw material for sulfuric acid production (Lens et al., 2002).

The main objective of this study is to develop an anaerobic/micro-aerobic bioreactor system for sulfate-rich wastewater treatment. In this study, mixed microbial culture was collected from a wastewater treatment plant, acclimatized in presence of sulfate and a series of batch experiments were performed to assess the effects of various parameters on sulfate reduction. A packed bed reactor (PBR) was setup for sulfate reduction studies in anaerobic condition, whereas an upflow reactor system was fabricated to operate in microaerobic condition. The use of toxic compound such as phenol as co-substrate and sole carbon source for sulfate reduction was also studied in the PBR. The results indicated that simultaneous removal of phenol and sulfate could take place in the PBR. This is the first detailed study on effects of phenol as well as phenol as sole source of carbon during sulfate reduction. The utilization of phenol in sulfate reduction would serve the dual benefit of removing both phenol and sulfate along with reducing the cost of providing another carbon source. Bacterial isolation and identification studies were also carried out to isolate and identify the predominant species present in the PBR. Finally, experiments were conducted to optimize the dissolved oxygen level, air flow rate, and hydraulic retention time (HRT) in the microaerobic reactor with the aim of obtaining maximum elemental sulfur formation at lowest HRT and air flow rate.

Organization of the Thesis

The thesis has been organized into five chapters. The current **Chapter 1** presents the general introduction to the present work while the literature that supports the present study is presented in **Chapter 2.** Details of the materials and methods adopted in the present study along with the reactor configurations and operating conditions are

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discussed in details in **Chapter 3. Chapter 4** presents the results and discussions of sequential studies carried out on batch reactors, PBR, and the microaerobic reactor. The key conclusions drawn from these studies and discussion on the future scope of work are presented in **Chapter 5**.

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Chapter 2

LITERATURE REVIEW AND SCOPE OF THE STUDY

2.1 Sulfate

Sulfate is one of the most abundant anions found in the environment. It is a common constituent of many natural water and wastewater, and present, sometimes, in high concentrations. Wastewater generated from various industrial activities such as pulp and paper industries, mining and mineral processing, production of explosives, scrubbing of flue gases, petrochemical industries, galvanic processes, battery, paint and chemical manufacturing, food processing (molasses, seafood, edible oil), and pharmaceutical industries. (Lens et al., 1998) are the main source of most anthropogenic emissions. Other industrial activities involved in the production of fertilizers, dyes, glass, soaps, textiles, fungicides and leather also release high sulfate bearing effluents (Masigol et al., 2012). Certain industrial effluents may contain large concentration of sulfate (Table 2.1) while domestic sewage contains typically less than 500 mg/L.

2.2 Effects of sulfate

Sulfate becomes a pollutant if it is released in excess quantity, leading to various environmental hazards and impacts upon its discharge into the natural environment. It is only mildly hazardous in comparison with other toxic metals and for this reason many countries have not set any guidelines for sulfate in drinking water. However, at concentrations above 600 mg/L, the taste of water gets affected and can have laxative effects (Silva et al., 2012). High sulfate levels in tailings (piles or dumps) from coal and some metal-bearing ores (especially those rich in pyrite and chalcopyrite) are readily oxidized by water and oxygen, resulting in acid drainage creating several problems in coal and ore producing countries (Masigol et al., 2012). Excessive quantities of released sulfate can lead to pollution of the surface and ground water supplies posing health threat to various life forms and therefore it needs to be treated before being discharged in order to maintain its level within the permissible limits (Moon et al., 2013). In the aquatic environment, the natural sulfur cycle would be

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altered due to release of excessive sulfate and sulfide formed due to sulfate reduction. Sulfate ions also leads to increase in the conductivity and corrosion potential of receptor water bodies as they are one of the main contributors of mineralization of water (Silva et al., 2010). These anions promote the corrosion and scaling in pipes, structures and equipment; fouling and deposition in boilers; and acidification of soils and blockage of soil pores, retarding irrigation or water drainage (Bowell, 2000).

Table 2.1 Industries producing sulfate rich wastewater

Wastewater source	Sulfate (mg/L)	Reference
Mining	20,800	Bai et al. (2013)
Tannery industry	2500-3000	Galiana-Aleixandre et al. (2011)
Chemical industry	180000-284000	Sarti and Zaiat (2011)
Molasses fermentation	7616	Zhang et al (2009)
Drug industry	500-600	Rao et al. (2007)
TNT (trinitrotoluene) manufacturing process	5400	Lens et al. (1998)
Electroplating industry	2000	Song et al. (1998)
Galvanic industry	200 – 50000	Tichy et al. (1998)
Mining industry	100 – 20000	Banks et al. (1997)
Citric acid	2500-4500	Colleran et al. (1995)
Flue gas scrubbing	1000 – 2000	Dijkman (1995)
Alcohol production	2900	Lens et al. (1995)
Sea food processing	2100-2700	Mendez et al. (1995)
	600	
Textile industry	2690	Kabdasli et al. (1995)
Pulp & paper industry	200-700	Habets and de Vegt (1991)
	1200-1500	

Though, sulfate is non-toxic, however the reduced form hydrogen sulfide is toxic and corrosive in nature. Sulfate itself does not pose a threat to the environment as sulfate is a chemically inert, non-volatile and non-toxic compound (Shin et al., 1995). However, the anaerobic reduction of sulfate to sulfide by SRB may have undesirable and/or detrimental effects. In the absence of dissolved oxygen and nitrate, sulfate is converted to sulfide (H_2S) by acting as a source of oxygen or electron acceptor. The release of H_2S creates odor and corrosion problems (Sawyer CN et al., 2009) of the

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sewer pipes in the long run thus hampering the functioning of the sewage systems. In addition, H_2S is fatally toxic to humans, causing death within 30 minutes at gaseous concentrations of only 800 - 1000 mg/L, and instant death at higher concentrations (Speece, 1983).

2.3 **Permissible limit of sulfate**

The upper concentration limit of sulfate in water intended for human consumption is recommended at 250 mg/L (U.S.EPA, 1992; WHO, 1996) whereas the general standards for discharge effluents is limited upto 1000 mg/L (MoEF, 1986). The BIS 10500 (2012) states that maximum concentration of sulfate in drinking water should not exceed 200 mg/L. Environmental agencies in many countries have set maximum values varying between 250 and 500 mg/L in both mine drainages and industrial effluents (Silva et al., 2012).

2.4 **Sulfate removal technologies**

Normally sulfate containing wastewater can be treated using physicochemical and biological methods. However, biological treatment is preferred due to the overlying limitations of separation and appropriate disposal of the solid phase, relatively high cost and energy consumption involved in physicochemical methods (Silva et al., 2002). In the biological treatment, sulfate rich wastewater is treated under anaerobic conditions (Dries et al., 1998; Percheron et al., 1997).

2.4.1 Chemical precipitation

The removal of sulfate by precipitation may be carried with the use of gypsum (lime or limestone), barium sulfate or ettringite $[Ca_6Al_2(SO_4)_3(OH)_{12}\cdot 26H_2O]$. Partial sulfate ions removal was accomplished through precipitation with lime (Rubio et al., 2009). However, this process has a very low practical efficiency as the produced $CaSO_4$ is high soluble. Similarly, even though barium precipitation is highly efficient the loophole lies in the high cost of barium compounds and the toxicity concern of any residual Ba^{2+} ions that may be present in solution. Ettringite precipitation can also reduce sulfate concentrations to within regulatory limits without utilizing any toxic element like barium, but an alkaline pH is required for effective sulfate removal. The

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main drawback of all precipitation processes is also the generation of the huge volume of sludge.

2.4.2 Membrane processes

Membrane technologies such as reverse osmosis and electrodialysis are also applied to treatment of sulfate rich waters with the predominance of the former (Silva et al., 2012). However, both processes require pre-treatment to prevent fouling and microbial growth. In addition to this, the cost of treating huge amount of water is not economically viable.

2.4.3 Biological sulfate removal

Biological sulfate removal is a cost effective alternative for the expensive and sometimes complex physicochemical removal methods for the treatment of low organic strength and high sulfate rich wastewater (Maree et al., 1991). This treatment process generally comprises of two steps starting with the biological sulfate reduction to sulfide in the first step. In the second step, the sulfide produced is then oxidized to elemental sulfur (S^0).

2.5 **Biological sulfate reduction**

The biological sulfate reduction process is mediated in dissolved oxygen deficient environment by a group of microorganisms known as sulfate reducing bacteria (SRB). Though many microbes generate H_2S metabolically, sulfate often being the primary source of that H_2S, the process is normally a small-scale one involving the incorporation of sulfur into cell protein and its subsequent degradation by catabolic and autolytic processes (Postgate, 1965).

Under anaerobic conditions, heterotrophic SRB use sulfate as the terminal electron acceptor for the degradation of electron donors like various organic compounds and hydrogen (Elferink et al., 1994). In the absence of dissolved oxygen and nitrate, sulfate is converted to sulfide by acting as a source of oxygen or electron acceptor as shown in Eq. 2.1.

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$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$ 2.1

$\Delta G° = + 166.79$ kJ/mol

Sulfate after getting activated to adenosine-phosphosulfate (APS), is reduced to sulfite, which is reduced to sulfide as the final end product, in the above mentioned process. Sulfate transport in SRB has been proposed to be driven by a proton symport, which follows chemi-osmotic principles of transport. However, sulfide moves across membranes by diffusion and not by an active transport process (Cervantes J.Francisco. et al., 2006). Once within the cytoplasm, the sulfate is reduced to sulfide in a series of reactions driven by various enzymes. The reduction of sulfate to sulfide in dissimilatory sulfate reduction is mediated by three enzymes which occur within the cell cytoplasm (Hansen, 1994).The pathway as shown in Figure 2.1 is comprised of the following four steps catalyzed by membrane bound enzymes (Brunner and Bernasconi, 2005):

Step 1: Transfer of sulfate inside the bacterial cell

Step 2: Activation of internal sulfate to adenosine 5′ phosphosulfate (APS) with adenosine triphosphate (ATP) mediated by enzyme ATP sulfurylase.

Step 3: Reduction of APS to sulfite by APS reductase

Step 4: Finally reduction of sulfite to sulfide by sulfite reductase

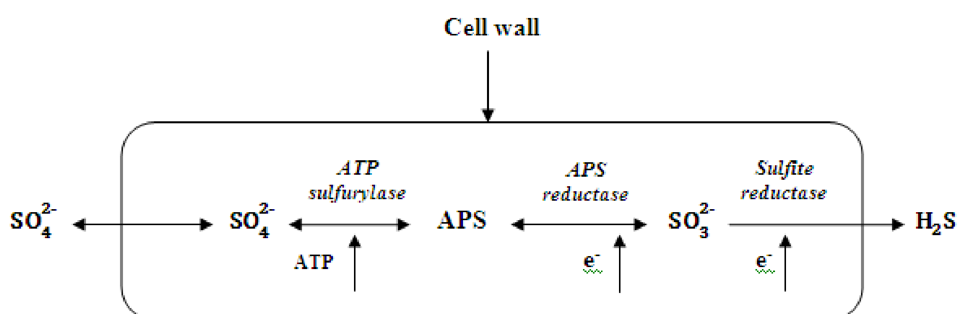

Figure 2.1 Pathway for dissimilatory sulfate reduction (Shen and Buick, 2004)

The similarity between assimilatory and dissimilatory sulfate reductions lies in presence of enzyme ATP sulfurylase leading to formation of APS which is mediated by APS reductase to reduce to sulfite. The main difference lies in the reversible transition from external sulfate to sulfite in dissimilatory sulfate reduction as compared to assimilatory sulfate reduction. Sulfite has been conceived to be an

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intermediate of sulfate reduction in sulfate reducing bacteria like *Desulfovibrio sp*, as well as in other microorganisms and higher plants (Ishimoto and Yagi, 1961). Till date two mechanisms have been proposed to describe the reduction of sulfite to sulfide: (i) by direct reduction of sulfite to sulfide without the formation of any intermediate compound, and (ii) through the formation of trithionate and thiosulfate. In case of the first mechanism, the direct reduction of sulfite to sulfide takes place with the reduction of six electrons, leading to the formation of sulfide through a single step only and is catalyzed by sulfite reductase enzyme (Fukui and Takii, 1994). In the second mechanism, reduction of sulfite to sulfide through the trithionate pathway takes place through two steps as shown in Figure 2.2. The first step involves reduction of sulfite to trithionate catalyzed by trithionate reductase with the reduction of 2 electrons (Kobayashi et al., 1974; Widdel et al., 1992). In the second step, trithionate is converted to thiosulfate with the reduction of two electrons in the presence of enzyme thiosulfate reductase (Kim and Akagi, 1985; Kobayashi et al., 1972; Kobayashi et al., 1974).

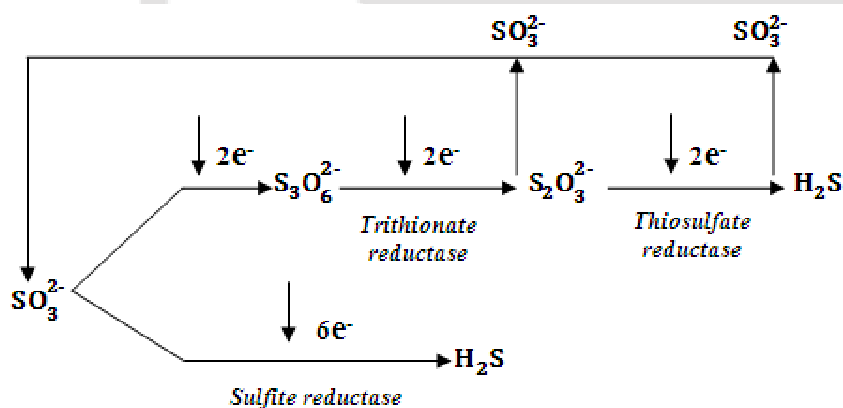

Figure 2.2 The proposed sulfite reduction pathways (Shen and Buick, 2004)

Trudinger and Loughlin (1981) reported that trithionate and thiosulfate formation can take place by chemical reactions in the culture medium with high sulfite concentrations or abiotically due to reaction of sulfite and sulfide (Widdel et al., 1992). They have also reported that neither trithionate nor thiosulfate is obligatory intermediates in the sulfite reduction pathway. However, formation of thiosulfate (Fitz and Cypionka, 1990) and trithionate (Kobayashi et al., 1969) as intermediates in the reduction of sulfite by *Desulfovibrio vulgaris* has been reported. Findley and Akagi

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(1970) have even provided evidence about the generation of both sulfur atoms of thiosulfate from sulfite, reduction of the outer sulfur atom to sulfide, and regeneration of the inner sulfur atom back to sulfite during thiosulfate reduction. Trithionate and thiosulfate formation as intermediates with whole cells of sulfate reducing bacterium *Desulfovibrio desulfuricans* supports the trithionate pathway of sulfite reduction (Fitz and Cypionka, 1990). Thus, the trithionate pathway of sulfite reduction may be a fully functional biochemical process (Shen and Buick, 2004).

2.6 **Bacterial community structure:**

Dissimilatory sulfate reduction process which utilize sulfate ions as electron acceptors for anaerobic respiration is mediated by sulfate reducing bacteria and Archaea (Widdel, 1988). The SRB can be grouped into seven phylogenetic lineages, five within the Bacteria and two within the Archaea based on comparative analysis of 16S rRNA sequences (Muyzer and Stams, 2008). Maximum sulfate-reducers are found within the Deltaproteobacteria (~ 23 genera) which includes the typical sulfate reducer *Desulfovibrio*, followed by the Clostridia (*Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa* genera) which are low G+C gram-positive SRB (Shen and Buick, 2004). Only thermophilic SRB occur within Nitrospirae (*Thermodesulfovibrio* genus), Thermodesulfobacteria (*Thermodesulfobacterium* genus) and Thermodesulfobiaceae (*Thermodesulfobium* genus). On the other hand, within the Archaea, SRB is divided into the Euryarchaeota (*Archaeoglobus* genus) and the Crenarchaeota (*Thermocladium* and *Caldirvirga* genera).

SRB are capable of utilizing sulfate as an electron acceptor for growth and convert it to sulfide. Sulfate reducers can be divided into two metabolic groups on the basis of their substrate utilization. The first group comprised of those species that are capable of complete oxidation of substrates to carbon dioxide while the second group includes those that oxidize their substrate to acetate and not completely to carbon dioxide Representatives of the genera *Desulfomonas, Desulfococcus, Desulfobacter, Desulfosarcina*, *Desulfotomaculum, Desulfonema, Desulfoarculus, Desulfoacinum, Desulforhabdus, Desulfomonile,as well as Desulfotomaculum acetoxidans, Desulfotomaculum sapomandens* and *Desulfovibrio baarsii*, are capable of degrading organic compounds (e. g. acetate) completely (Madigan et al., 2009; Postgate, 1984;

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Tang et al., 2009; Widdel, 1988). *Desulfobulbus* and *Desulfovibrio ,Desulfomicrobium, Desulfobotulus, Desulfofustis, Desulfotomaculum, Desulfomonile, Desulfobacula, Archaeoglobus, Desulfobulbus, Desulforhopalus* and *Thermodesulfobacterium* are some of the SRB which are not complete oxidizers (Madigan et al., 2009; Tang et al., 2009). SRB have the ability to utilize a broad range of electron donors, including lactate, propionate, acetate, and hydrogen (Widdel et al., 1992). Lactate can be consumed both by complete oxidizers as well as incomplete oxidizers, while hydrogen can be utilized more by incomplete oxidizers and very less by most complete oxidizers.

2.7 **Factors influencing sulfate reduction**

2.7.1 pH

Sheoran et al. (2010) reported that SRB has two threshold inhibition levels, one for the undissociated H_2S and the other for the total sulfide. The state of sulfide solely depends on the pH of the environment as shown in Figure 2.3. Most of the SRB are reported to be neutrophilic (Widdel, 1988) and prefer an environment having pH between 7.5-8. However, various acid tolerant species have also been seen to thrive for sulfate reduction at pH value as low as 3.8 (Kimura et al., 2006), while some species have been found to be alkaliphilic and the highest pH seen to support the growth of SRB has been reported to be 10 (Pikuta et al., 2003). Below pH of 5, activities of SRB reduce considerably whereas at neutral pH their activity is enhanced. The inactivity of SRB at low pH is mainly attributed to the acidification of the cytoplasm which inhibits the formation of a proton motive force. At a pH less than 7.2, undissociated H_2S is dominant and it will reach the threshold limit while at a pH above 7.2, the total sulfide is responsible for the inhibitory effect (Perry et al., 1984). At pH of 8.5, the HS^- further dissociates into the sulfide dianion (S^{2-}) form and becomes the predominant sole species at pH value above 10 (Tang et al., 2009; Visser, 1995).The SRB are less sensitive to total sulfide when the pH is increased from 6.8 to 8.0 and more sensitive to the undissociated sulfide concentration.

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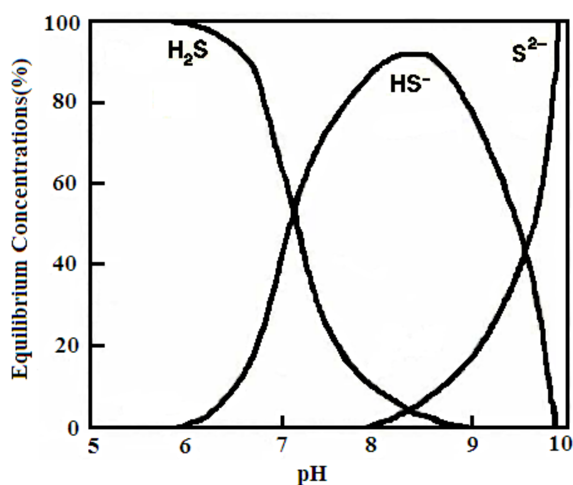
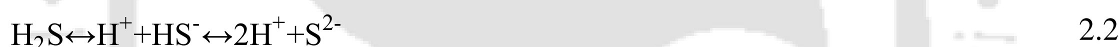


Figure 2.3 Prevalent forms of sulfide at different pH values (Lens et al., 1998)

At low pH, the produced hydrogen sulfide exists in undissociated form and as the pH increases it dissociates into HS^- and S^{2-}. The dissociation process is given by reaction presented in Eq. 2.2.



$$H_2S \leftrightarrow H^+ + HS^- \leftrightarrow 2H^+ + S^{2-} \quad 2.2$$

Most sulfidogenic bioreactors have been operated around neutral pH. In order to survive, SRB require a pH in the range of 5–8 (Willow and Cohen, 2003) and outside this range the rate of microbial sulfate reduction generally declines. Low pH (<5) normally inhibits sulfate reduction and increases the solubility of metal sulfides (Dvorak et al., 1992). Below pH of 4, bioreactors have been less successful; however, Elliott et al. (1998) reported the presence of SRB activity at a pH of 3.0 in an anaerobic upflow bioreactor. Kolmert and Johnson (2001) reported the growth of a mixed acidophilic SRB culture in a medium with a pH of 3.0 thus supporting the view by Postgate (1984) that mixed SRB cultures are more tolerant to extreme conditions than pure culture. Sulfate reduction has also been reported to occur at a pH of 10 (Pikuta et al., 2003), however significant reduction rates have only been shown until a pH of 8.0 wherein a volumetric activity of 25 SO_4^{2-} g/L/d was reported (van Houten et al., 1995).

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2.7.2 Electron donors/carbon source

Lens et al. (1998) reported the diversity of SRB in their carbon source utilization and the metabolic activities. The carbon and energy source provides the energy for the growth and maintenance of SRB based on the reaction given Eq. 2.1. The electrons required for the sulfate reduction are generated by the oxidation of a carbon source (e.g, lactate, acetate, and propionate). A schematic representation of the sulfate reduction metabolism coupled with the utilization of carbon source is shown in Figure 2.4.

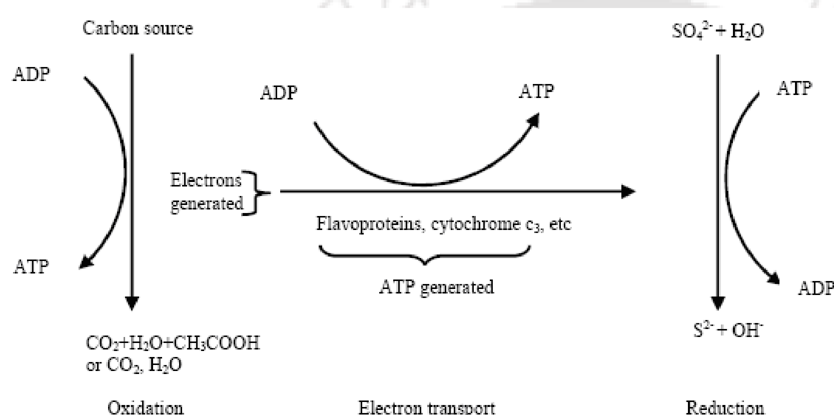

Figure 2.4 Schematic representation of sulfate reduction coupled to utilization of an organic compound (Postgate, 1984).

The ATP produced, using the energy released from oxidation of the organic carbon is utilized for the reduction of sulfate to sulfide. In most instances, the electron donor and the carbon source are the same compound. Only when hydrogen is used as the electron donor, CO_2 is used as the carbon source. A minimum COD/SO_4^{2-} mole ratio of 0.67 is required for achieving theoretically possible removal of sulfate (Choi and Rim, 1991). Various organic compounds including such as sewage sludge, leaf mulch, molasses animal and manure have been used as carbon sources in addition to the low molecular weight organic compounds. Some of the commonly used electron donors are hydrogen, formate, methanol, ethanol, molasses, lactate, acetate, propionate, butyrate, sugar, hydrocarbons and organic waste (Liamleam and Annachhatre, 2007). The advantages and disadvantages of different carbon sources are shown in Table 2.2.

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Table 2.2 Electron donors and carbon sources for SRB (Liamleam and Annachhatre, 2007)

Carbon Source	Advantage	Disadvantage
Hydrogen	More efficient utilization by SRB than methanogenesis.	Only a few anaerobes can grow with hydrogen as sole energy source
Acetate	-	SRB cannot completely oxidize acetate even with excess sulfate levels
Methanol	Readily available and cost effective	Low growth rate of SRB
Ethanol	SO_4^{2-} conversion efficiency	Slow growth rate of SRB, produces acetate.
Molasses	Low cost , ready availability	Accumulation of non-biodegradable content reduces biomass activity and COD removal. High VFA generation
Lactate	Most SRB can utilize it	Complete lactate oxidation not achieved by some SRB species
Sugar	Easily degraded under anaerobic conditions.	Costly
Hydrocarbons	-	Free energy change low as estimated for the methane oxidation
Organic waste	Cost effective.	Very less utilization rate

2.7.3 COD/SO_4^{2-} ratio

COD/SO_4^{2-} ratio appears to be a key factor in the regulation of sulfate reduction as it determines the competition between SRB and methanogens for monomeric (e.g sugar, amino acids) and H_2 or acetate compounds (Sarti et al., 2009). In addition, COD/SO_4^{2-} also determines the electron flow during sulfate reduction and methanogenesis. It has been reported theoretically that conversion of 1 mol of sulfate requires 0.67 mol of COD or electron donor (Choi and Rim, 1991; Omil et al., 1998). The amount of organic matter required by the biomass for sulfate reduction is not available in abundance when this ratio is decreased. Under such case, external source of organic matter, preferably carbon source/electron donor needs to be added. Conversely, the sulfate reduction is also hampered when this ratio is increased as the electron transport to the sulfate reducing bacteria decreases. In fact, when the ratio increases beyond a certain value, there is competition between methane formers and

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sulfate reducers for acetate. Choi & Rim (1991) indicated that sulfate reducers and methane formers are very competitive at a ratio of 1.7 to 2.7 and observed that methane producers dominate at ratio higher than 2.7 while sulfate reducers dominate at ratio lower than 1.7. Chou et al.,(2008) studied the competition reaction kinetics of SRB and MB at different COD/SO_4^{2-} ratios by finding out the values of mass fraction of SRB and MB i.e f(SRB) and f(MB), respectively. They found out that f(SRB) continued to be higher up to a COD/SO_4^{2-} of 1.3 indicating that SRB could outcompete MB for bacterial growth. However as the ratio was increased upto 2 and 3 the mass fraction of MB became more than mass fraction of SRB. Various studies carried out at different COD/SO_4^{2-} ratio along with the sulfate and organic matter removal efficiency is shown in Table 2.3.

Table 2.3 Sulfate and COD removal of different reactors at different COD/SO_4^{2-} ratio

Reactor type	**COD/SO_4^{2-}**	**COD removal efficiency**	**SO_4^{2-} removal efficiency**	**Reference**
SBBR	1-1.5	48-95	84-98	Archilha et al (2010)
ABR	6	>85	96.8	Vossoughi et al (2003)
UASB	0.67-1.5	100	94	Velasco et al (2008)
Batch	2.6	92.6	>92	Cao et al (2011)
EGSB	6	>65	>85	de Smul et al (1999)
FBR	1.17	87	91	Thabet et al (2009)
SBBR	3.67±0.19	32	99	Sarti and Zaiat (2011)
UASB	1	67.4	85.6	Rodriguez et al (2012)
UASB	6.67	-	>95	Sipma et al (1999)
CSTR	1.2	99±0.6	86±0.5	Oyekola et al (2010)
CSTR	2	N.A	99	Zhao et al.(2010)
Upflow hybrid reactor	1.3	>90	>90	Sabumon (2008)
Batch	0.7	>85	>90	
Batch	1.6	N.A	97.4	Wang et al (2008)
Continuous	2.7	N.A	>94.6	

Note: SBBR - Sequential batch biofilm reactor; UASB - Upflow anaerobic sludge blanket reactor; FBR - Fixed bed reactor; EGSB - Expanded granular sludge bed reactor; ABR - Anaerobic baffled reactor; CSTR - Continuous stirred tank reactor.

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2.7.4 Temperature

Sulfate reducers can grow over a wide range of temperature. Some can thrive at temperature as low as 5°C (Sahinkaya, 2009) while others have been reported to grow at temperature above 50°C (Lopes et al., 2007; Rosnes et al., 1991). Sulfate reducing bacteria can be classified into mesophiles (growth temperature <40°C), moderate thermophiles (growth temperature 40-60°C) and extreme thermophiles (>60°C). But most of the studies conducted so far in laboratory scale for sulfate reduction show that majority of the sulfate reducers such as *Desulfobacter hydrogenophilus, Desulfobacter curvatus, Desulfovibriolatus, Desulfovibrio vibrioformis and Desulfovibrio halotolerans* are mesophilic in nature. Arrhenius plot has been used in order to gain an insight of the adaptation of bacteria for sulfate reduction in low temperature regions like marshy areas, deep sea, and sediments.

Temperature also affects the bio-kinetic parameters involved in sulfate reduction. For instance, decay rate, k_d ($time^{-1}$), increases beyond a certain range of temperature. The temperature may cause the denaturation of proteins and enzymes, which are involved in sulfate reduction, thus preventing bacterial growth and leading to death phase. The decrease in the value of half saturation constant, Ks (mg/L), with temperature represents enhanced affinity of the bacterial enzymatic system for the substrate available for growth.

Ingvorsen et al (2003) investigated the effect of temperature on sulfate reduction on concentrated sludge and native sludge . They found out that the exponential phase was attained after 6 hours when temperature was 20°C as against 20 hours when temperature was 5°C. Studies by Pallud & Cappellen (2006) and Sawicka et al.(2012) on samples obtained from marshes and sediments show that the sulfate reduction rates increase with increase of temperature from 20°C to 30°C. The E_a and Q_{10} values found in the studies showed that the temperature range is optimum for sulfate reduction. de Smul et al (1999) found that the optimum sulfate reduction rate was maintained at a temperature of 33°C with the ESGB reactors fed with ethanol and ethylene glycol. In addition to that, they also observed the suppression of overall sulfidogenic activity in contrast to methanogenesis which became active once the temperature was increased to 55°C. Similar results were also found by Sulaiman Al-

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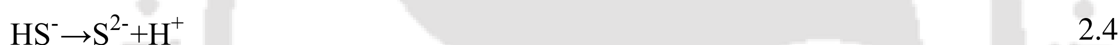
Zuhair (2008) and Moosa et al (2002) where fastest drop in sulfate concentration was observed at temperature of 35°C. Studies by Moosa et al (2005) on anaerobic sulfate reduction across a temperature of 20-35°C observed that the values of bio-kinetic parameters namely specific growth rate (μ_m) and yield (Y) did not change with temperature while K_s declined to a value of 0.949 from 0.016 kg dry weight/m^3 and k_d value increased from 0.008 to 0.038 h^{-1}.

2.7.5 Sulfide

The toxicity of sulfide is regarded to depend on pH because in the pH range of 6-8, sulfide exists as in the form of HS^- while at a pH lower than 6, un-dissociated hydrogen sulfide becomes the dominant sulfide species (Moosa and Harrison, 2006). The chemical reactions taking place for the sulfide species is governed by the equations given below:

$$H_2S \rightarrow HS^- + H^+ \qquad 2.3$$



$$HS^- \rightarrow S^{2-} + H^+ \qquad 2.4$$

The total hydrogen sulfide is found out as below:

$$H_2S_{total} = H_2S_{aq} + HS^- \qquad 2.5$$

Two theories have been postulated so far for sulfide inhibition. As per one of the theories, the undissociated sulfide molecule can pass through the cell membrane, making the cell inactive by destroying the bacterial proteins (Postgate, 1984; Speece, 1983) and interfering with the metabolic coenzymes by formation of sulfide bond (Parkin and Owen, 1986). The other theory states that due to the precipitation of heavy metals the sulfate reducing bacteria are deprived of the essential trace nutrients used as cofactors and hence their growth gets limited (Bharathi et al., 1990).This theory is applicable when there are heavy metals in the system. It was, however, seen that the sulfide toxicity is reversible and the normal cell growth and sulfate reduction rates are attained as soon as sulfide is removed from the system (Krishnanand and Parkin, 1996; Okabe et al., 1995).

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Table 2.4 summarizes the findings from various studies on the effects of sulfide on the sulfate reducing bacteria at different concentration levels.

Table 2.4 Toxicity levels of sulfide on Sulfate reducing bacteria

Organism	**Reactor**	**Sulfide inhibition level (mg/L)**		**Reference**
		Un-dissociated sulfide (mg/L)	**Dissolved sulfide (mg/L)**	
Mixed culture	CSTR	290	-	Moosa & Harrison (2006)
		-	1000	
Mixed culture	Continuous	-	1000	Icgen and Harrison (2006)
Wet granular sludge	UASB	-	100	Lopes et al (2010)
Desulfovibrio Desulfuricans	Batch culture	-	251	Okabe et al (1995)
	Continuous chemostat	-	250	
Mixed culture	Ethanol-lactate fed FBR	-	613.44	Nevatalo et al (2010)
Desulfovibrio Desulfuricans	Bacterial culture		34.08 (1mM)	Truong et al (2013)
Desulfovibrio Desulfuricans	Chemostat	-	212±23	Okabe et al (1992)
Granular Sludge	UASB	-	115	Lopes et al (2007)
SRB growing on lactate and sulfate	-	-	547	Reis et al (1992)
Mixed culture	Chemostat	-	150-200	Krishnanand and Parkin (1996)
AMD treatment sludge	Serum vials	302.6	1781 (27.4mmol/L)	O'Flaherty et al.,(1998)
Anaerobic hybrid reactor sludge	Packed up-flow hybrid reactor	258.4	2736.5(42.1mmol/L)	O'Flaherty & Colleran (1999)

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2.7.6 Heavy metals

The capacity of various heavy metals to react with the functional groups of enzymes and deactivating them in the process which results in toxic effects on microorganisms such as SRB. The heavy metals are even capable of substituting essential ions on cellular sites causing denaturation of proteins (Cabrera et al., 2006). The main criterion on which the removal ability of the SRB depends is the metal concentration in solution which may lead to decrease in metabolic activity of the bacteria or even death when the metal concentration is very high. The toxicity concentrations of heavy metals for SRB have been reported to range from a few mg/L to as high as 100 mg/L (Sani et al., 2001). Martins et al (2009) reported that the variation of the metal species also plays a very important role in imparting toxicity to the SRB. For instance, the less mobile arsenate As (V) is more toxic than arsenite As (III), while inorganic species are more toxic as compared to their methylated counterparts (Turpeinen et al., 1999). Various resistance mechanisms such as sequestration or transformation to other chemical species have been observed with different organisms in order to tolerate the toxic effect of the metal ions (Valls and Lorenzo, 2002).

Jong and Parry (2003) reported that sulfate reduction decreases with increasing initial concentrations of metals. High metal concentration results in increasing the metal toxicity leading to reduction in metabolic activity of SRB. The other effect of increasing metal concentration could be the partial blockage of the reactor's sand-bed leading to severe mass transfer limitations. The toxic concentrations of some heavy metals as reported by Hao et al.(1994) employing a mixed culture of SRB for studying the effects of heavy metals on sulfate reduction are Zn (25–40 mg/L), Pb (75–80 mg/L), Cu (4–20 mg/L), Cd (>4–20 mg/L), Ni (10–20 mg/L) and Cr (60 mg/L). The sulfate removal IC50 (concentration causing 50% inhibition of SRB sulfate removal efficiency) for Cu was reported to be 156 mg/L (Song et al., 1998) in contrast to 1.02 mg/L as reported by Sani et al. (2001) who used *D. desulfuricans* strain along with a specific metal toxicity medium containing constituents that did not result in any abiotic precipitation of metal ions. Table 2.5 summarizes the findings from various studies on the toxicity levels of various metals. This comparison shows that the chemical and physicochemical properties of the environment surrounding the SRB play an important role in determining the level of metal toxicity and inhibition in

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SRB (Jong and Parry, 2003). In the studies conducted by Martins et al.(2009),the inhibition of sulfate reducing ability of the SRB was significant in the presence of zinc concentration of 150 mg/L and copper concentration of 80 mg/L.

Table 2.5 Toxicity levels of various metals on the sulfate reducing bacteria

Metal	SRB	Toxic concentration (mg/L)	Reference
Zinc	Mixed culture	150	Martins et al (2009)
	Mixed culture	25-40	Hao et al.(1994)
	Desulfovibrio vulgaris	20	Cabrera et al (2006)
	Desulfomicrobium sp.	>125	Azabou et al.(2007)
Copper	Mixed culture	80	Martins et al (2009)
	Mixed culture	4-20	Hao et al.(1994)
	Desulfovibrio vulgaris and *Desulfovibrio* sp.	9	Cabrera et al (2006).
	Desulfovibrio desulfuricans G20	2	Sani et al. (2001)
	Desulfomicrobium sp.	>10	Azabou et al.(2007)
Lead	Mixed culture	75-80	Hao et al.(1994)
Iron	*Desulfomicrobium* sp.	>60	Azabou et al.(2007)
Chromium	Mixed culture	60	Hao et al.(1994)
	Desulfovibrio vulgaris and *Desulfovibrio* sp.	>15	Cabrera et al (2006)
Nickel	Mixed culture	10-20	Hao et al.(1994)
	Desulfovibrio vulgaris and *Desulfovibrio* sp.	>8.5	Cabrera et al (2006)
Cadmium	Mixed culture	4-20	Hao et al.(1994)
Manganese	*Desulfovibrio vulgaris* and *Desulfovibrio* sp.	>10	Cabrera et al (2006)

2.8 **Bio-reactors on sulfate rich wastewater treatment**

Different bioreactor configurations have been reported in literature for anaerobic reduction of sulfate. Some of the common bioreactor configurations include continuous stirred tank reactors (Herrera et al., 1997; Moosa et al., 2005; Moosa et al., 2002); membrane reactors (Chuichulcherm et al., 2001); packed bed reactors (Chang et al., 2000; Jong and Parry, 2003); and up-flow anaerobic sludge blanket reactors (Colleran et al., 1994; Sanchez et al., 1997). These bioreactors can be classified into two main groups based on the mixing regime of the influent (Figure 2.5). The bioreactors with completely mixed regime can be subdivided into CSTR and MBR

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based on the biomass retention characteristic of the reactor. Biomass retention is especially important in sulfidogenic bioreactors, which are characterized with low growth rate of anaerobic microorganisms, as biomass retention increases biomass concentration. In case of incompletely mixed or gradient type bioreactor, the bioreactor can be categorized into PBR and UASB based on the use or non-use of the carrier material, respectively. These reactors with gradient mixing regime are mainly used for soluble, low suspended solid wastes (Jhung and Choi, 1995). In case of these bioreactors, the activity of the bioreactor is determined by the activity of the biomass and the biomass concentration. Brief descriptions of these bioreactors are given in the subsequent sub-sections.

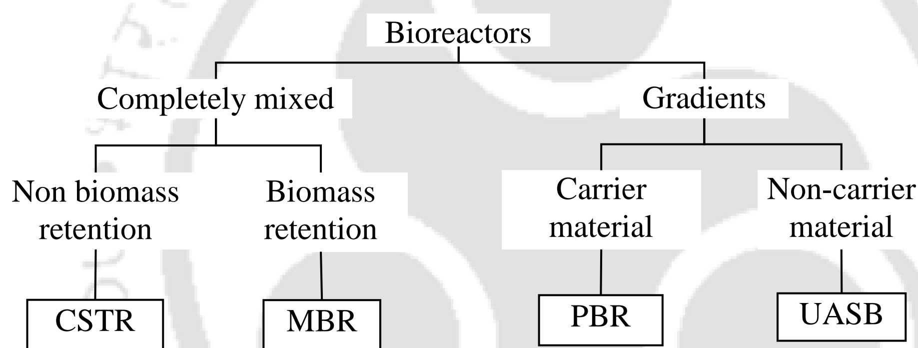

Figure 2.5 Reactors used for sulfate reduction (Kaksonen, 2004)

2.8.1 Continuous stirred tank reactors (CSTR)

Continuous stirred tank reactors use a mechanical stirrer to stir the liquor resulting in a completely mixed system. However, CSTR is subjected to washout of biomass (Speece, 1983). Biomass retention has been enhanced by employing sedimentation systems and cationic flocculants (White et al., 1995). The performance of CSTR in treating sulfate rich wastewater with different substrate and under varying volumetric loading rates has been investigated by various researchers (Table 2.6). Moosa et al. (2002) investigated the effects of initial sulfate concentration and its volumetric loading on the kinetics of reaction and activity of sulfate-reducing bacteria. The

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increase in initial concentration of sulfate in the range 1.0–10.0 kg/m^3 enhanced the reaction rate from 0.007–0.17 $kg/m^3/h$. In case of reactor with a feed sulfate concentration of 1.0 kg/m^3, the maximum bacterial concentration of 0.98 g/L was observed at a dilution rate of 0.005 h^{-1}. The maximum reduction rate in this set of experiments was 0.007 $kg/m^3/h$, achieved at a retention time of 3.75 days. Moosa et al.(2005) studied the effects of temperature on the kinetics of anaerobic sulfate reduction in continuous bioreactors using acetate as an electron donor. Linear increase in reduction rate of sulfate was observed when the temperature was varied between the range of 20 to 35 °C, and volumetric loading rate was increased from 0.08 to 0.1 $kg/m^3/h$. The increasing reaction rate showed a lower dependence on volumetric loading rate in the range 0.1 - 0.15 $kg/m^3/h$. Further increase in volumetric loading rate above 0.15 $kg/m^3/h$ was accompanied by wash out of bacterial cells and a sharp decrease in reaction rate. Despite a similar pattern for dependency of reaction rate on volumetric loading at all temperatures tested, the magnitude of reaction rate was influenced by temperature, with a maximum rate of 0.075 $kg/m^3/h$ observed at 35 °C.

2.8.2 Membrane reactors (MBR)

Membrane reactors are combination of a membrane process with a suspended growth reactor and are relatively new in the field of sulfate reduction. The advantage of this configuration is that almost complete biomass retention can be obtained which is especially useful in slow growing processes (Bijmans, 2008). Membrane bioreactors commonly adopt a biomass retention system relying on the difference in density between the sludge and the reactor liquor, resulting in settling or floatation of the sludge. Vallero et al. (2005) investigated the sulfate reducing potential of anaerobic membrane reactor in salt rich wastewater using a 6L submerged anaerobic membrane bioreactor (SAMBaR) inoculated solely with *Desulfobacter halotolerans* (Table 2.6). The SAMBaR was fed with acetate and ethanol at organic loading rates up to 14 g COD/L/day in excess of sulfate (COD/SO_4^{2-} of 0.5) and operated at pH 7.2±0.2 and a HRT from 8 to 36 h. A sulfate reduction rate up to 6.6g SO_4^{2-}/L/d. was achieved in the

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SAMBaR at a HRT of 9 h including the backflow of permeate used for back flushing. The constant high specific sulfate reduction rate of 5.5 g SO_4^{2-}/g VSS/day showed that the low amount of biomass (0.85 g VSS/L) present in the reactor at the end of the experiment limited the performance of the SAMBaR. Mizuno et al.(1998) investigated the biological sulfate removal in the acidogenic bioreactor with an ultra-filtration membrane system at 35ºC using sucrose as the sole organic substrate. The efficiency of sulfate removal by sulfate reduction reached about 100% in the membrane bioreactor, and 55 to 87% of sulfide was removed from the permeate by membrane filtration. When the sulfate concentration is increased, acetate and 2-proponol levels also increases significantly while *n*-butyrate and 3-pentanol levels show a downward trend. This could be attributed to the role played by the sulfate-reducing bacteria as an acetogenic bacterium consuming volatile fatty acids and alcohols as electron donors under sulfate-rich conditions. This shows that the acidogenesis and sulfate reduction proceed simultaneously in the membrane bioreactor.

2.8.3 Upflow anaerobic sludge blanket reactor (UASB)

The UASB reactor was developed for methane production from highly concentrated organic wastewater (Hulshoff Pol et al., 1998). It is a robust system in which the produced methane gas provides the mixing of the reactor liquor. However, in sulfate reducing reactors mixing depends solely on the upflow of the waste stream, since the gases produced during sulfate reduction stay mainly in solution (Bijmans, 2008). Lens et al. (2001) investigated the effect of the superficial liquid upflow velocity on the acidifying and sulfate reducing capacity of thermophilic (55°C; pH 6.0) granular sludge bed reactors treating partly acidified wastewater. Synthetic wastewater containing starch, sucrose, lactate, propionate and acetate and a low sulfate concentration (COD/SO_4^{2-}) ratio of 10 was fed at a HRT of about 5 h and volumetric organic loading rates (OLR) ranging from 4.9 to 40.0 g COD/L/day. At the end of the

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experiment, the sulfate level of the influent was slightly increased to a COD/SO_4^{2-} ratio of 8. When imposing an OLR of 40.0 g COD/L/day, the acidification efficiency dropped to 80% and the sulfate reduction efficiency decreased to 50% in the UASB reactor producing acetate and propionate. At the higher organic loading rates, propionate was converted to n-butyrate and n-valerate. The effluent sulfide concentration was always below 200 mg/L, of which about 90% was present as undissociated H_2S (under the given conditions--pH 5.8-6.1 and 55°C).

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Table 2.6 Performance of UASB, CSTR and MBR used for treating sulfate rich wastewater

Bacterial group	Substrate	Reactor Type	Temp. (°C)	Feed pH	Volumetric reduction rate (g/L/h)	Reference
Mixed SRB	acetate	CSTR	35	8±0.2	0.076	Moosa et al. (2005)
Mixed SRB	acetate	CSTR	35	8±0.2	0.184	Moosa et al. (2002)
Aeration tank sludge	sucrose	CSTR	35	6.4-7	-	Chen and Lin 2003
Activated sludge	molasses	CSTR	30	4.5-5.5	-	Ren et al. (1997)
Desulfobacter halotolerans	acetate, ethanol	MBR	33±1	7.2±0.2	0.276	Vallero et al. (2005)
Sulfate reducing bacteria	sucrose	MBR	35	6-6.5	-	Mizuno et al. (1998)
Granular methanogenic sludge	acetate	UASB	32±1	8.3	0.584	Muthumbi et al. (2001)
Mixed culture	methanol	UASB	30		0.016	Weijima et al. (2003)
Mixed sludge	Starch, sucrose, lactate, acetate, propionate	UASB	55	6	-	Lens et al. (2001)
Mixed sludge	Sucrose, propionate, butyrate	UASB	55	6	-	Sipma et al. (1999)

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2.8.4 Packed bed reactors (PBR)

In this reactor, a carrier material is used to obtain well settable biomass by biofilm formation on the carrier material in contrast to granulation in a UASB. The carrier material provides a large surface area for bio-film formation (Speece, 1983). The use of different packed-bed reactors with various combinations of carrier material, carbon source and bacterial group is reported in literature for treating sulfate rich wastewater (Table 2.7). Chen et al. (1994) used a packed-bed bioreactor using sea sand as carrier matrix to study the kinetics and stoichiometry of sulfide formation. In this reactor, lactate was used as a carbon source and the SRB species *Desulfovibrio desulfuricans* as inoculum. At the volumetric loading rate of 0.138 g/L/h, the maximum volumetric reduction rate achieved was 0.015 g/L/h. Waybrant et al.(2002) investigated the effect of packing reactive mixtures which were basically waste products. Two up-flow packed-bed bioreactors containing two different reactive mixtures were used: first reactor containing leaf mulch, sawdust, sewage sludge, and wood chips and the second reactor containing leaf mulch and sawdust. The maximum volumetric reduction rates achieved in the first and second columns were 0.003 and 0.005 g/L/h, respectively. Elliott et al.(1998) conducted experiments in a packed-bed bioreactor to investigate the effect of pH on the anaerobic sulfate reduction. The column was packed with sand and the pore volume was 783 ml. In this study, Postgate Medium B without iron sulfate was pumped through the column at a rate of 0.6 ml/min. This bioreactor was operated at a given pH until a steady state was achieved. After attaining the steady state, the pH of the feed was lowered step by step. The pH of the feed was initially adjusted to 4.5 and then it was decreased to 4.0, 3.5, 3.25 and 3.0 under continuous flow conditions. The bioreactor removed 45.1%, 44.6%, 35.5%, 38.3% and 14.4% of initial sulfate at pH 4.5, 4.0, 3.5, 3.25 and 3.0, respectively. Chang et al.(2000) demonstrated that solid waste materials including oak chips (OC), spent oak from shiitake mushroom farms (SOS), spent mushroom compost (SMC), sludge from a wastepaper recycling plant (SWP) can be used as electron donors and immobilization matrices to treat ARD. The bioreactors were inoculated with an

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anaerobic digester fluid. The feed sulfate concentration was 2.58 g/L and total dissolved metal concentrations were 500 mg/L iron, 100 mg/L zinc, 50 mg/L manganese and 50 mg/L copper. Experiments were conducted with temperature maintained at 25°C and the pH of the medium adjusted to 6.8. At a volumetric loading rate of 0.005 g/L/h, the highest volumetric reduction rate of 0.005 g/L/h was achieved in the bioreactor packed with sludge from wastepaper recycling plant. Kolmert and Johnson (2001) investigated the tolerance of mixed SRB culture to acidic environment in an up-flow packed-bed bioreactor, using porous glass beads as a carrier matrix. The average volumetric reduction rates of 0.010–0.013 g/L/day were achieved in bioreactors containing mixed culture of acidophilic and neutrophilic SRB with a feed pH of 4.0. Kolmert and Johnson (2001) reported that sulfate reduction occurred at a pH of 3.0 but with a lower rate. Jong and Parry (2003) used an up-flow packed-bed bioreactor with sand as carrier matrix for anaerobic reduction of sulfate with mixed culture of SRB. Feed contained 2.5 g/L sulfate and 10 mg/L of each Al, As, Cu, Zn, Ni and Fe metals. The highest volumetric reduction rate of 0.019 g/L/h was observed at a volumetric loading rate of 0.155 g/L/h at 25°C. Foucher et al.(2001) successfully used CO_2 and H_2 as carbon and energy source to treat Chessy mine drainage in an upflow packed-bed bioreactor with a special packing to provide good mass transfer between hydrogen and liquid. The pH of the feed was 2.55 and the sulfate concentration was 5.8 g/L and metals like Fe^{2+}, Fe^{3+}, Zn, Cu, Al, Mn, Co, Ni and Pb were present in concentrations of 1470, 70, 320, 160, 210, 5.5, 0.06, 0.4 and 0.5 mg/L, respectively. Although the feed sulfate concentration was 5.8 g/L, a part of the effluent stream was recycled and the concentration of sulfate in the inlet stream was reduced to 0.6–0.8 g/L. The maximum flow rate employed was 900 ml/h (residence time of 0.9 days), and the corresponding volumetric reduction rate achieved was 0.2 g/L/h. Lin and Lee (2001) studied anaerobic sulfate reduction in a fixed bed bio-film column bioreactor. The Plastic Ballast rings were chosen as the supporting media for bio-film formation. The feed sulfate concentration was 0.9 g/L. The reactor volume was 42.65 L, which yields a HRT of 2.5 days. The reactor temperature was controlled at 35°C. The conversion achieved was 98%.

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Table 2.7 Performance of continuous packed bed reactors used for treating sulfate rich wastewater

Bacterial group	Reactor type	Carrier matrix	Temp. (°C)	Feed pH	Carbon source	HRT (h)	Volumetric reduction rate(g/L/h)	Reference
Mixed SRB	PBR	Sand	22	7	lactate	4.01	0.228	Baskaran and Nemati (2006)
Mixed SRB	PBR	Pool filter silica sand	25	4.5	lactate	16.16	0.019	Jong and Parry (2003)
Mixed SRB	PBR	Special packing	25	2.55	H_2&CO_2 + sodium acetate	21.6	0.20	Foucher et al. (2001)
Mixed acidophilic or neutrophilic SRB	PBR	Porous glass beads	-	4	ethanol+ lactic acid+ glycerol	49.3	0.022	Kolmert and Johnson (2001)
Anaerobic digester fluid	PBR	Sludge from wastepaper recycling plant(SWP)	25	6.8	SWP	480	0.005	Chang et al. (2000)

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In continuous bioreactors (bioreactors with continuous mode of operation), application of freely suspended cells require a high residence time to prevent cell washout. In other words, a continuous reactor with freely suspended cells has to be operated at low flow rate and high residence time. In an immobilized cell bioreactor, the biomass residence time becomes uncoupled from the hydraulic residence time; therefore it is possible to operate the reactor at high flow rate without cell washout. The bio-film formed in the immobilized cell bioreactors also offers more resistance to extreme conditions such as low pH, and high metal concentrations.

The CSTR is a completely mixed system and usually used in fundamental studies on sulfate reduction processes, while the lack of biomass retention and high energy requirements limits its industrial applications.

In UASB, due to biomass granulation no packing or carrier material is required which reduces the startup costs as compared to other reactors. However, biogas production may require extra instrumentation adding up to the capital costs in addition to a long start-up period requirement. Moreover, significant wash-out of sludge during the initial phase of the process is likely and the reactor needs skilled operation.

High sulfate reduction efficiency can be achieved in MBR. However, the fouling of the membrane was observed at higher concentration of sulfate. Frequent cleaning of the membrane is required to continuously operate the membrane bioreactor resulting in increased expenditure in operation of the reactor.

Packed bed reactors offer the advantages of simplicity of construction, elimination of mechanical mixing, better stability at higher loading rates, and capability to withstand large toxic shock loads and organic shock loads. The reactors can recover very quickly after a period of starvation. The main limitation of this design is that the reactor volume is relatively high compared to other high rate processes due to the volume occupied by the media. Another constraint is clogging of the reactor due to increase in biofilm thickness and/or high suspended solids concentration in the wastewater. A major advantage is that the technology has comparatively less investment requirements when compared to an anaerobic filter or a fluidized bed

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system. Among notable disadvantages, it has a long start-up period along with the requirement for a sufficient amount of granular seed sludge for faster startup.

2.9 **Sulfide oxidation to elemental sulfur**

The major problem associated with the anaerobic treatment of sulfate-rich wastewater is the production of sulfide. The sulfide so produced is an undesirable product as it is reported to severely impair methanogenesis (Khanal and Huang, 2003), emanates unpleasant odor, causes corrosion of materials, affects human health and lowers the quality of biogas especially when sulfide content of biogas is above 0.7% by volume (Reis et al., 1988). Sulfide is one of the more toxic pollutants having a characteristic "rotten eggs" odor perceptible in fresh air in a dilution of 0.002 mg/L of air (Buisman et al., 1989). Different sulfide removal techniques exist, including chemical precipitation as well as gas scrubbing in combination with chemical or biological oxidation processes (Burgess et al., 2001). The high cost of operation and sludge disposal problems constitute the main drawbacks of physical–chemical method. The biological sulfate removal process can be made more effective if the sulfide produced can be converted to some other harmless and useful product such as elemental sulfur. Partial oxidation of sulfide to elemental sulfur is a cheap alternative which also allows reclamation of sulfur as it is non-soluble and can be removed from the wastewater (Jensen and Webb, 1995). When the reactor liquid is aerated partially in an aeration unit, well settling S^0 particles were formed under autotrophic conditions (Janssen et al., 1997). The biological sulfur cycle also supports the conversion of the sulfide to elemental sulfur by partial oxidation with the introduction of limited amount of air/oxygen (Figure 2.6).

Under conditions with limited availability of oxygen, sulfur is the major end product of the sulfide oxidation, whereas under fully oxygenated condition, sulfide will be completely oxidized to sulfate (Cirne et al., 2008). Elemental sulfur production is favorable because it is neither inhibitory nor highly soluble, forming a solid precipitate that may produce dense sludge which settles well. The Gibbs free energy, $\Delta G°$ kJ/mol) calculated for the reactions involved in sulfide oxidation suggests that the reactions are feasible (Table 2.8).

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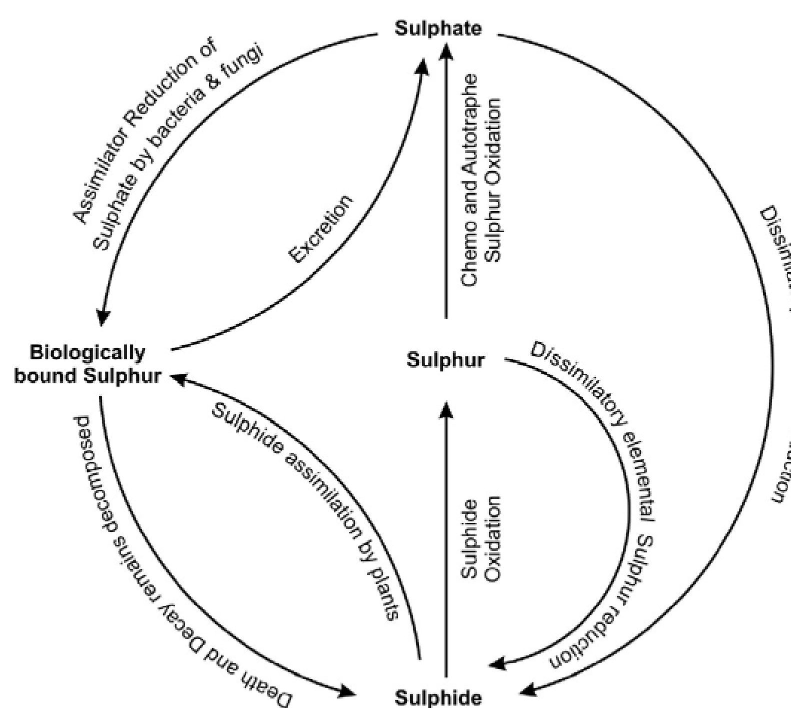

Figure 2.6 Biological sulfur cycle (Sheoran et al., 2010)

Table 2.8 Gibbs free energy values for the reactions involved in sulfide oxidation. ΔG° (kJ/mol) values for the individual compounds for calculation are referred from Lide (2004), Thauer et al.,(1977), Stumm and Morgan,(1996) and Rossini et al.,(1952)

Reaction	ΔG°' (kJ/mol) pH = 7.0	ΔG°' ((kJ/mol) pH = 7.8	ΔG°' (kJ/mol) pH = 8.4
$H_2S+0.5O_2 \rightarrow S^o+H_2O$	-203.8	-203.8	-203.8
$HS^-+0.5O_2 \rightarrow S^0+OH^-$	-209.1	-204.7	-201.2
$S^{2-}+0.5O_2+2H^+ \rightarrow 2S^0+H_2O$	-237.1	-227.6	-220.8
$H_2S+2O_2 \rightarrow SO_4^{2-}+2H^+$	-791.2	-800.0	-807.2
$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$	-796.7	-801.2	-804.6
$S^{2-}+2O_2 \rightarrow SO_4^{2-}$	-824.1	-824.1	-824.1

ΔG°' = Standard Gibbs free energy for the reaction at pH = 7, 7.8 and 8

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2.10 **Mode of micro-aerobic regulation**

Direct introduction of oxygen/air into ‘anaerobic’ bioreactor systems for sulfide removal has been investigated previously during treatment of sulfate-rich wastewater. By regulating the oxygen dosing, micro-aerobic conditions can be maintained in anaerobic reactors to maintain an acceptable reducing environment for anaerobic microorganisms to degrade the organic matter (Fox and Venkatasubbiah, 1996; Khanal and Huang, 2003; Zitomer and Shrout, 2000).

Biological hydrogen sulfide treatment processes is more favored nowadays compared to other traditional physico-chemical processes as it is less expensive and requires less or no chemicals (Lens and Hulshoff Pol, 2000; Syed et al., 2006). The utilization of hydrogen sulfide in the presence of oxygen as electron acceptor by sulfur oxidizing microorganisms to obtain energy is the basis of biological sulfide removal (Diaz et al., 2011). It has been investigated that the mechanism of sulfide removal, takes place not only chemically but also biologically (Kleinjan et al., 2006), and is believed to begin with the formation of polysulfides, which may be further oxidized to sulfate (Steudel, 1996). According to Kelly et al. (1997),the biological oxidation is proposed to take place in stages through several intermediates as shown in Eq.2.6

$$HS^- \rightarrow S^0 \rightarrow S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow SO_4^{2-} \quad 2.6$$

Biogas containing hydrogen sulfide from anaerobic treatment of high sulfate wastewater can be reduced effectively both in fed-batch reactors (van der Zee et al., 2007), and in continuous reactors (Fox and Venkatasubbiah, 1996; Khanal and Huang, 2003; Zitomer and Shrout, 2000) by providing limited oxygen supply. Microorganisms such as *Thiomicrospira* sp. and *Thiobacillus* sp. are capable of performing sulfide oxidation even in anaerobic conditions like those in the anaerobic sludge digester depending on the oxygen availability (Tang et al., 2009). Pure cultures acclimatized to hydrogen sulfide, oxygen, and nutrients are utilized in bio- scrubbers (Janssen et al., 2001) and biotrickling filters (Goncalves and Govind, 2009; Ramirez

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et al., 2009) to remove hydrogen sulfide biologically. In order to make biological hydrogen oxidation more cost effective, micro-oxygenation of the digester can be done as an alternative as the sludge already contains some sulfide oxidizing bacteria (Abatzoglou and Boivin, 2009). By supplying air or pure oxygen under micro-aerobic conditions to the headspace (Díaz et al., 2010) and the limited air to the sludge recirculation (Sridevi et al., 2012) of full-scale digesters for treatment of sewage sludge, removal of hydrogen sulfide in the biogas was observed. Introduction of 4–6% of air calculated according to the total biogas production in the headspace of anaerobic digesters treating agricultural wastes have been practiced to reduce the hydrogen sulfide in the biogas below 200 ppmv (Weiland, 2010). Depending on the sludge or biogas recirculation and the oxygen supply point (headspace or liquid phase) removal of only hydrogen sulfide from the biogas or the total dissolved sulfide could be observed. More than 98% removal efficiency was achieved in micro-aerobic conditions. Sludge recirculation resulted in the removal of hydrogen sulfide from the biogas while dissolved sulfide also gets removed with bio-gas recirculation (Diaz et al., 2011). Micro-aerobic supply of oxygen or air is thus a very practical and feasible method for hydrogen sulfide removal from anaerobic digesters without causing much harm to the anaerobic digestion process (Díaz et al., 2010; Diaz et al., 2011).

Oxygen or air was introduced either directly into the reactor (van der Zee et al., 2007; Zhou et al., 2007; Zitomer and Shrout, 2000) or into the combined flow of effluent and biogas, right before this mixture entered a reservoir acting as a gas/liquid separator (Khanal and Huang, 2003). Studies have been carried out different mode of oxygen dosing. For instance, Khanal and Huang (2003) applied an ORP system to monitor the oxygen dosing taking into account that the ORP varies linearly with the logarithm of oxygen concentration. The intrusion of oxygen, even at a level well beyond the detection limit of commercially available oxygen probe (0.1 mg/L), can be easily sensed by the ORP measurement. Chuang et al.(2005) used DO and ORP sensors in a floated bed micro-aerobic reactor for a moderate degree of oxidation of

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hydrogen sulfide. van der Zee et al. (2007) introduced a low airflow of 0.7–0.9 m^3/m^3 /day, corresponding to a super-stoichiometric ratio of 8– 10 mol O_2 per mol. S.Diaz et al.(2010) maintained micro-aerobic conditions using the regulated flow of pure oxygen with a Cole-Parmer EW-32660-26 mass flow controller from an oxygen cylinder; when air was employed as an oxygen source and was injected into the headspace. A flow rate of 1.8 ± 0.1 N mL/min representing ~0.25 NL of oxygen per L of feed sludge was provided to the sludge digesters to provide micro-oxygenation (Diaz et al., 2011). A controlled and continuous air injection (0.19 L/min) given at 40% volume of an up-flow hybrid sulphidogenic reactor affected sulfide oxidation inside the reactor and enhanced the sulfate reduction efficiency (Sabumon, 2008). Xu et al.(2012) achieved sulfate removal efficiency of 81.5% and S^0 recovery of 71.8% in an integrated sulfate reducing and sulfate oxidizing EGSB bioreactor under micro-aerobic conditions by providing dissolved oxygen concentration of 0.10 – 0.12 mg/L adjusting the aeration flow rate of a separate 5 L vessel used as an aeration unit.

2.11 **Bioreactors employed for microaerobic process**

Khanal and Huang (2003) operated chemostats with working volume of 4L at 35±0.5°C and a HRT of 15 days was maintained in a complete mixing condition by biogas recirculation at a flow rate of 3–4 L/min through a cadet pump (Cole Palmer, Model 7530-65). Krishnakumar et al.(2005) used a novel aerobic bioreactor, the reverse fluidized loop reactor (RFLR) (US Pat. No.6,544,421) with biofilm carrier particle for recovering sulfur from aqueous sulfide at an HRT around 90 minutes. The air supply into the reactor was regulated with an on–off controller to maintain the redox potential required levels. Chuang et al (2005) operated a system consisting of an upward-flow anaerobic sludge blanket (UASB) reactor and a floated bed micro-aerobic reactor packed with elastic porous carriers maintained at dissolved oxygen below 0.5 mg/L. An average of 70±6% of sulfate was transformed to hydrogen sulfide in UASB reactor followed by the oxidation of most of the sulfide to elemental sulfur and sulfate in micro-aerobic reactor. At a HRT of 2.8 h, sulfide was almost

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completely removed in the microaerobic reactor. Diaz et al.(2010) studied the microaerobic removal of hydrogen sulfide in biogas from sludge using a 200 L digester with HRT of ~20 days under varying conditions of oxygen, air and nitrate. Hydrogen sulfide content was reduced from 15,811 mg/N m^3 to less than 400 mg/N m^3 when oxygen was supplied (0.25 N m^3/m^3 feed) while introduction of air (1.27 N m^3/m^3 feed) successfully removed more than 99% of the hydrogen sulfide content, with a final concentration of ~55 mg/N m^3. Diaz et al. (2011) studied the performance of two pilot-plant digesters with an HRT of ~20 d micro-oxygenated at a rate of 0.25 NL per L of feed sludge. The digesters were able to achieve a removal efficiency of more than 98%. The supply of oxygen to the headspace was found to be the optimal dosing point resulting in elemental sulfur formation due to the presence of different sulfide-oxidizing bacteria. Xu et al. (2012) reported the successful operation of an integrated SRB + SOB expanded granular sludge bed (EGSB) reactor under microaerobic condition. At DO level of 0.10–0.12 mg/L, the sulfate removal efficiency reached 81.5% and the recovery of S^0 peaked at 71.8%, which is the highest reported removal efficiency. At DO level higher than 0.30 mg/L, activities of SRB were inhibited leading to failure of the SRB + SOB reactor. The findings from the various studies on performance of microaerobic reactors used for treating sulfate rich wastewater have been summarized in Table 2.9.

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Table 2.9 Performance of microaerobic reactors used for treating sulfate rich wastewater

Type of reactor	Reactor volume (L)	Influent Sulfate (g/L)	COD/ Sulfate ratio	% Sulfate Removal efficiency	S^0 recovered	Carbon source	Oxygen introduction	DO (mg/L)	Aeration level (L/d)	Temp (°C)	HRT (h)	Reference
Down flow fluidized bed reactor	2.3	3.9 6.2	0.66 0.67	75 77	52 54	Lactate	Filtered air at bottom of reactor		2.28 3.42	30	24	Celis-García et al. (2008)
EGSB	4	1±0.1	3	81.5 94.6	71.8 62.5	Lactate	Separate vessel for aeration to maintain DO	0.08-0.1 0.1-0.12	14.4 28.8	30±1	18	Xu et al (2012)
Chemostat	4	1 3 5	10 3.33 2	43.4 22.4 59	Dissolved sulfides UD	Glucose	Recycled biogas stream (pure O_2)		-230 to -180 ORP	35±0.5	360	Khanal and Huang, (2003)
Pilot plant reactor	250	2.2	42.7-21.8	-	-	Sludge	Pure O_2 Headspace	~0.25 NL of oxygen per L of feed sludge	1.8 ± 0.1 NmL/ min	35±1	480	Diaz et al (2011)

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2.12 **Phenol as carbon source for sulfate reduction**

Phenol is a man-made as well as a naturally occurring aromatic compound and an important intermediate in the biodegradation of natural and industrial aromatic compounds (Schie and Young, 1998). Phenol is a known bactericidal compound at high concentrations and for non-adapted cultures it may prove to be toxic or inhibitory even at low concentrations. Although biodegradation of phenol can occur both aerobically and anaerobically, it is toxic to microorganisms even at relatively low concentrations of tens of milligrams per liter, specially unacclimatised microorganisms (Tay et al., 2001). In addition, phenol can be growth inhibitory even to those species that have the metabolic capacity of using it as a growth substrate (Hill and Robinson, 1975). Anaerobic degradation of phenolic compounds has been reported for sludge samples, methanogenic enrichment cultures or facultative anaerobic, nitrate reducing *Pseudomonas* strains (Schink and Pfennig, 1982; Szewzyk et al., 1985; Young and Rivera, 1985). Li et al.(1995) reported the effective removal of phenolic and benzoate aromatic compounds by anaerobic biofilm processes. Treatment of phenol derivatives found in synthetic chemicals, pesticides, and coal-conversion wastewater by using an activated carbon anaerobic biofilm process have also been studied (Young and Rivera, 1985). Basic studies on phenolic compounds degradation suggested that anaerobic bacteria possessed the metabolic capacity to degrade phenol to CO_2. Anaerobic degradation of phenol based on the analogy with anaerobic benzoate pathway proposed for *Paracoccus denitrificans* by Williams and Evans, (1975) leads to the formation of acetyl CoA or succinyl CoA as the degradation product (Sridevi et al., 2012). Acetic acid was the major end product of phenol metabolism and almost 2 moles of acetic acid were produced per mole of phenol degraded by SRB isolated from swine manure utilizing phenol as the sole source of carbon and energy (Boopathy, 1995). Haggblom and Young (1995) reported simultaneous degradation of 4-chlorophenol and SO_4^{2-} by sulfidogenic consortia grown on either 2-, 3-, or 4-chlorophenol as the only source of carbon and energy for over 5 years. The sulfidogenic consortium also utilized phenol, 4-bromophenol, and 4-iodophenol in addition to 4-chlorophenol. The SO_4^{2-} reducers *Desulfococcus multivorans*, *Desulfosarcina variabilis* and *Desulfonema magnum* enriched and isolated with benzoate, also use one or two of the three hydroxybenzoates (Widdel,

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1983; Widdel et al., 1983; Widdel and Pfennig, 1981).Bak and Widdel (1986) reported the anaerobic degradation of phenol and phenol derivatives by *Desulfobacterium phenolicum* strain Ph01. Several other SRB have also been isolated including spore-forming *Desulfotomaculum* strain Groll from freshwater ditch and *Desulfovibrio* sp. from swine manure (Boopathy, 1995). Simultaneous removal of phenolic compounds and SO_4^{2-} follows metabolic pathways that includes biodegradation of phenolic compounds to simple organic acids by phenol-utilizing bacteria (PUB) while SRB utilized simple organic compounds as the electron donor and SO_4^{2-} sulfate as the terminal electron acceptor for SRB growth (Lin and Lee, 2001). The overall reaction on phenol utilization with SO_4^{2-} reduction in an anaerobic biofilm reactor as suggested by Lin and Lee (2001) is as follows:

$$C_6H_5OH + 5H_2O \rightarrow 3CH_3COOH + 2H_2 \qquad 2.7$$

$$CH_3COOH + SO_4^{2-} \rightarrow 2CO_2 + S^{2-} + 2H_2O \qquad 2.8$$

Based on the above reaction stoichiometry, the PUB is responsible for the conversion of phenol to acetate and H_2 (Eq. 2.7) while SRB uses acetate as an electron donor and SO_4^{2-} as an electron acceptor (Eq. 2.8) for their growth. Thus, SRB mineralizes acetate to carbon dioxide and reduces SO_4^{2-} to sulfide, making acetate and SO_4^{2-} the limiting substrates for SRB growth.

Though phenol and its compounds are toxic, it can however be effectively utilized as a source of carbon and energy and degraded by several bacterial species to even innocuous CO_2. However, proper acclimation is required for effective biodegradation of phenol. Thus, phenol and its compounds (pollutants) can serve as sources of carbon and electron donor to reduce oxyanions, which can serve as electron acceptor for heterotrophic microorganisms in biochemical reactions.

Anaerobic treatment processes have been successfully reported in recent studies for removal of aromatic compounds such as benzoate and phenolic compounds (Wang and Loh, 1999). Wang et al.(1986) treated phenol containing wastewater in an

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anaerobic expanded bed granular activated carbon (GAC) reactor. Phenol removal in a complete mixing type three-phase fluidized bed containing both biofilm and suspended sludge was evaluated (Hirata et al., 1998). Industrial wastewater from pulp and paper, and oil refinery industries in addition to groundwater and leacheate contain high concentrations of sulfate along with aromatic compounds such as phenol simultaneously (Hirata et al., 1998; Maree et al., 1991). These wastewater are highly toxic and may contaminate the receiving waters when present in high concentrations (Lin and Wu, 2011). Thus the simultaneous removal of both sulfate and phenolic wastewater is very much essential. However, very limited studies have been undertaken on simultaneous removal of phenol and SO_4^{2-} except the study carried by out by Chen et al.(2012) reported in an abstract.

2.13 **Design of Experiments**

Design of experiments is a statistical design technique involving variation of the levels of influential variables simultaneously instead of varying only one variable and its level at a time in conventional experimentation. The statistical interpretation of the results are in the form of analysis of variance (ANOVA), student's *t*-test, p-value, and *F*-value which give better understanding of the factor effects and their interaction on a given response. Statistical designs of experiments are necessary for systematic investigations requiring only a low number of experiments, and to interpret results in a meaningful manner (Montgomery, 2008). The factorial experimental design methodology involves changing all variables from one experiment to the next. The reason for this is that variables can influence each other and the ideal value for one of them can depend on the values of the others. This interaction between variables is a frequent phenomenon.

2.14 **Factorial Design of Experiments**

The 2^k design is particularly useful in the early stages of experimental work, when there are many factors likely to be investigated. It provides the smallest number of runs with which '*k*' factors can be studied in a complete design. Since there are only two levels for each factor, it is assumed that the response is approximately linear over the range of the factors level chosen. The statistical model for a 2^k design would

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include *k* main effects, (K/2) two factor interactions,(K/3) three factor interaction and so on and one *k* -factor interaction. That means, for 2^k design, the complete model would contain k^{2-1} effects. The levels of the factors may arbitrarily be called "low" and "high" and denoted by "–" and "+", respectively. For example, in 2^2 design, two factors *viz*. A and B at two levels are chosen. The experimental design consists of treatment combinations denoted by "a", "b", "c", and "ab". A suitable number of runs carried out to replicate the levels of variables at their center point (0) provide an estimate of the residual error associated with the experiments and also the curvature of the response. Three degrees of freedom is associated with the four treatment combination in 2^2 design, which in turn consists of two degrees of freedom associated with main effects of A and B, one degree of freedom associated with interaction effect between A and B (Montgomery, 2008).

2.15 **Analysis of Variance**

Analysis of variance (ANOVA) is a collection of statistical models and their associated procedures in which the observed variance is partitioned into components due to different explanatory variables. In general terms, ANOVA explains any variation in the statistically derived model and significance of the model parameters. The model parameters, usually indicated in ANOVA, are the main effects, interaction effects and error terms, and their significance in the model is represented by Fischer '*F*' and associated P values. Normally, larger *F* and lower P values of a model term in ANOVA indicate a higher significance of the term involved in the process. The other items in ANOVA table are degrees of freedom (df), sum of squares (SS), and mean squares (MS). The MS value of a model term in an ANOVA table is obtained by dividing SS over df and its *F* value is obtained by dividing MS due to the model term by MS due to error.

2.16 **Student 't' Test**

A *t*-test is any statistical hypothesis test in which the test statistic has a Student's *t* distribution if the null hypothesis is true. A test of the null hypothesis is that the means of two normally distributed populations are equal. Given two data sets, each characterized by its mean, standard deviation and number of data points, one can use

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't' test to determine whether the means are distinct, provided that the underlying distributions can be assumed to be normal.

There are 2 main types of t-tests, namely

a) Independent groups t-test, which is used when we intend to compare two independent groups on a certain variable which is measured only once.
b) Repeated measures t-test, which is used when the same parameters are being tested on two occasions (so the dependent variable is measured twice) and we want to know whether the outputs on the two occasions were different. . The t-test indicated by the p-value (usually, the 0.05 level) is required to judge the statistical significance, so as to accept or reject null hypothesis.

2.17 **Response surface methodology (RSM)**

Response surface methodology is a collection of mathematical and statistical techniques useful for modeling and analysis of problems in which a response of interest is influenced by several variables. The eventual objective of RSM is to determine the operating optimum conditions for the system or to determine a region of the factor space in which operating requirements are satisfied. The response surface is usually represented by a three dimensional response surface and contour plots. In most of the RSM formulated problems, the form of relationship between the response and the independent variables is not known. Hence, the first step of exploring the relationship lies in finding a suitable approximation for a true functional relationship between response and set of independent variables. If the response is well modeled by a linear function of the independent variables, then approximating model is the first order model in the form of:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 \ldots\ldots\ldots.. + \beta_k X_k + \boldsymbol{\varepsilon}$$

And, if there is any curvature in the system, then a polynomial of higher degree must be used, for example like

$$\mathrm{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j + \boldsymbol{\varepsilon}$$

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Where ,

Y, is the response

β_0, is the offset term

k, factor variables

β_i, is the ith linear coefficient

β_{ii} , is the ith quadriatic coefficient

β_{ij} is the ij^{th} interaction coefficient

ε is the error.

The method of least squares is used to estimate the parameters of the polynomial thus formulated. The next step involves performing response surface analysis using the fitted surface.

Some of the steps involved in the application of RSM as an optimization technique are as follows (Bezerra et al., 2008): (1) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; (2) the selection of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (4) the evaluation of the model's fitness; (5) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (6) obtaining the optimum values for each studied variable.

If the fitted surface is an adequate approximation of the true response function, then analysis of the fitted surface will be approximately equivalent to analysis of the actual system. The model parameters can be estimated most effectively if proper experimental designs are used to collect the data.

2.18 **Summary of literature review**

Biological sulfate removal is widely used for removing sulfate from sulfate rich wastewater. The microorganisms involved in this process can adjust very well to different environmental conditions like pH, and temperature. Lower sludge production along with the generation of bio-film in attached growth processes like

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PBR, and membrane bio reactors makes it a more appropriate method to isolate the involved microorganisms from toxic environments. The choice of a suitable electron donor and process performance of a reactor for sulfate treatment depends upon the availability and effectiveness as well as operational costs involved. The recovery of sulfide to elemental sulfur is a great trend which makes it as one of the emerging technologies for sulfate removal.

Phenol, though, is a pollutant but can be utilized as a carbon source for sulfate reduction, wherein phenol also get degraded. Few studies have been carried out on the simultaneous removal of sulfate and phenol. Thus, study on sulfate reduction with phenol as co-substrate and sole carbon source, and micro-oxidation of generated sulfide to elemental sulfur would be very much helpful for practical applications.

2.19 **Aim and Scope of the Study**

The review of literature shows that biological sulfate reduction is an effective treatment option for sulfate removal from wastewater. However, the main demerit lies in the generation of sulfide in this process which needs to be addressed. If the generated sulfide could be managed properly then the biological sulfate reduction process will be a cost effective option when compared with the other available physico-chemical treatment options.

The main objective of the present investigation is to develop an anaerobic/microaerobic bioreactor system for treatment of SO_4^{2-} rich wastewater with simultaneous conversion of the generated sulfide into elemental sulfur. In order to achieve the above goal, the scope of the study includes:

1. Collection, acclimatization and performance evaluation of mixed microbial culture to evaluate the influence of various parameters such as pH, temperature, sulfate concentration and different carbon sources on sulfate reduction potential.

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2. Fabrication of a biofilm packed bed reactor (PBR) and evaluation of the optimum HRT and COD/SO_4^{2-} ratio for effective sulfate reduction in anaerobic environment.
3. Investigation on elemental sulfur formation in anaerobic condition.
4. Investigation on the effectiveness of toxic wastes (e.g., phenol) as an alternative to non-toxic and conventional carbon sources such as lactate, dextrose, and acetate.
5. Development of an empirical model to optimize sulfate reduction process when phenol is used as a co-substrate as well as sole carbon source using Response Surface Methodology (RSM).
6. Investigation on sulfate reduction potential in the PBR under sulfate shock loading conditions.
7. Fabrication of a microaerobic reactor and evaluation of optimum oxygen supply, dissolved oxygen level and HRT for efficient elemental sulfur formation in a separate stage microaerobic reactor.
8. Develop a suitable methodology for separation of solid elemental sulfur particles from effluents of microaerobic reactor.
9. Isolation and identification of bacterial species in the PBR.

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Chapter 3

MATERIALS AND METHODS

3.1 Materials

In this section various materials and equipment used and methodologies in the present investigation are being discussed.

Distilled water was used for preparation of the standard solutions. For preparation of feed solution, distilled water was used in the case of the batch studies while for the continuous flow through reactor studies, tap water was used whose characteristics are given in Table 3.1.

All glassware used in this study was from Borosil. All glasswares were kept immersed overnight in dilute HCl (0.1 N) followed by washing with tap water and then distilled water. The washed glassware were then kept in oven at 70-75˚C for about 4 hours for drying. All chemicals used in the present study were either of analytical reagents (AR) grade or laboratory reagents (LR) grade. Table 3.2 shows the list of various instrument used in this study.

Table 3.1 Characteristics of tap water used in the present study

Parameter	Concentration	Parameter	Concentration
pH	8.10±0.05	Iron	0.10±0.01 mg Fe^{2+}/L
Conductivity	0.225±0.005 mmho/cm	Fluoride	0.325±0.05 mg F^-/L
Turbidity	1.10±0.05 NTU	Nitrate	5.50±0.5 mg NO_3^-/L
Hardness	94±5 mg/L as $CaCO_3$	Ammonia	0.2±0.05 mg NH_4^+/L
Alkalinity (total)	50±10 mg $CaCO_3$/L	Phenol	-ND-
Chloride	4.55±0.05 mg Cl-/L	Sodium	4.25±0.01 mg Na^+/L
Sulfate	24.75±0.10 mg SO_4^{2-}/L	Potassium	2.00±0.01 mg K^+/L
Solids	-ND-	Calcium	10.75±0.05 mg Ca^{2+}/L

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Table 3.2 Instruments and equipment used in the present investigation

Instrument/ Equipments	**Parameters tested/measured**	**Model/ Manufacturer/ Specification**
2ML magnetic Stirrer	Mixing	REMI
Autoclave	Sterilization	Equitron
COD Digestor	COD	Hach DRB 200
Digital Nephelo-Turbidity Meter	Sulfate	132, Systronics
DO meter	DO	DO 32 A, DKK-TOA Corporation,Japan
Hot air oven	Drying	Ikon
Muffle Furnace	MLVSS	Tonco-PLT-125, India
pH meter	pH	Thermo Scientific
Centrifuge	Remove settleable solids	R-24 REMI
Shaking incubator	For mixing and temperature control	Labtech
Spectrophotometer		169, Systronics
Weighing balance	Weight	SL-234, Denver Instrument
Field Emission Scanning electron microscope	For images of bacteria and PUF particles	Sigma, Carl Zeiss
Temperature controlled water bath	Maintain constant temperature	LAUDA
Mass flow controller	Maintain airflow	Model: Smart II Mass flow, Brooks Instrument, USA

3.2 **Analytical methods**

In this section, different techniques and methods have been described which have been followed in the present investigation. In general, standard techniques as given in Standard Methods for the Examination of Water and Wastewater (APHA, 2005) have been followed unless otherwise stated.

3.2.1 pH

pH was measured using a digital pH meter .The instrument was calibrated periodically with standard buffer solutions of pH 4.1, 7.0 and 10. 0.

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3.2.2 Chemical oxygen demand (COD)

Chemical oxygen demand was determined by closed refluxed method as per the Standard Methods (APHA, AWWA, WEF, 2005). Digestion was carried out in a COD digester (HACHDRB 200) fitted with a temperature controller and timer. Reagents were prepared as per the standards methods (APHA, AWWA, WEF, 2005). The vials used as digestion vessels were of 10 ml capacity. The digestion solution consisted of 1.5 ml of 0.016 M potassium dichromate solution, 2.5 ml of sample or diluted sample and 3.5 ml of digestion reagent. Refluxing was done for 2 hours at a temperature of 150˚C. Digested samples were cooled down to room temperature and titrated against 0.1 M Ferrous Ammonium Sulfate (FAS). The FAS solution was standardized daily against standard 0.1 M $K_2Cr_2O_3$ digestion solution.

3.2.3 Sulfate

The concentration of sulfate was measured using the turbidimetric method (APHA, 2005) . When barium chloride is added to a solution containing sulfate, barium sulfate precipitation takes place according to the reaction (Eq. 3.1)

$$SO_4^{2-} + BaCl_2 \rightarrow BaSO_4^{2-} + Cl_2 \quad 3.1$$

Around 15 ml of effluent sample were pre-treated with 0.2 mL of $ZnCH_3COONa$(1N) and 0.1 mL of NaOH (6N) in order to fix the sulfide present (Sabumon, 2008) and centrifuged at 8000 rpm for 5 minutes. A suitable portion of the centrifuged sample was made upto 50 ml in an Erlenmeyer flask and then 10 ml of the buffer solution was added, making the volume 60 ml. After adding one-fourth of a spoonful of $BaCl_2$ crystals, the solution was mixed uniformly with the help of magnetic stirrer for 1 minute at constant speed. After stirring, turbidity measurement of the solution was done in a nephelometer. A calibration curve using standard sulfate solution was prepared. Concentrations of sulfate in the samples were determined using a similar procedure and the prepared calibration curve is shown in Figure 3.1.

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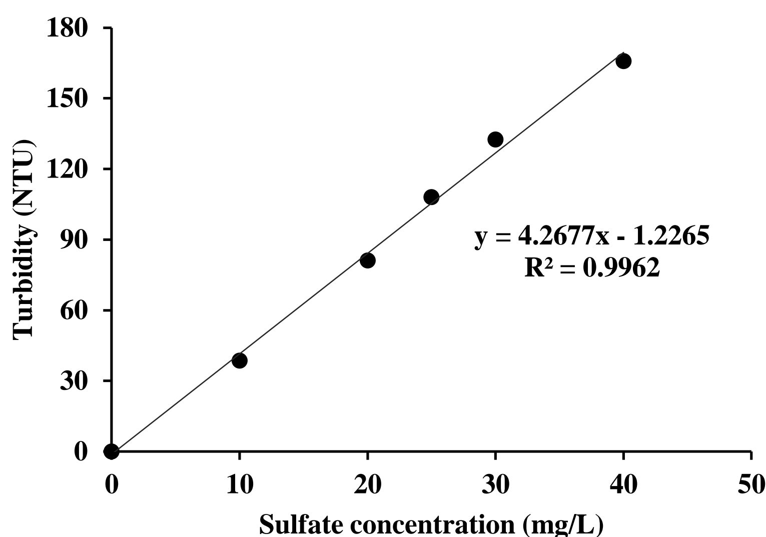

Figure 3.1 Calibration curve for sulfate determination

3.2.4 Sulfide

Dissolved sulfide was determined spectrophotometrically using the procedure followed by Ralf Cord-Ruwisch (1985). When copper reagent consisting of HCl (50 mmol/L) and $CuSO_4$, (5 mmol/L) was added to a solution containing sulfide, copper sulfide precipitates according to Eq.3.2. The absorbance of the resulting mixture can be measured at 480 nm and it is proportional to the sulfide concentration.

$$CuSO_4 + H_2S \rightarrow CuS + H_2SO_4 \qquad 3.2$$

Procedure

a) 0.1ml of the sample was removed by a pipette from the culture vessel while 4 ml of the copper reagent was magnetically stirred (1000 rpm.) in a test tube.
b) The sample was rapidly injected into the stirring reagent.
c) Immediately after mixing for 5s, the absorbance was measured at 480nm in a spectrophotometer.
d) The mixture of 0.1ml distilled water and 4 ml copper reagent served as blank.
e) Using the absorbance of the standard solutions of different concentrations a calibration curve was prepared (Figure 3.2.).

Concentration of sulfide in the samples taken from the bioreactors was determined following the above procedure.

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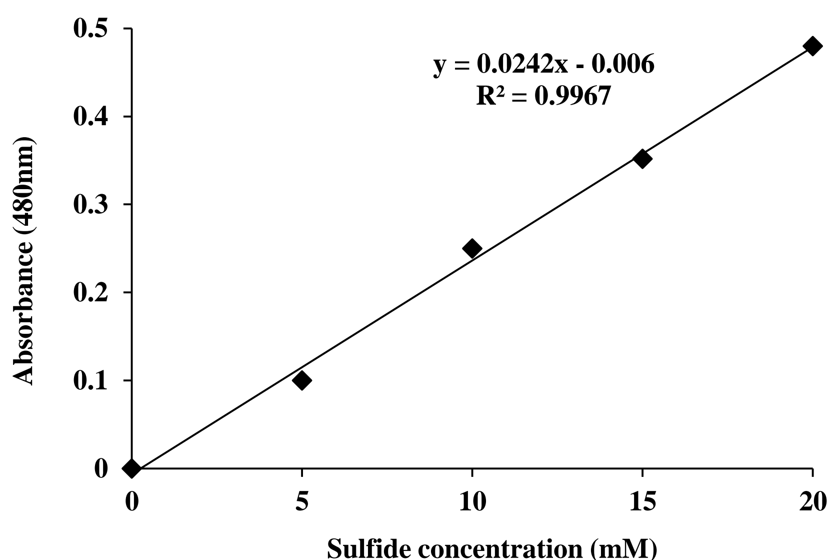

Figure 3.2 Calibration curve for sulfide determination

3.2.5 Phenol

Phenol was determined according to the 4-aminoantipyrine colorimetric method as specified in the Standard Methods (APHA, 2005).

100 mL of the sample or a portion containing not more than 0.5 mg phenol was diluted to 100 mL in 250 mL beaker.100 mL distilled water blank and a series of 100mL phenol standards containing 0.1, 0.2, 0.3, 0.4, and 0.5 mg phenol was prepared. Sample, blank, and standards were treated as follows:

a) 2.5 mL 0.5N NH_4OH solution was added and pH was immediately adjusted to 7.9 ± 0.1 with phosphate buffer.
b) 1.0 mL 4-aminoantipyrine solution was added and mixed well.
c) Finally, 1.0 mL K_3Fe_6 solution was added and mixed well.
d) After 15 min, the absorbance of sample and standards were measured against the blank at 500 nm.

Using the absorbance of the standard solutions of different concentrations a calibration curve was prepared as shown in Figure 3.3. Concentration of phenol in the samples taken from the bioreactors was determined using a similar procedure.

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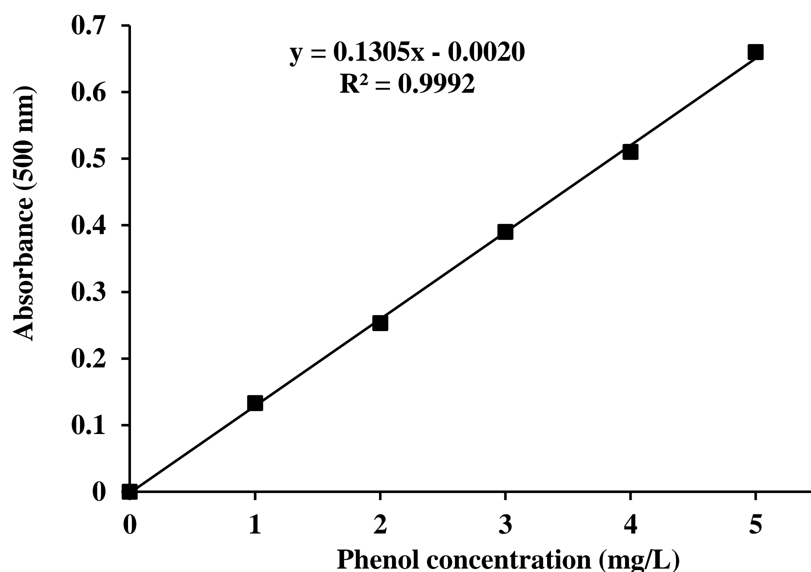

Figure 3.3 Calibration curve for phenol determination

3.2.6 Metabolic Intermediate analysis

For identification of intermediates formed during phenol biodegradation, the samples were analyzed using LC-MS (AGILENT Technologies LC-MS system). A capillary AGILENT 1260 INFINITY CAPILLARY SYSTEM C-18 column (4.6 X 250 mm length) was used for the separation of product intermediates. The mobile phase was a mixture of acetonitrile–water-formic acid (1:1:0.1 v/v) filtered through Millipore syringe filter.

3.2.7 Microscopic methods

Fragments of polyurethane foam (PUF) containing the microbial bio-film were sampled at various sampling points of the packed bed reactor and cut into small thin pieces. The sample was mounted on aluminum stubs and then coated with gold using sputter coater (Edward, UK). The stubs were then introduced into the specimen chamber of LEO 1430vp scanning electron microscope for scanning.

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3.2.8 Porosity Determination of Polyurethane Foam

The packed bed reactor was packed with Polyurethane form (PUF) for bio-film development. Porosity and void volume of PUF were determined as follows: PUF material density analysis was evaluated by weighing a sample of known volume. The porosity of samples was estimated by determining the sample to volume ratio. The volume of the sample was determined with a graduated cylinder (1 L). The sample was weighed with an analytical balance before water was added to fill the void space volume. Air bubbles were dislodged by periodically tapping the cylinder. The saturated sample weight was then determined and percent porosity was calculated from the following relationship.

$$\% \text{ porosity} = \frac{\text{Void space volume}}{\text{Volume of the sample}}$$

$$\% \text{ porosity} = \frac{\text{Weight of [(sample+cyclinder+water)-(sample+cylinder)]}}{\text{density of water X volume of water}}$$

3.3 **Experimental methodologies**

3.3.1 Seed sludge and its acclimatization

Sludge (MLSS of 20.5 g/L and MLVSS of 16.5 g/L) was collected from a wastewater treatment plant located at IIT Guwahati; India and acclimatized. Fresh medium was replaced every week and oxygen free nitrogen was purged after medium replacement. A glass aspirator bottle of (2.5 L working volume) which is discussed as reactor R0 in the subsequent sections was used for anaerobic acclimatization of 1 L of the collected sludge after removing the suspended particles such as twigs and then the final volume was made to 2 L with addition of synthetic feed dissolved in tap water. Table 3.3 gives the composition of the synthetic feed used in the study using dextrose as the carbon source. Initially 100 mg/L sulfate was added as $MgSO_4.7H_2O$ which was steadily increased to 1000 mg/L through the acclimatization period. To maintain the anaerobic condition in the culture flask, oxygen free nitrogen was purged at regular

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intervals. The culture was then acclimatized, over a period of two and half month, to reduce sulfate starting from 100 mg/L upto a concentration of 1000 mg/L. The same acclimatized sludge was used for inoculation in the packed bed reactor.

Table 3.3 Composition of sulfate containing synthetic wastewater

Components	g/L		Trace metal solution	g/L
KH_2PO_4	0.5		$NaMo_4.2H_2O$	0.03
K_2HPO_4	0.1		$CaCl_2.2H_2O$	0.05
$NaHCO_3$	0.5-0.8		$CoCl_2.6H_2O$	0.02
NH_4Cl	0.3		KCl	0.05
Carbon source	#variable		$MgCl_2.6H_2O$	0.05
$MgSO_4$. $7H_2O$ as Sulfate	As required		$NiSO_4.6H_2O$	0.01
			Sodium ascorbate	0.05
			Sodium thioglycollate	0.05

#carbon source such as dextrose, lactate, formate, acetate and ethanol were used for the different experiments as required.

3.3.2 Biomass support material

Polyurethane foam (PUF) procured from the local market was used as the supporting material for microbial growth. PUF was cut into cube sizes of approximately 2.5 cm × 1.5 cm × 1.5 cm. The cubes were washed twice with double de-ionized water, autoclaved (20 min, 120 °C), rewashed, and dried overnight at 70 °C in a hot air oven before being used as bio-support material in the PBR. The sponge cubes had a porosity of around 0.78 as estimated from the ratio of void space volume and sample sponge volume. Approximately, 240 g of oven dried PUF was placed inside the reactor to get a packed volume of 5.5 L.

3.4 **Reactor configuration and experimental set-up**

In this section, detailed description on the experimental set-up, reactor specification and feeding and operating conditions are given. This is subdivided into two parts. The first part describes the reactor specification and configuration with the operating conditions in both batch and fed-batch mode. The second part deals with the details of

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the PBR and the microaerobic reactor. The photographs and schematic diagrams of the reactor set-up are also provided.

3.4.1 Batch and fed-batch reactors

Several parameters affecting SO_4^{2-} reduction were investigated through batch and fed batch experiments, including the effect of pH, temperature, initial SO_4^{2-} concentration, suspended and attached growth system, and different carbon sources on sulfate reduction efficiency.

The operating and feeding conditions of the reactors used in the experiments are given in Table 3.4. All the reactors operated in batch and fed-batch mode were anaerobic suspended growth systems utilizing the mixed microbial consortia, except reactor R2 which was an anaerobic attached growth system.

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Table 3.4 Operating conditions of the batch and fed batch reactors used in the different experiments.

Reactor	Carbon source	Influent		HRT (days)	Temp. (°C)	Mode of operation
		SO_4^{2-} conc.(mg/L)	COD/SO_4^{2-}			
R0	Dextrose	100-1000	1.5	-	28	Batch
BR1		1000	1.5	8		
BR2		1000	1.5		20,25,30, 35,40	
BR3		500,700,800,1000,1200	1.5		28	
R1		1000	1.5, 1.2,1	7.5, 5	30	Fed-batch
R2						
C1	Acetate	1000	1.5	6		
C2	Dextrose					
C3	Ethanol					
C4	Formate					
C5	Lactate					
R3		1000,1200	1.5, 1.2,1	7.5, 5		

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3.4.2 Operating conditions of batch reactors

A) Reactor R0

A glass aspirator bottle of (2.5 L working volume) named R0 was used for anaerobic acclimatization of 1 L screened seed sludge which was made to final volume of 2 L with addition of synthetic feed dissolved in tap water. Initially, the mixed culture was grown in feed medium starting with low initial concentration of sulfate. The influent pH was maintained at 7±0.2 with the addition of $NaHCO_3$ (0.5-0.8 g/L).Initially, 100 mg/L sulfate was added as $MgSO_4.7H_2O$ which was steadily increased to 1000 mg/L through the acclimatization period. To maintain the anaerobic condition in the culture flask oxygen free, nitrogen was purged at regular intervals. The culture was then acclimatized, over a period of two and half month, to reduce sulfate starting from 100 mg/L upto a concentration of 1000 mg/L.

B) Reactors BR1, BR2 and BR3

The batch reactor sets used in the study of the effect of pH, temperature, initial SO_4^{2-} concentration were named as BR1, BR2 and BR3, respectively. All experiments in this study were performed in triplicate sets of 150 ml Erlenmeyer flasks containing 100 ml of media containing SO_4^{2-} with dextrose as the carbon source. Acclimatized culture of 200 mg/L (as MLVSS) was added as inoculums in these experiments. Media containing 1000 mg/L SO_4^{2-} with COD/SO_4^{2-} ratio of 1.5 were adjusted to seven different pH values namely 4, 5, 6, 7, 8, 9 and 10 separately by using 0.1M HCl and 0.1M NaOH solutions. After the pH adjustment, each flask was purged with nitrogen gas for 5 minutes to maintain anaerobic condition in the culture flasks. The effect of temperature on SO_4^{2-} reduction was also studied at five different temperatures between 20˚C and 40˚C. Similarly, the effect of initial SO_4^{2-} concentration on the efficiency of the mixed culture was investigated with five different SO_4^{2-} concentrations ranging from 500 mg/L to 1200 mg/L, maintaining COD/SO_4^{2-} ratio of 1.5 and initial pH of 7±0.2. All the flasks were incubated at 28°C in an incubator shaker at an arbitrarily chosen agitation speed of 140 rpm and samples were withdrawn at regular time intervals, centrifuged and analyzed for residual SO_4^{2-} concentration.

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C) Reactors R1 and R2

Two 250 ml Erlenmeyer flask were used as batch reactors R1 and R2 which were operated continuously for around 70 days to study SO_4^{2-} and COD reduction efficiency of the acclimatized sludge under different operating conditions. About 350 mg/L of the acclimatized sludge (as MLVSS) was added to each reactor. Both the reactors were started with initial SO_4^{2-} concentration of 1000 mg/L and, and COD of 1500 mg/L. The study was done in the fed batch mode maintaining an HRT of 7.5 days in Phase I, replacing 100 ml of treated wastewater with 100 ml of fresh feed at an interval of every 3 days. The HRT was further reduced to 5 days in Phase II by replacing 100 ml of fresh feed at an interval of every 2 days, while keeping all the remaining conditions same. The COD in both the reactors was reduced stepwise to 1200 mg/Lin Phase III and then finally to 1000 mg/L in Phase IV.

R1 was operated in suspended growth mode while R2 was a packed bed system where about 5 g of polyurethane foam (PUF) cubes were used as supporting material for microbial growth. Both R1 and R2 were kept in an incubator shaker at a constant temperature of 30±0.2°C and 140 rpm.

D) Reactors C1, C2, C3, C4 and C5

Five different carbon sources such as acetate, dextrose, ethanol, formate, and lactate used were in reactors C1, C2, C3, C4 and C5, respectively. The study was done in the fed batch mode in 150 ml Erlenmeyer flask with 100 ml as working volume, maintaining a HRT of 6 days, replacing 50% of fresh feed every 3 days with the simultaneous removal of 50% of the media so that there would be minimum or no inhibition of sulfate reduction due to lack of carbon sources. Nitrogen gas was purged for about 5 minutes after replacement of the feed each time so as to remove any dissolved oxygen and maintain anaerobic conditions. Each of the carbon sources was added at an initial concentration of 1500 mg/L COD with initial SO_4^{2-} concentration of 1000 mg/L. About 10 % v/v of the enriched culture was added as the inoculum in these experiments. In all these experiments, the initial pH of the media was set to 7, temperature to 30±0.2°C, which was found to be the optimum range, and 180 rpm.

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E) Reactor R3

250 ml capacity Erlenmeyer flask fed batch reactor (R3) was operated with lactate as carbon source with initial SO_4^{2-} concentration of 1000 mg/L and COD of 1500 mg/L maintaining a HRT of 7.5 days in Phase I and further reducing it to 5 days in Phase II. About 350 mg/L of the acclimatized sludge (as MLVSS) was added to the reactor. The COD in the reactor was reduced stepwise to 1200 mg/L in Phase III and then finally to 1000 mg/L in Phase IV. The SO_4^{2-} concentration was further increased to 1200 mg/L in Phase V so as to reduce the COD/SO_4^{2-} ratio further down to 0.8 and study the effect on SO_4^{2-} and COD removal.

3.5 **Flow through reactors**

A packed bed bioreactor (PBR) was used to study the performance of the mixed microbial culture on sulfate reduction under continuous mode of operation. An upflow suspended reactor was also fabricated for the treatment of the effluent from the PBR in microaerobic condition. This section mainly deals with the detailed description of different components of the PBR reactor and the reactor set-up. Description of the microaerobic reactor is also discussed in this section.

A) Packed bed bioreactor (PBR)

A laboratory scale packed bed bioreactor (PBR) was fabricated using a Perspex cylinder of overall height 90 cm and internal diameter 11.3 cm (area of cross section = 100 cm^2) as the main reactor unit and polyurethane foam (PUF) as supporting material for microbial growth. The schematic diagram of the PBR system and photograph of the PBR are shown in Figure 3.4 and Figure 3.5, respectively. Detailed specification of the reactor and its various components are presented in Table 3.5.

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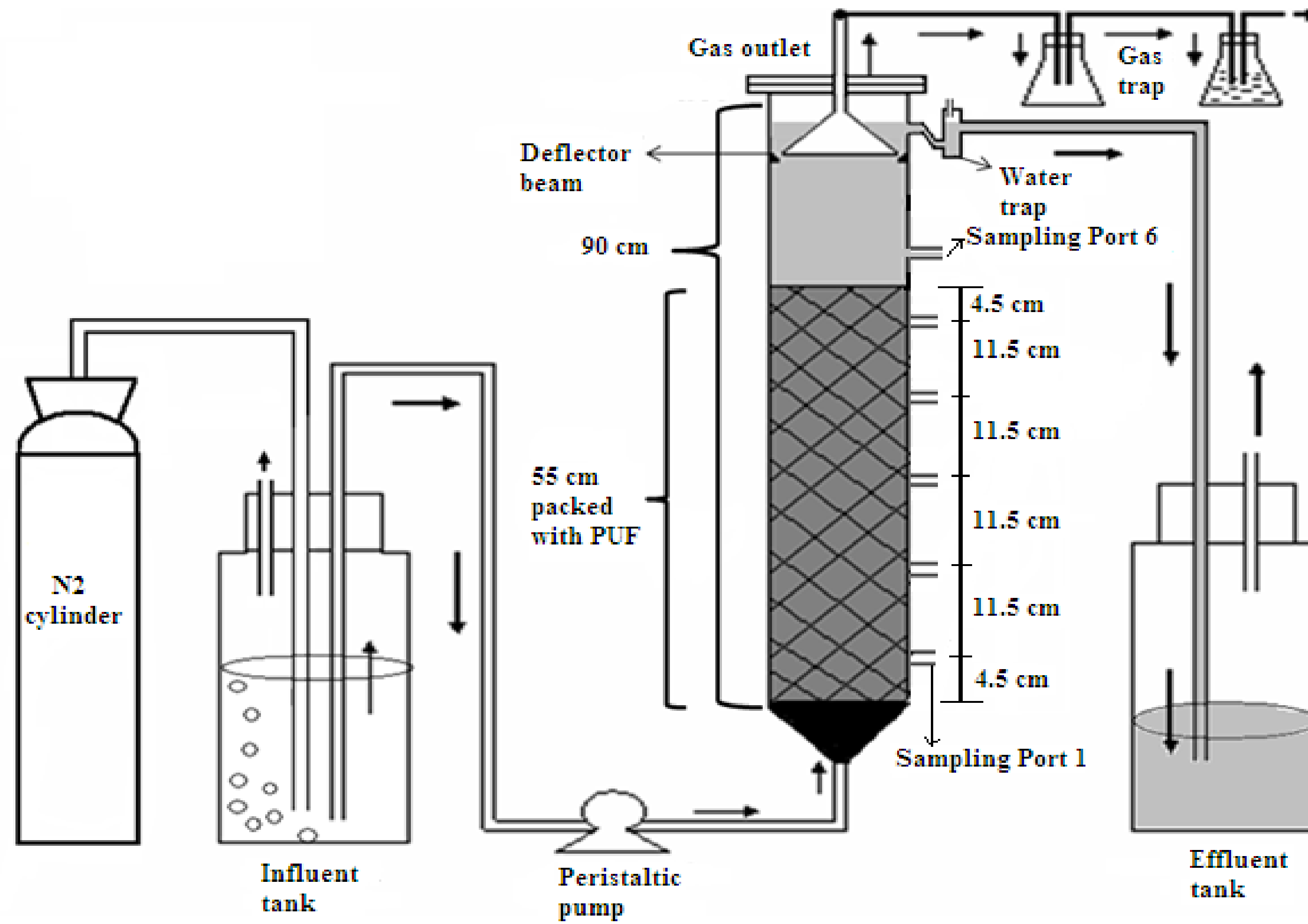

Figure 3.4 Schematic diagram of PBR system

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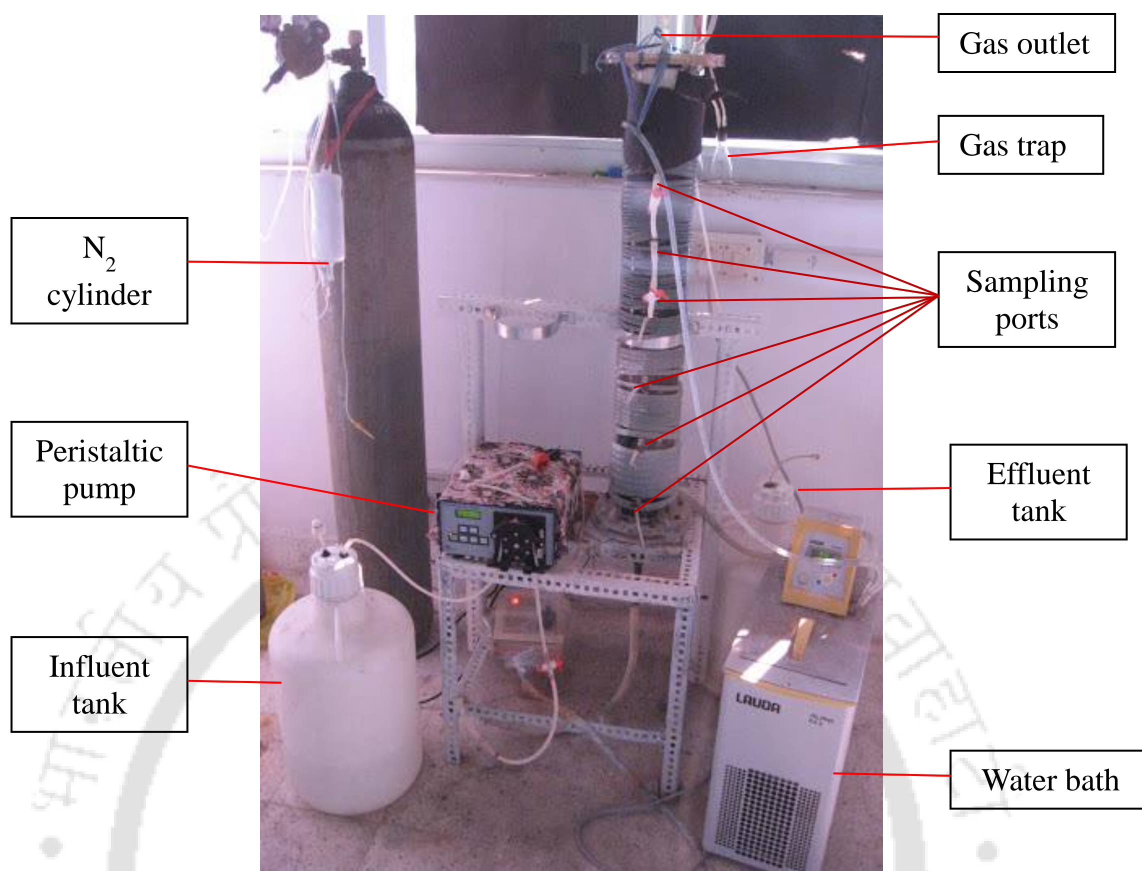

Figure 3.5 Photograph of the PBR system

Table 3.5 Detailed specifications of the reactor

Item	Description
Column height:	90 cm
Column inner-diameter:	11.3 cm
Liquid volume:	8.5 L
Packed bed height:	55 cm
Packed-bed volume:	5.5 L
Packing media:	Polyurethane foam (PUF)

The PBR was inoculated with 1000 ml of mixed microbial consortia having MLVSS of around 10.65 g/L acclimatized with sulfate rich wastewater in a batch flask by means of a peristaltic pump. Any biomass washed out of the reactor along with the effluent was recycled back. During the start up phase, the PBR was fed continuously with influent having concentration of 1000 mg/L SO_4^{2-} and 1000 mg/L COD for 30 days, maintaining a HRT of 48 h at a temperature of 30±0.2 °C. HRT calculation was

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based on packed volume of reactor, which is 5.5 L. The void volume of the reactor was not taken under consideration for calculation of HRT. Approximately, 240 g of dried PUF was placed inside the reactor to occupy 55 cm of packed bed. The bioreactor was wrapped with plastic pipes serving as water jacket, through which constant temperature water was circulated from a thermostat controlled water bath (RA8, Lauda, Germany) to maintain the temperature of the reactor content at 30±0.5 ^{0}C. The influent was purged with N_2 from a nitrogen cylinder to expel out DO, if any. The DO was kept below 10 ppb as measured in DO meter (Model: DO 32 A, DKK-TOA Corporation, Japan).

The discussion on PBR operation has been divided into four phases. The operation of the reactor along with the operating conditions in each phase is discussed in the following sub-sections.

3.5.1 Phase I of PBR operation: Startup of the reactor and optimization of COD/SO_4^{2-} and HRT with lactate as sole carbon source

After inoculation of the PBR with the acclimatized mixed microbial consortia, the reactor was fed continuously with influent having concentration of 1000 mg/L sulfate and 1000 mg/L COD for 30 days maintaining a HRT of 48 h. Any biomass washed out of the reactor along with the effluent was recycled back. After the start-up phase, performance of the PBR under different feeding and operating conditions, namely influent COD/SO_4^{2-} ratio, HRT, and influent SO_4^{2-} concentration were studied. The overall operating schedule of PBR in Phase I is given in Table 3.6.

The PBR was operated at a HRT of 30 h at constant influent COD of 1000 mg/L but with different influent SO_4^{2-} concentrations of 1000, 1200, 1400, 1600 and 1500 mg/L, with an aim to study the effect of COD/SO_4^{2-} ratio on sulfate reduction efficiency. COD/SO_4^{2-} ratio were thus varied from 1, 0.8, 0.7, 0.62 and 0.67 respectively. The effect of HRT was studied at COD/SO_4^{2-} ratio of 0.67 with initial SO_4^{2-} concentration

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of 1500 mg/L. Steady state conditions were assumed when the effluent concentrations of SO_4^{2-} and COD were varied by less than 10% after a period of operation equal to a minimum of three residence times (HRT) after each feeding and/or operating conditions were changed (Oyekola et al., 2009). Once the PBR reached the steady state condition, the PBR was operated at HRTs of 30, 24, 20, and 18 h to study the effect of variation of HRT on the performance of the PBR. After optimization of COD/SO_4^{2-} ratio and HRT, the SO_4^{2-} loading rate was increased by increasing influent SO_4^{2-} concentration to 1800 mg/L and 2200 mg/L.

Partially treated samples were collected from all the six sampling ports of the PBR for profile sampling, once the reactor reached the steady state operating conditions. The schedule of profile sampling is given in Table 3.6. Samples were analyzed mainly for SO_4^{2-}, COD and pH. However, when the influent SO_4^{2-} concentration was increased to 2200 mg/L, analysis of profile samples (day 262) were done for sulfites and thiosulfate as well.

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Table 3.6 Operating schedule of PBR in Phase I

Days of operation	Purpose of operation	HRT(h)	Influent		Remarks
			Sulfate(mg/L)	COD/SO_4^{2-} atio	
1-30	Startup	48	1000	1	
31-99	Optimization of COD/SO_4^{2-} ratio	30	1000	1	Profile sampling (Day 34)
			1200	0.8	Profile sampling (Day 50)
			1400	0.7	Profile sampling (Day 78)
			1600	0.63	Profile sampling (Day 89)
			1500	0.67	Profile sampling (Day 99)*
96-166	Optimization of HRT	30*	1500	0.67	Profile sampling (Day 99)*
		24			Profile sampling (Day 103)
		18			Profile sampling (Day 118)
		20			Profile sampling (Day 132)
		24#			Profile sampling (Day 163)#
167-234	Increase in influent SO_4^{2-} concentration	24	1800	0.67-0.7	Profile sampling (Day 232)
235-320			2200		Profile sampling (Day 262)
321-335			2000		

* The profile sampling at 30 h HRT is used commonly at both COD/SO_4^{2-} ratio optimization and HRT optimization

After 20 h HRT, the PBR was operated back at 24 h HRT

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3.5.2 Phase II of PBR operation: Effect of phenol as co-substrate on sulfate reduction

The PBR was operated for 227 days with the introduction of phenol in a stepwise manner from 25 mg/L to 350 mg/L, keeping total COD (due to both lactate and phenol) constant. The reactor was also operated at varying COD concentration (thus varying COD/SO_4^{2-} ratio) by addition of phenol without changing lactate or SO_4^{2-} concentration in the influent. The lactate COD was kept constant at about 1480 mg/L while the phenol concentration was changed stepwise starting from 100, 150, 250 and finally to 350 mg/L. Operating schedule of the PBR in Phase II is given in Table 3.7.

After running the PBR for 320 days with the different feeding and operating conditions, it was fed with varying phenol concentrations of 150, 250 and 350 mg/L and lactate (as COD) of 667, 905, and 1143 mg/L. A total of 13 experiments as shown in Table 3.8 with 5 replicates (k) at the center point and simulations based on RSM were carried out to obtain the optimum values. A lactate COD of 1300 mg/L and phenol concentration of 250 mg/L were chosen as the center point (0).

Table 3.7 Operating schedule of PBR in Phase II

<table>
<tr><th rowspan="2">Days of operation</th><th colspan="4">Influent</th></tr>
<tr><th>Sulfate (mg/L)</th><th>COD (mg/L)</th><th>Lactate COD (mg/L)</th><th>Phenol (mg/L)</th></tr>
<tr><td>1-9</td><td rowspan="12">2000</td><td rowspan="7">1480</td><td>1422</td><td>25</td></tr>
<tr><td>10-32</td><td>1375</td><td>50</td></tr>
<tr><td>35-55</td><td>1227</td><td>100</td></tr>
<tr><td>56-99</td><td>1135</td><td>150</td></tr>
<tr><td>100-132</td><td>1020</td><td>200</td></tr>
<tr><td>133-192</td><td>905</td><td>250</td></tr>
<tr><td>193-227</td><td>680</td><td>350</td></tr>
<tr><td>228-246</td><td>1680</td><td rowspan="4">1480</td><td>100</td></tr>
<tr><td>247-263</td><td>1795</td><td>150</td></tr>
<tr><td>264-291</td><td>2025</td><td>250</td></tr>
<tr><td>292-321</td><td>2255</td><td>350</td></tr>
<tr><td>322-372</td><td colspan="3">Optimization of lactate COD and phenol using RSM</td></tr>
</table>

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Table 3.8 CCD with various Un-coded and coded values

Run Order No.	Un-coded values		Coded values	
	Lactate COD (mg/L)	Phenol conc.(mg/L)	Lactate COD (mg/L)	Phenol conc.(mg/L)
1	1582.84	250	+α	0
2	1100	150	-1	-1
3	1300	108.579	0	-α
4	1500	150	+1	-1
5	1500	350	+1	+1
6	1300	250	0	0
7	1017.16	250	-α	0
8	1300	250	0	0
9	1100	350	-1	+1
10	1300	391.421	0	+α
11	1300	250	0	0
12	1300	250	0	0
13	1300	250	0	0

3.5.3 Phase III of PBR operation: Phenol as sole source of carbon

The PBR was operated with phenol as the only carbon source and lactate was totally removed from the influent feed. The operating schedule in Phase III of the PBR operation is given in Table 3.9. Initially, the phenol concentration was maintained at 350 mg/L and SO_4^{2-} concentration at 2000 mg/L at a HRT of 24 h. The HRT was then increased stepwise from 24 to 30 and finally to 36 h. Once the phenol removal efficiency stabilized, HRT was then increased further to 48 h to investigate if the efficiency could improve further. As there was no significant improvement in phenol removal efficiency even after 10 days of reactor operation, the HRT was again decreased to 36 h. After operating the PBR at 36 h HRT with steady results, the phenol concentration was increased stepwise to 450, 550, and 750 mg/L, maintaining constant SO_4^{2-} concentration of 2000 mg/L throughout the study.

After running the PBR for 160 days with the different operating conditions, different experimental runs were carried out following the design based on RSM (Table 3.10). These experiments were carried out to investigate the operating optimum values of HRT and phenol concentration require for maximum SO_4^{2-} and phenol removal. The

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PBR was operated under each condition for at least 10 days and the SO_4^{2-} and phenol removal efficiency was taken as the average of each run value.

Table 3.9 Operating schedule of PBR in Phase III (phenol as sole carbon source)

Days of operation	**Influent**		**HRT (h)**
	Sulfate (mg/L)	**Phenol (mg/L)**	
1-34	2000	350	24#
35-54			30
55-65			36
66-79			48
80-89		350	36
90-116		450	
117-146		550	
147-160		750	
161-193		Optimization of phenol and HRT using RSM	

Microaerobic study was done with air supply for 5 hrs at different flow rates.

Table 3.10 Experimental runs following a full factorial design

Run Order No.	**Un-coded values**			**Coded values**	
	Phenol conc. (mg/L)	**HRT (h)**		**Phenol conc. (mg/L)**	**HRT (h)**
1	550	53		0	+α
2	550	36		0	0
3	550	19		0	-α
4	267	36		-α	0
5	550	36		0	0
6	834	36		+α	0
7	550	36		0	0
8	750	48		+1	+1
9	550	36		0	0
10	350	24		-1	-1
11	550	36		0	0
12	750	24		+1	-1
13	550	36		0	0
14	350	48		-1	+1

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3.5.4 Phase IV of PBR operation: Shock loading condition

The key problem of any industrial wastewater treatment involves irregular organic and pollutant loading due to change in process conditions. When concentrations of its pollutants change abruptly, any industrial wastewater treatment plant faces the problem of sudden organic and pollutant loading conditions. Shock loading generally refers to sudden increase of the pollutant concentration resulting in accumulation of the substrate leading to inhibition and the degree of inhibition depends on overall microbial activity and the extent of shock loading (Veeresh et al., 2005). Reactor stability to shockloading is one of the most important aspects of design of reactor in view of the variable nature of industrial wastes. Experiments were thus carried out by subjecting the PBR to shock loading conditions in the form of sulfate loading rate in order to address this problem (Table 3.11).

In order to study the effect of sulfate loading rate (SLR) on sulfate removal rate (SRR) of the PBR, sulfate concentrations of 3000, 5000 and 7000 mg/L were fed to the PBR stepwise at three different HRT of 24, 36 and 48 h keeping the COD/SO_4^{2-} of 0.7±0.02 in each condition. After every shock load, the sulfate concentration was reduced to 2200 mg/L at 24 h HRT before the next concentration was increased so as to check if the PBR can be brought back to its normal condition.

Table 3.11 Operating schedule of PBR in Phase IV (Shock loading condition).

Days of operation	Purpose of operation	HRT(h)	Influent	
			Sulfate(mg/L)	COD/SO_4^{2-} ratio
1-30	Lactate as carbon source	24	2000	0.7±0.02
31-91	Sulfate shock loading effects	24,36,48	3000	
			5000,	
			7000	
92-172	Normal conditions and sequence microaerobic study	24	2000	

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B) Microaerobic reactor

3.5.5 Batch studies on sulfide oxidation

Batch studies were carried out in 1 L aspirator bottles to determine the chemical and biological components of sulfide oxidation. For the chemical oxidation, 1 L medium containing 420 mg/L of sulfide was prepared by adding Na_2S in deoxygenated water while for the biotic study 1 L effluent from the PBR containing around 420 mg/L of sulfide was collected in the aspirator bottle anaerobically. Oxygen in the form of air was added in both the bottles to maintain oxygen to sulfide (R_{OS}) molar ratio of 0.6 and the volume of oxygen to be added to the headspace of each bottle was calculated using the method suggested by Johnston and Voordouw (2012).

The volume of oxygen to be added to the headspace of each bottle was calculated as below, based on the sulfide concentration in the aqueous phase using 41.2 mM as the concentration of gaseous oxygen at 23°C and 1 atm. Both the bottles were kept at room temperature without shaking for 30 hours and sampling was done from time to time for the sulfide.

$$V = (\text{mM } O_2 \text{ wanted in solution} \times 180 \text{ mL headspace})/41.2 \text{ mM}$$

3.5.6 Continuous reactor studies on sulfide oxidation

The microaerobic reactor was fabricated using a perspex cylinder of overall height 60 cm and internal diameter 10 cm. The micro aerobic reactor had a working volume of 4.12 L. Figure 3.6 and Figure 3.7 show the schematic diagram and photograph of the anaerobic/microaerobic bioreactor system, respectively.

Microaerobic study was first carried out with phenol as the sole carbon source, at phenol concentration of 350 mg/L at 24 h HRT. Different airflow rate of 5 ml/min,10 ml/min and 15 ml/min, respectively were supplied for 5 h to the microaerobic reactor, which was already filled up to study the sulfide oxidation to elemental sulfur.

Different air flow rate from the air cylinder controlled through the mass flow controller was pass on to the microaerobic reactor in continuous mode when the PBR was operated at SO_4^{2-} concentration of 2000 mg/L with lactate as carbon source after the shock loading study was over.

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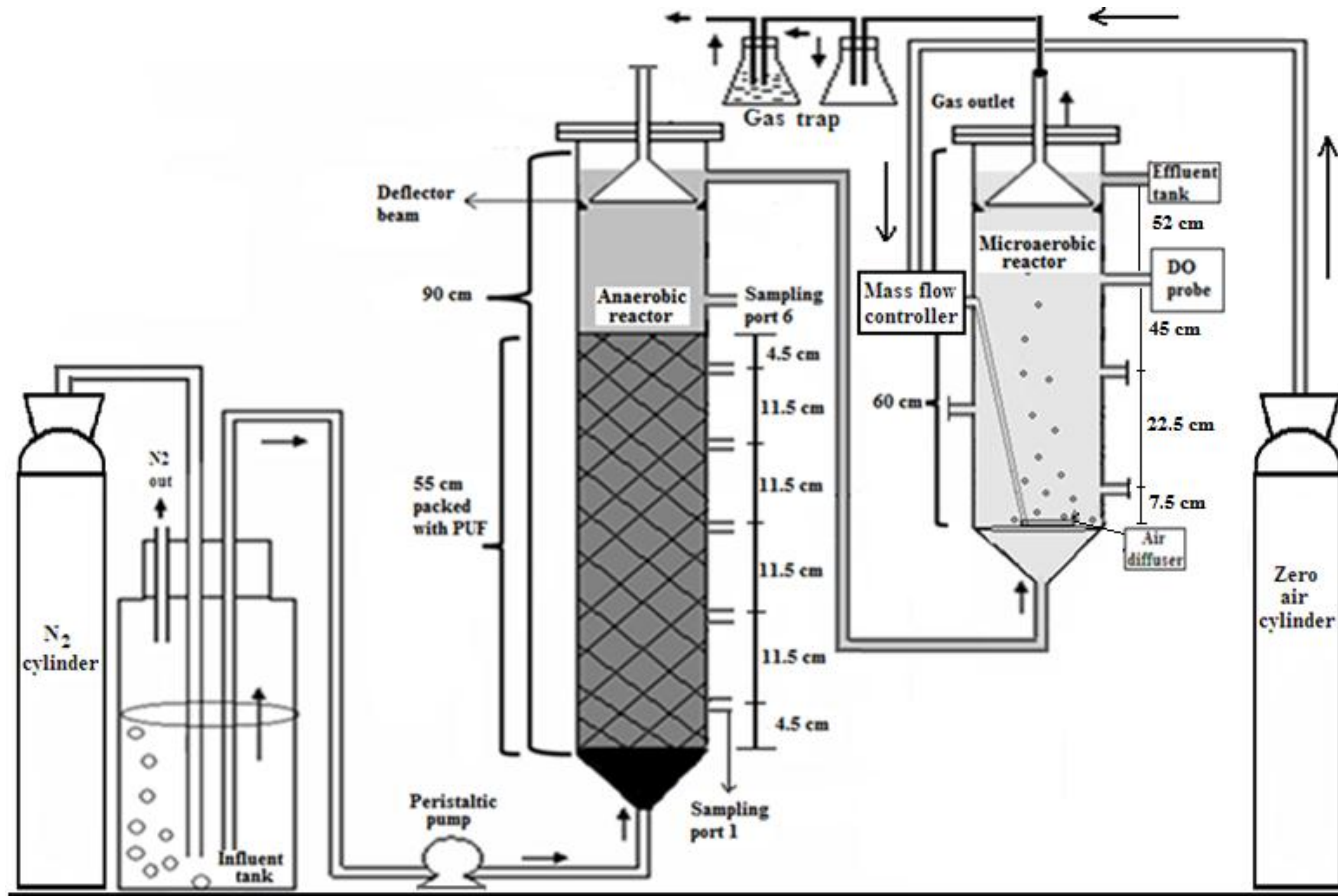

Figure 3.6 Schematic diagram of the anaerobic/microaerobic bioreactor system

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Figure 3.7 Photograph of the anaerobic/microaerobic bioreactor system

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3.6 **Separation of elemental sulfur**

Sulfur formed in a microaerobic reactor are in the form of colloidal particles which may eventually agglomerate to form flocs, preferably after addition of a flocculant (Lens and Hulshoff Pol, 2000). Use of flocculants to separate sulfur particles will not only add to the cost of treatment but the sludge will also contain flocculants along with the sulfur particles. The direct quantitative measurement of S^0 production was not available as sizable portion of S^0 remained in the granule suspension inside the reactor. The performance of gravity settling, and centrifugation were investigated in the present study to investigate separation efficiency of sulfur particles from the effluents of the microaerobic reactor.

3.6.1 Sedimentation

The effluent of microaerobic reactor was collected in 2 L graduated measuring cylinders and covered with parafilm to avoid further oxidation with atmospheric oxygen and allowed to settle. Interface heights at different time interval was noted for further analysis.

3.6.2 Centrifugation

About 10 ml of effluent from the microaerobic reactor was collected in 15 ml centrifuge vials after the visible formation of elemental sulfur and then centrifuged at different rotation speed for 5 minutes. The turbidity of the supernatant was then measured in a nephelometer and compared for the different rotational speeds.

3.7 **Bacterial community**

3.7.1 Microbial community in batch reactor

One percent of the microbial culture from reactor R0 after the acclimatization phase was sub cultured twice using a fresh medium for isolating the predominant species present in the acclimatized mixed consortia. The culture was also plated on a solid medium and incubated in an anaerobic jar containing the media with 1.5 g/L agar containing an Anaerogas pack (Hi Media) to provide anaerobic environment. The isolated pure bacterial strain, which was named r3, was sent to Genie (Bangalore,

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India) for 16S rDNA sequence analysis, and later the result was submitted to GenBank database to carry out similarity search for nucleotides by online Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). The neighbor-joining phylogenetic tree was constructed using Robust Phylogenetic Analysis for the Non-Specialist to represent the relationship between the strainr3 (JN600615.1) and related genera (Dereeper et al., 2008).

3.7.2 Isolation and identification of microbial community in the PBR

The microbial culture obtained from PUF particles from different reactor heights after about 65 weeks of reactor operation was plated on solid medium containing the media with 1.5 g/L agar and incubated in an anaerobic jar containing an Anaerogas pack (Hi Media) to provide anaerobic environment. The three visible colonies PB2, PB7 and PB8 on the plates were transferred to fresh nutrient broth medium and incubated further in the anaerobic jar. The bacterial pellet obtained by centrifuging the fully grown cultures at 10,000 rpm was resuspended with bacterial lysis buffer. The subsequent bacterial DNA was extracted with the DNA purification kit (GeneiPure^{TM} Bacterial DNA Purification Kit, Merck) as per the manufacturer's instructions. The extracted DNA obtained from each of the strains was amplified by the polymerase chain reaction (PCR) (Applied Biosystems, 2720 Thermal Cycler) with a universal primer for identification of the bacterial isolates using the 16S rDNA technique. The nucleotide sequence of the primers was as follows: primer 3A F, 5'-AGAGTTTGATCCTGGCTCAG-3' and primer 3B F, 5'-CGGCTACCTTGTTACGACTT-3'. The amplification mixture was then added with PCR Master Mix kit (Merck, Bangalore, India) under condition of holding at 94°C for 3min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1.3 min, with final extension at 72°C for 10 min. The reaction mixture was subsequently cooled to 4°C. The PCR products were verified on 1.3% agarose gel electrophoresis. The amplified PCR product was sequenced at Scigenom Labs (Kochi, India) and the result was submitted to the National Center for Biotechnology Information (NCBI) database to search for similar nucleotides using

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the online Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). The neighbor-joining phylogenetic tree was constructed using Robust Phylogenetic Analysis for the Non-Specialist (Dereeper et al., 2008) to represent the relationship between the isolated strains and related genera. The morphology of the bacterial strains was analyzed using a scanning electron microscope (Zeiss Sigma, USA).

3.7.3 Terminal- Restriction Fragment Length Polymorphism (T-RFLP) analysis

The primary goal of the t-RFLP analysis is to test the environmental samples using 16S rDNA as well as 16s rDNA amplification from community and restriction enzyme digestion (TaqI, HaeIII). The other objective of performing T-RF analysis (presence/absence of peaks and area of peaks) is to determine bacterial species richness, diversity index, community structure and subsequent comparison of profiles between environmental samples. T-RF numbers have been widely used as indicators of microbial species richness in current environmental microbial studies using the T-RFLP technique (e.g. Denaro et al. 2005).

Samples from the PBR were collected when it was operated with lactate as the sole source of carbon (Phase I) as well as when phenol was substituted as the sole carbon source (Phase III) and sent to gene Ombio Technologies Private Limited, Pune for T-RFLP analysis.

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Chapter 4

RESULTS AND DISCUSSIONS

The discussions on the experimental results are made as per the following sequence in accordance with the aim and scope of this study:

4.1 Seed sludge collection and acclimatization

4.2. Batch study results

4.3. Performance of the PBR in Phase I

4.4. Performance of the PBR in Phase II

4.5. Performance of the PBR in Phase III

4.6. Performance of the PBR in Phase IV

4.7. Performance of the microaerobic reactor

4.8. Bacterial community study results

4.1 **Seed sludge collection and acclimatization**

Enrichment of the mixed consortium was carried out in reactor R0 by adding gradually increasing amount of SO_4^{2-} in synthetic wastewater from 100 to 1000 mg/L. The detailed enrichment phase is presented in Figure 4.1. It could be observed that SO_4^{2-} removal rate was improved at each and every stage of acclimatization. During the initial period of acclimatization and on complete depletion of the initial 100 mg/L SO_4^{2-} in the media, SO_4^{2-} concentration was increased stepwise till a final value of 1000 mg/L was achieved in the 110 day acclimatization period to check the efficiency of the consortium to further reduce the added SO_4^{2-} in the medium. With the increased in SO_4^{2-} concentration, the mixed consortium required more time for SO_4^{2-} reduction as compared to the lower concentration.

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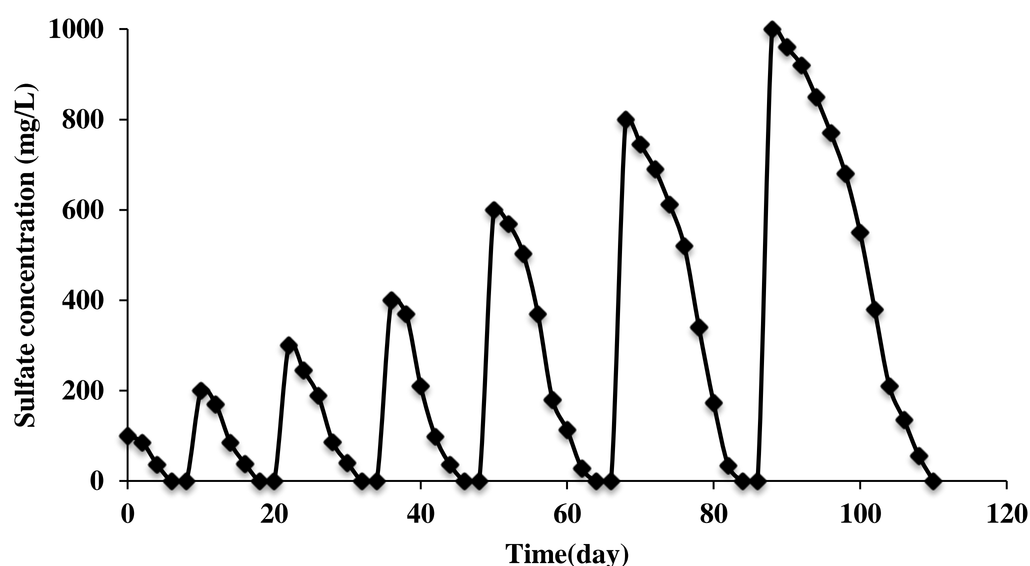

Figure 4.1 Sulfate reduction profile during the acclimatization period of the mixed consortia

4.2 **Batch study results**

4.2.1 Effect of pH, temperature and initial SO_4^{2-} concentration

Effects of pH, temperature and initial SO_4^{2-} concentration on sulfate reduction were performed in batch mode in reactor BR1, BR2 and BR3, respectively (Table 3.4). The results were fitted to zero order rate equation which gave the best fit and are shown in Figure 4.2, Figure 4.3 and Figure 4.4, respectively. SO_4^{2-} degradation rate coefficient (k_r) was estimated for different pH, temperature and initial sulfate concentration using the following equation.

$$C_0 - C = k_r t \qquad 4.1$$

Where, C_0 and C are the SO_4^{2-} concentration at initial and at time't' respectively.

Figure 4.2 shows SO_4^{2-} reduction profile of the mixed consortium over the pH range of 4.0 to 10.0, with initial SO_4^{2-} concentration of 1000 mg/L. It could be observed from Fig. 4.2 that the culture could substantially reduce in the pH range 4.0-10.0; the degradation efficiency, was considerably less at pH 4.0, 5.0, and 10.0 with values

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~3.7%, ~9.1% and ~7.5 %, respectively. The maximum degradation (~68.6%) of SO_4^{2-} was observed when the initial pH was 7.0 within 8 days with a k_r value of 87.86 mg/L/day. It has been reported that SRB require a pH in the range of 5–8 for survival and outside this range, the rate of microbial SO_4^{2-} reduction generally declines (Willow and Cohen, 2003). Low pH (<5) normally inhibits SO_4^{2-} reduction and increases the solubility of metal sulfides (Dvorak et al., 1992). Although, there are studies highlighting the presence and survival of SRB at pH < 3 (Kolmert and Johnson, 2001) and higher than 10 (Pikuta et al., 2003), though sulfate reduction rate was very slow. However, higher reduction rates have only been shown until a pH of 8.0 were a volumetric activity of 25 SO_4^{2-} g/L/day was reported (van Houten et al., 1995).

With respect to the effect of temperature on SO_4^{2-} reduction in the range 20–40°C, maximum degradation of 69.57 % was observed at 30°C with a k_r value of 91.83 mg/L/day (Figure 4.3). Results indicated that at 30°C and 35°C, SO_4^{2-} reduction by the culture was sufficiently high; on the other hand, reduction efficiencies were very less at 20°C and 40°C (inset of Figure 4.3). van Houten et al. (1995) reported that SO_4^{2-} reduction increases when the reaction temperature is increased from 20 to 32°C, when a mesophilic SRB culture was used. Moosa et al. (2002) conducted batch experiments with a mixed culture consisting of acid producers, methane producers, and SO_4^{2-} reducers and reported that SO_4^{2-} reduction rate increased with the increase in the reaction temperature from 20 to 35°C. Further increase of temperature to 40°C led to inactivity of bacteria. From the results of the present study, it can be inferred that the mixed culture is a mesophilic bacterial consortium preferring 30°C and pH 7.0 for SO_4^{2-} reduction.

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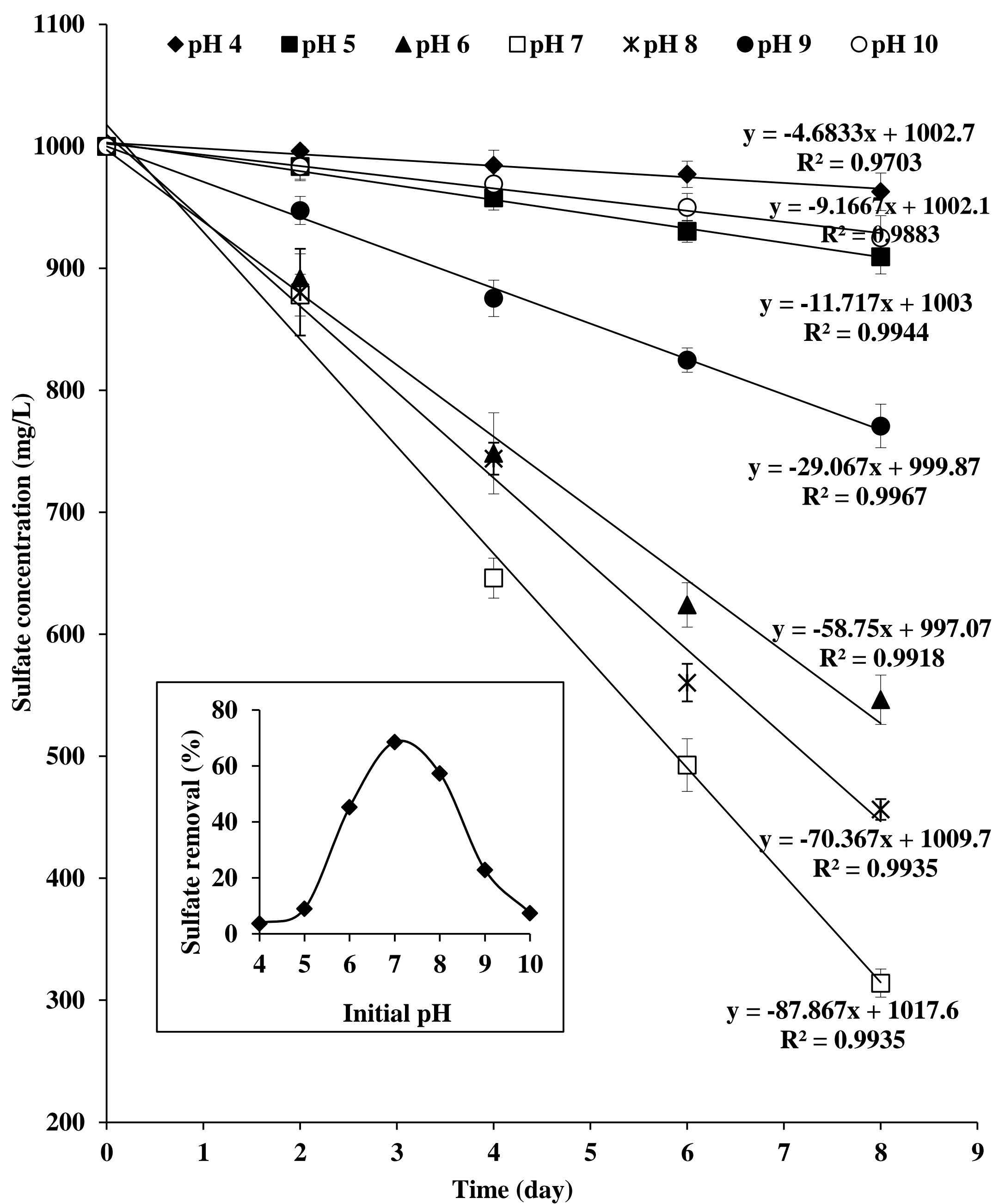

Figure 4.2 Performance of reactor BR1 on SO_4^{2-} reduction by the mixed consortium at different pH

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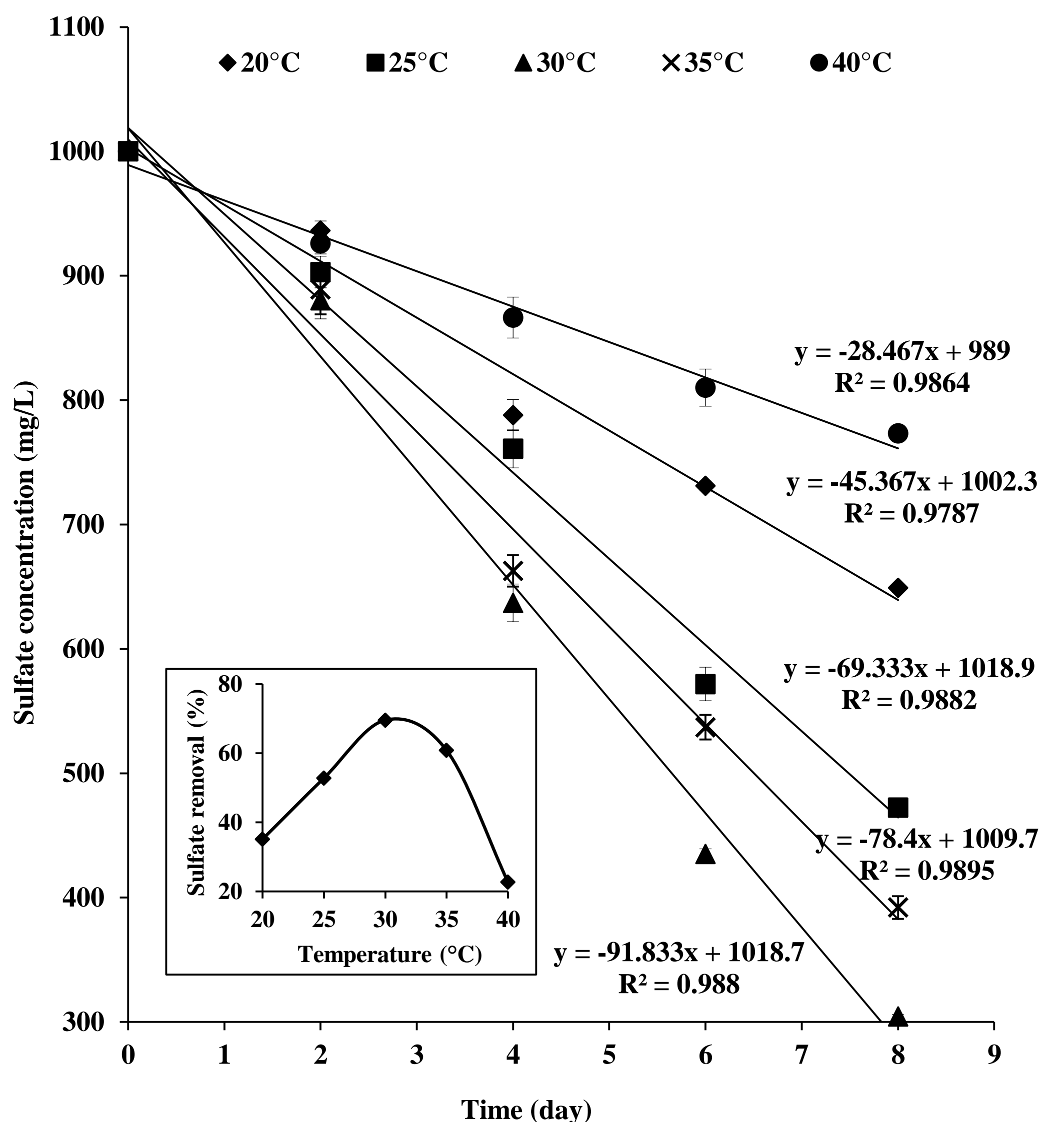

Figure 4.3 Performance of reactor BR2 on SO_4^{2-} reduction by the mixed consortium at different temperature

Figure 4.4 shows the time series profiles of SO_4^{2-} reduction by the mixed culture at different initial SO_4^{2-} concentration. It is clear from the profile that the time taken by the mixed culture to degrade SO_4^{2-} was dependent upon its initial concentration. From an initial SO_4^{2-} concentration of 500 mg/L, it took just around 4 days to reach below 150 mg/L while from the higher concentration of 1200 mg/L it took more than 8 days. Maximum k_r value of 98.13 mg/L/day was observed with the initial SO_4^{2-} concentration of 1200 mg/L.

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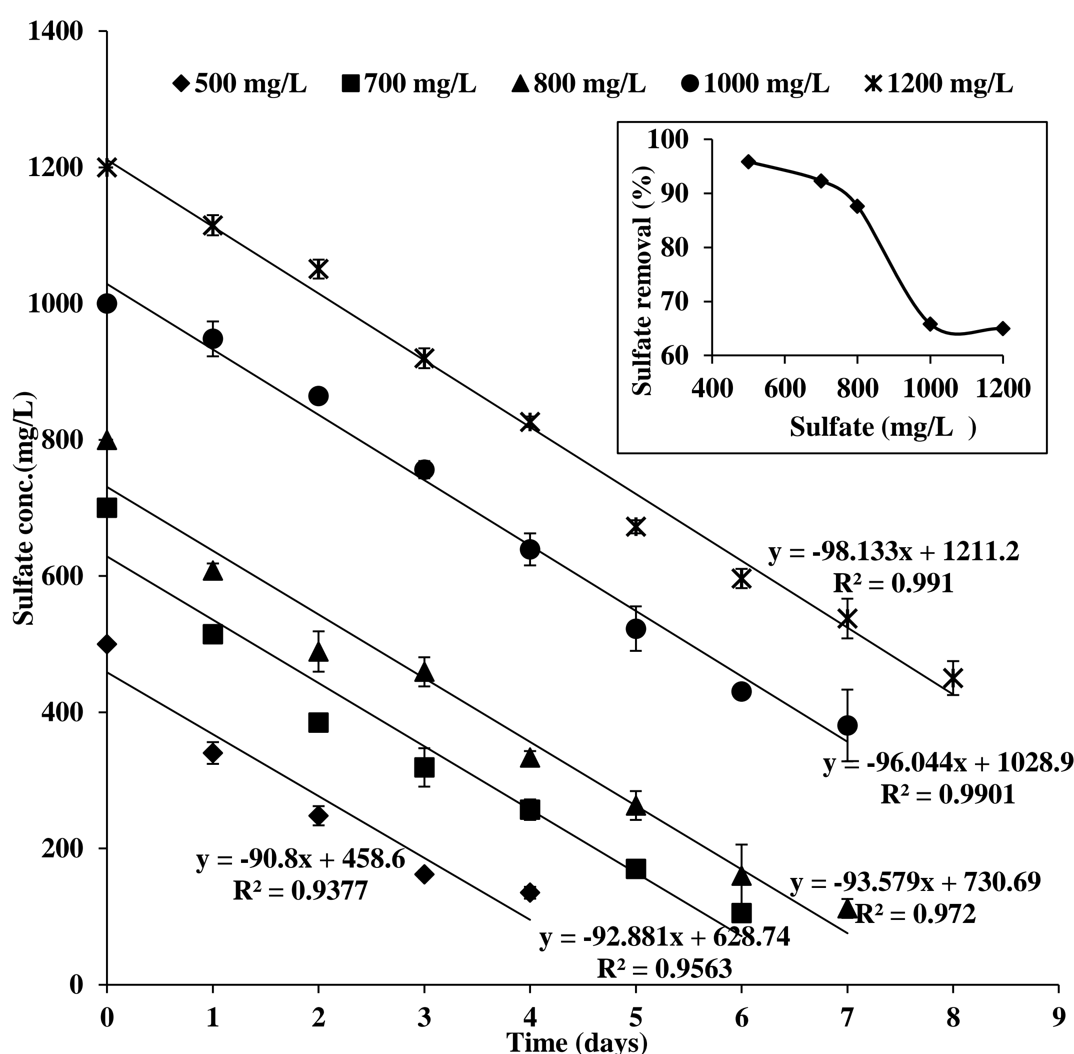

Figure 4.4 Performance of reactor BR3 on SO_4^{2-} reduction by the mixed consortium at different SO_4^{2-} concentration

4.2.2 Suspended and attached growth system

The performance of R1 and R2 are shown in Figure 4.5 and Figure 4.6, respectively. Both the reactors were started with a HRT of 7.5 days and initial SO_4^{2-}concentration of 1000 mg/Land COD of 1500 mg/L. At a HRT of 7.5 days in Phase I, R1 showed a maximum SO_4^{2-}reduction of almost 84% while that of reactor R2 was around 73%. This result indicated that in both R1 and R2 reactor systems SO_4^{2-} reduction was effectively going on even though the efficiency of R1 was slightly higher as compared to R2. In the case of R1, more than 98% SO_4^{2-} reduction took place in Phase II, Phase

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III, and Phase IV even when the COD was reduced to 1000 mg/Lin Phase IV. This may be due to the constant contact between the biomass and substrate in the case of the suspended growth system with continuous mixing conditions. However in the case of R2, the SO_4^{2-} reduction efficiency improved from around 78% in Phase II to around 88% in Phase III which is less in comparison with that of R1 in the same operating conditions. The lower efficiency may be attributed to the lesser growth rate of attached microorganisms in the packed bed system (R2) due to diffusion limitations as compared to that of the suspended microorganisms in the reactor R1 (Luc, 1994; Moghanloo et al., 2010). However once the biofilm was established, the attached growth reactor R2 gave similar efficiency with that of R1 which was observed in Phase IV. When HRT was reduced from 7.5 days to 5 days in both the reactors (Phase II), SO_4^{2-} reduction rate increased in both R1 and R2. This result indicates that with the increase in the SO_4^{2-} load of the feed media with decrease in HRT, SRB were able to outcompete the methanogens for COD utilization which correlates with the finding that at low feed rates the methanogenic bacteria degraded the COD as well as the SRBs as COD removal can take place through the methanogenic and the SO_4^{2-} reduction pathways in an anaerobic system (Greben H.A. and Maree J.P., 2000). Even after reducing the HRT, high amount of COD was left out in the effluent. In order to compensate for this, the influent COD was further reduced to 1200 mg/L in both R1 and R2 in Phase III of the reactor operation. SO_4^{2-} reduction was not hampered even with the reduction in COD concentration in both reactors R1 and R2. In Phase IV of both the reactor operation, the influent COD was further decreased to 1000 mg/L maintaining an influent COD/SO_4^{2-} ratio of 1.0. However, $SO_4^{2-}$was completely removed and as very less COD was left out in the effluent no further study was carried out with decrease in COD concentration.

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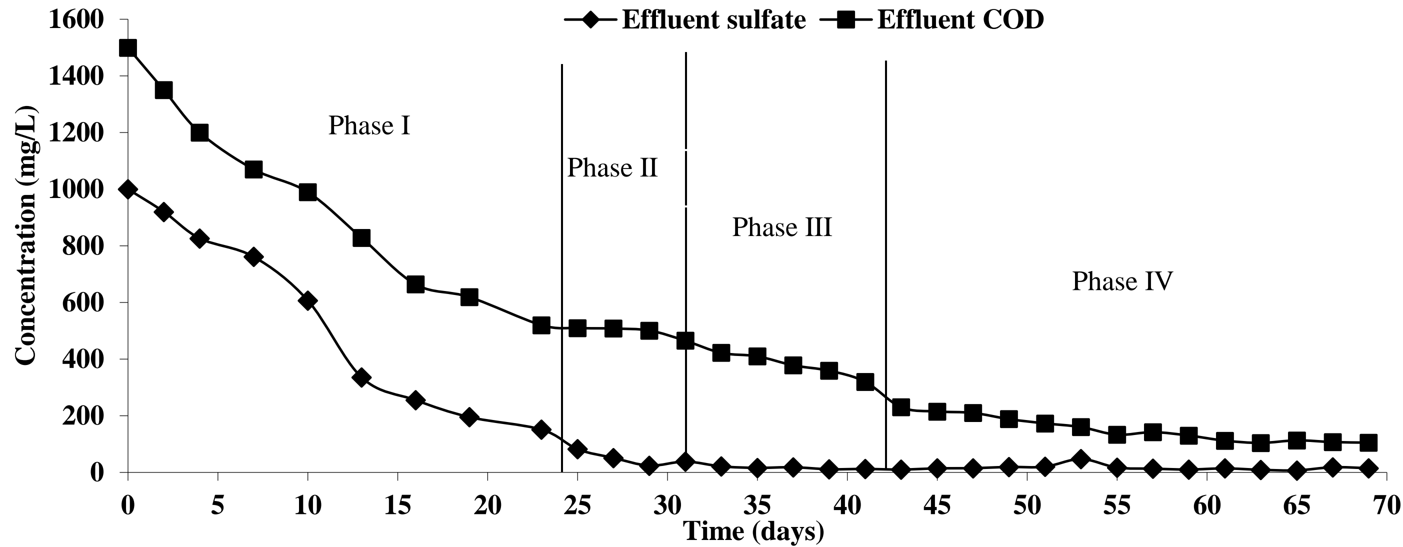

Figure 4.5 SO_4^{2-} reduction profile of reactor R1 with initial SO_4^{2-} =1000 mg/L. (Period I: HRT=7 days; COD=1500 mg/L, Period II: HRT=5 days; COD=1500 mg/L, Period III: HRT=5 days; COD=1200 mg/L, Period IV: HRT=5days; COD=1000 mg/L

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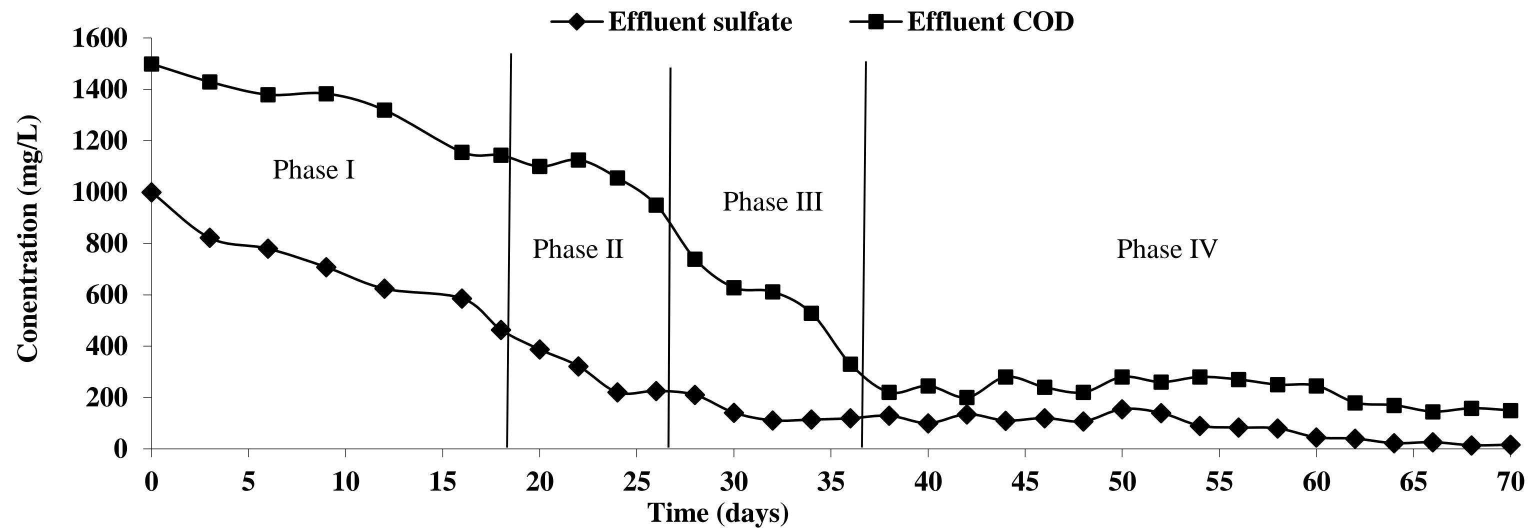

Figure 4.6 SO_4^{2-} reduction profile of reactor R2 with initial SO_4^{2-} =1000 mg/L. (Period I: HRT=7 days; COD=1500 mg/L, Period II: HRT=5 days; COD=1500 mg/L, Period III: HRT=5 days; COD=1200 mg/L, Period IV: HRT=5days; COD=1000 mg/L

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4.2.3 Effect of different carbon sources for SO_4^{2-} reduction

Among the five carbon sources used in reactors C1, C2, C3, C4 and C5, the mixed consortium showed the maximum reducing ability by utilizing lactate as the sole carbon source. The sulfate and COD removal with the five carbon sources is shown in Figure 4.7 and Figure 4.8, respectively. A maximum removal efficiency of 98% was observed when lactate was used as the carbon source. It may be because lactate promotes the growth of a wide variety of sulfate reducing bacteria leading to increased microbial diversity and treatment system resilence (Kaksonen, 2004; Oyekola, 2008). Sulfide toxicity has also been reported to decrease when lactate serves as the carbon source for biological sulfate reduction (Kuo and Shu, 2004). Only around 55% removal efficiency was observed in the case of using acetate as the sole carbon source after even 15 days of fed batch operation. The poor efficiency of the mixed consortium to reduce sulfate using acetate could be due to inability of the SRB to completely oxidize acetate even with excess sulfate levels (Lens et al., 2002). SRB are generally poor competitors of methanogenic archaea (MA) for acetate. Sugar is an effective electron donor that is easily degraded under anaerobic conditions. The anaerobic degradation pathway of sugar such as glucose, fructose, and dextrose is also similar to that of other organic compounds in which hydrogen is the interspecies (Liamleam and Annachhatre, 2007). Dextrose is also utilized effectively in our experiment for sulfate reduction. Most sulfate reducers using hydrogen as carbon source (e.g. *Desulfobulbus propionicus, Desulfovibrio baarsii*) are able to grow on formate (Widdel, 1988). Indeed, formate utilization is indicative of the presence of hydogenotrophic sulfate-reducing bacteria (de Smul et al., 1999). High sulfate removal efficiency in the experimental results indicates the presence and growth of sulfate reducers.

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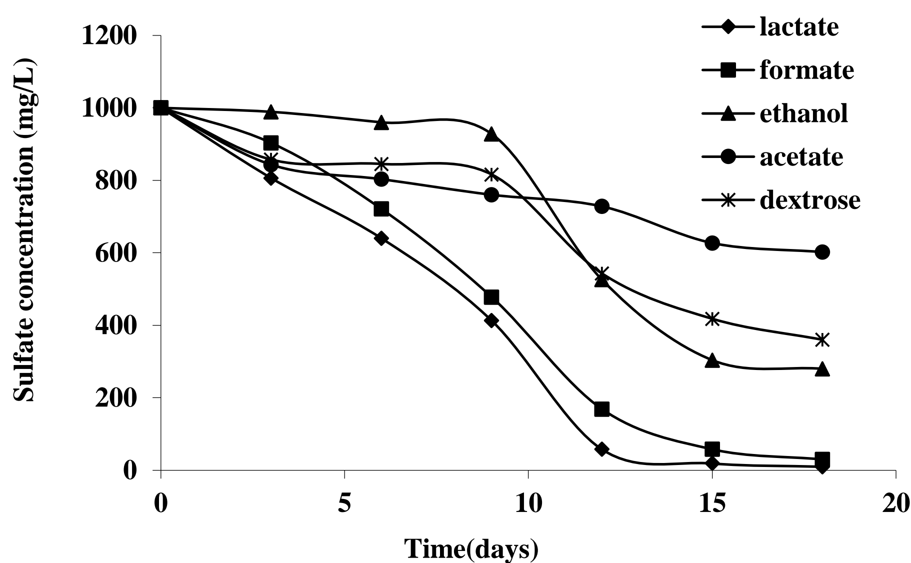

Figure 4.7 Performance of the mixed microbial culture on SO_4^{2-} reduction with the five carbon source

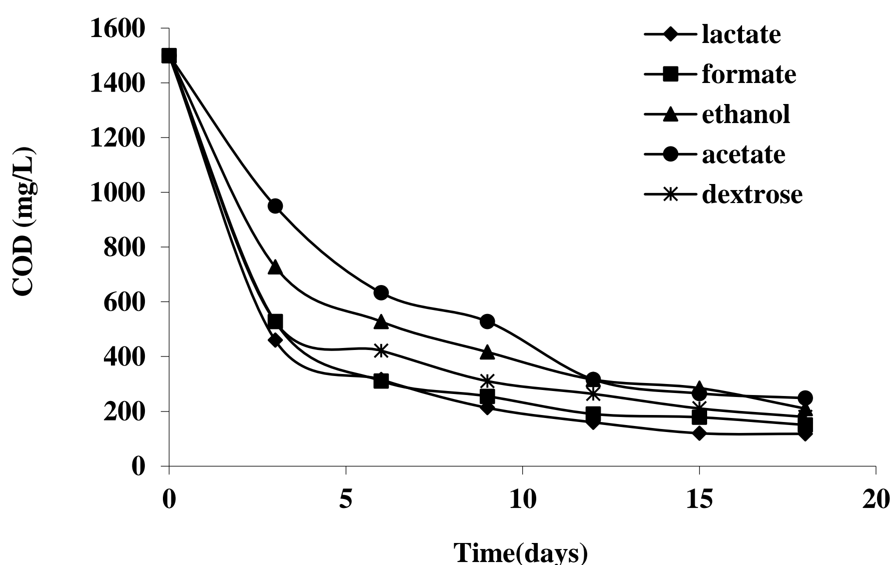

Figure 4.8 Performance of mixed microbial culture on COD removal with the five carbon source

The probable reaction mechanisms of sulfate reduction (Liamleam and Annachhatre, 2007) by the mixed consortium utilizing the five different carbon sources and Gibbs free energy calculated at pH=7 ($\Delta G°'$) are given below:

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For acetate,

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- \quad 4.2$$

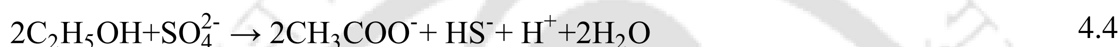
$\Delta G°' = -41.6 KJ/reaction$

For dextrose,

$$C_6H_{12}O_6 + 3SO_4^{2-} \rightarrow 3HS^- + HCO_3^- + 3H^+ \quad 4.3$$

$\Delta G°' = -572.2$ KJ/reaction

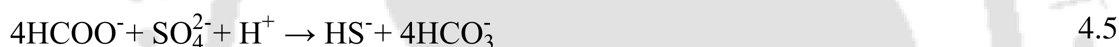
For ethanol,



$$2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O \quad 4.4$$

$\Delta G°' = -172.6$ KJ/reaction

For formate,



$$4HCOO^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^- \quad 4.5$$

$\Delta G°' = -106.1$ KJ/reaction

For lactate,

$$2CH_3CHOHCOO^- + 3SO_4^{2-} \rightarrow H^+ + 6HCO_3^- + 3HS^- \quad 4.6$$

$\Delta G°' = -185.4$ KJ/reaction

The reactions in the above equations 4.2, 4.3, 4.4, 4.5 and 4.6 show the energy required for the complete dissociation of the electron donors acetate, dextrose, ethanol, formate and lactate, respectively into hydrogen sulfide, bicarbonate and hydrogen ion. The degree of progress in the reaction depends on the classification of the bacteria along with the inhibitory effect of hydrogen sulfide. In most cases, the reaction will stop before it reaches complete dissociation. Based on the reactions, dextrose has the most energetic reaction so it would be the best electron donor and favorable carbon source for the bacteria. However, lactate was found to be the most

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efficient carbon source as it was capable of promoting the growth of a wide variety of sulfate reducing bacteria in the mixed microbial consortia in our study

The results were fitted to the linearized form of Monod's equation to determine the half velocity co-efficient and maximum substrate utilization rate of the carbon sources used in this study for SO_4^{2-} reduction.

$$\frac{\theta X}{S_0 - S_e} = \frac{K_s}{k} \cdot \frac{1}{S_e} + \frac{1}{k} \qquad 4.7$$

where

θ = HRT (days)

S_0 = Initial Final substrate concentration (mg/L)

S_e = Final substrate concentration (mg/L)

X = Biomass concentration (mg/L)

K_s = Half velocity constant (mg/L)

k= Maximum substrate utilization rate (day^{-1})

The half velocity constant (K_s) and the maximum substrate utilization rate (k) determined for the SO_4^{2-} reduction with the different carbon sources is given in Table 4.1. The maximum substrate utilization rate (k) for lactate which gave the maximum SO_4^{2-} removal efficiency was 0.063 day^{-1} and the half velocity constant (Ks) was found to be 386.17 mg/L as determined from Figure 4.9. The highest k value of lactate shows that the microbial consortia are capable of supporting high biomass growth subsequently leading to higher sulfate reduction.

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Table 4.1 Half velocity constant (Ks) and the maximum substrate utilization rate (k) determined for SO_4^{2-} reduction with the five carbon sources

Electron donor	Ks(mg/L)	k (day^{-1})
Lactate	386.17	0.063
Formate	215.8	0.042
Ethanol	302.01	0.035
Dextrose	532.59	0.055
Acetate	142	0.027

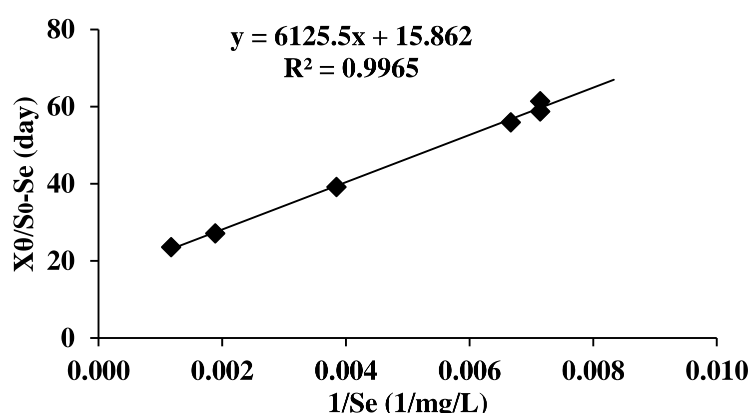

Figure 4.9 Biokinetic coefficients determined for the SO_4^{2-} reduction with the lactate as carbon source

4.2.4 Fed batch reactor with lactate as carbon source:

After the study on effects of different carbon sources on SO_4^{2-} reduction, reactor R3 was fed with lactate which was found to be the most efficient carbon source and the performance of R3 is shown in Figure 4.10. The reactor was started with HRT of 7.5 days in the initial Phase I with initial SO_4^{2-} concentration of 1000 mg/L and COD of 1500 mg/L. At HRT of 7.5 days, R3 showed a maximum SO_4^{2-} reduction of almost 90% after 18 days of reactor operation. This result indicated that SO_4^{2-} reduction was effectively going on in reactor R3 where lactate was used as the sole carbon source and the removal efficiency was better as compared to reactor R1 and R2 with dextrose as the carbon source. The removal efficiency of SO_4^{2-} was almost complete with the

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decrease of HRT to 5 days in Phase II. This might be due to the increase in the SRB population utilizing lactate as the carbon source with the increase in SO_4^{2-} load due to decrease in HRT. As large amount of COD was left out in the effluent, the COD in Phase III was reduced from 1500 to 1200 mg/L. With this reduction in the COD/SO_4^{2-} ratio, the SO_4^{2-} removal efficiency was not hampered. In the next phase (Phase IV), the COD/SO_4^{2-} ratio was further decreased to 1.0 by adjusting influent COD to 1000 mg/L. The removal efficiency of SO_4^{2-} was not reduced even with this COD concentration and still some amount was left out in the effluent. Therefore, in the next phase (Phase V), the influent SO_4^{2-} concentration was increased to 1200 mg/L. The SO_4^{2-} was almost completely removed even when COD/SO_4^{2-} was reduced to 0.8 in Phase V and very less COD was left out in the effluent.

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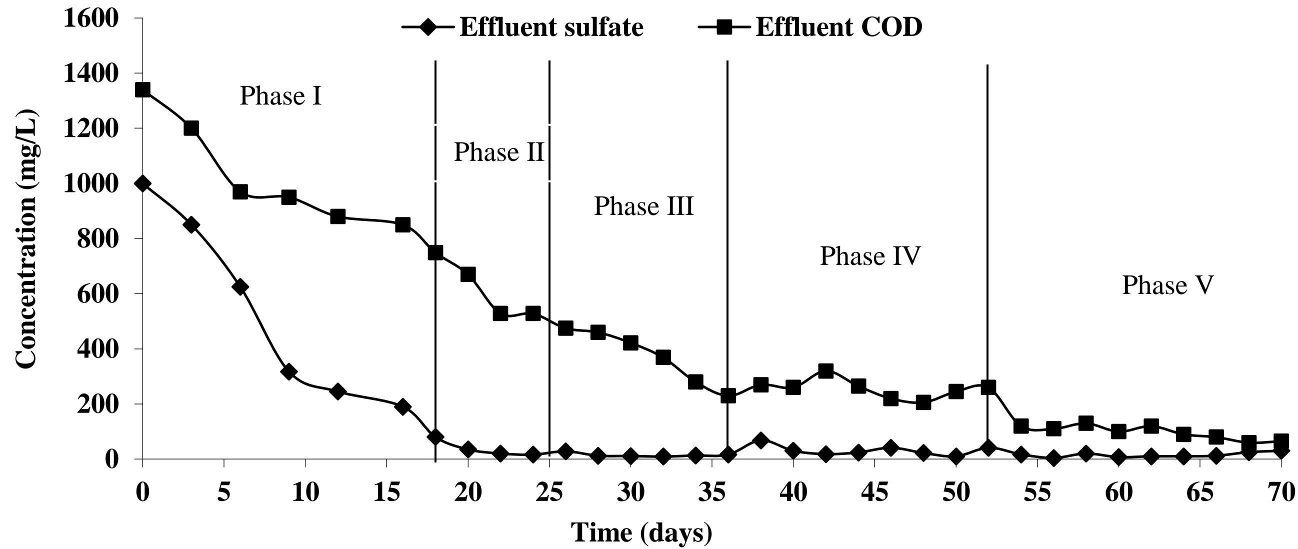

Figure 4.10 SO_4^{2-} reduction profile of reactor R3 with lactate as the carbon source. (Period I: HRT=7 days; SO_4^{2-} =1000 mg/L; COD=1500 mg/L, Period II: HRT=5 days; SO_4^{2-} =1000 mg/L; COD=1500 mg/L, Period III: HRT=5 days; SO_4^{2-} =1000 mg/L; COD=1200 mg/L, Period IV: HRT=5days; SO_4^{2-} =1000 mg/L; COD=1000 mg/L, Period V: HRT=5days; SO_4^{2-} =1200 mg/L; COD=1000 mg/L)

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4.3 **Performance of the PBR in Phase I: Startup of the reactor and optimization of COD/SO_4^{2-} and HRT with lactate as sole carbon source**

4.3.1 PBR startup

The reactor operated first for a period of 30 days in batch mode (close loop) for biofilm establishment with periodic replacement of 2.8 L of the nutrient medium every 24 h. Apart from the permanent characteristic odor of the produced hydrogen sulfide and the black colour of the PUF particles, the establishment of the biofilm was indirectly checked by the systematic pH monitoring of the liquid phase (neutral pH values) and the analytical determination of sulfate concentrations. Figure 4.11 shows the FESEM image PUF particle before acclimation and after acclimation. Following the first days of operation in batch mode, sulfates were reduced to a satisfactory percentage of 80% of the initial sulfate concentration fed to the reactor.

After the steady state results in terms of SO_4^{2-} reduction was obtained, the PBR was operated with SO_4^{2-} concentration of 1000 mg/L and COD also of 1000 mg/L at a HRT of 30 h. The optimization studies of COD/SO_4^{2-} ratio and HRT were carried out thereafter. Overall performance of PBR in terms of SO_4^{2-} and COD removal as well as pH profile after startup period (first 30 days) is shown in Figure 4.12. Results of the profile samples taken on day 262 (influent SO_4^{2-} = 2200 mg/L) is discussed separately.

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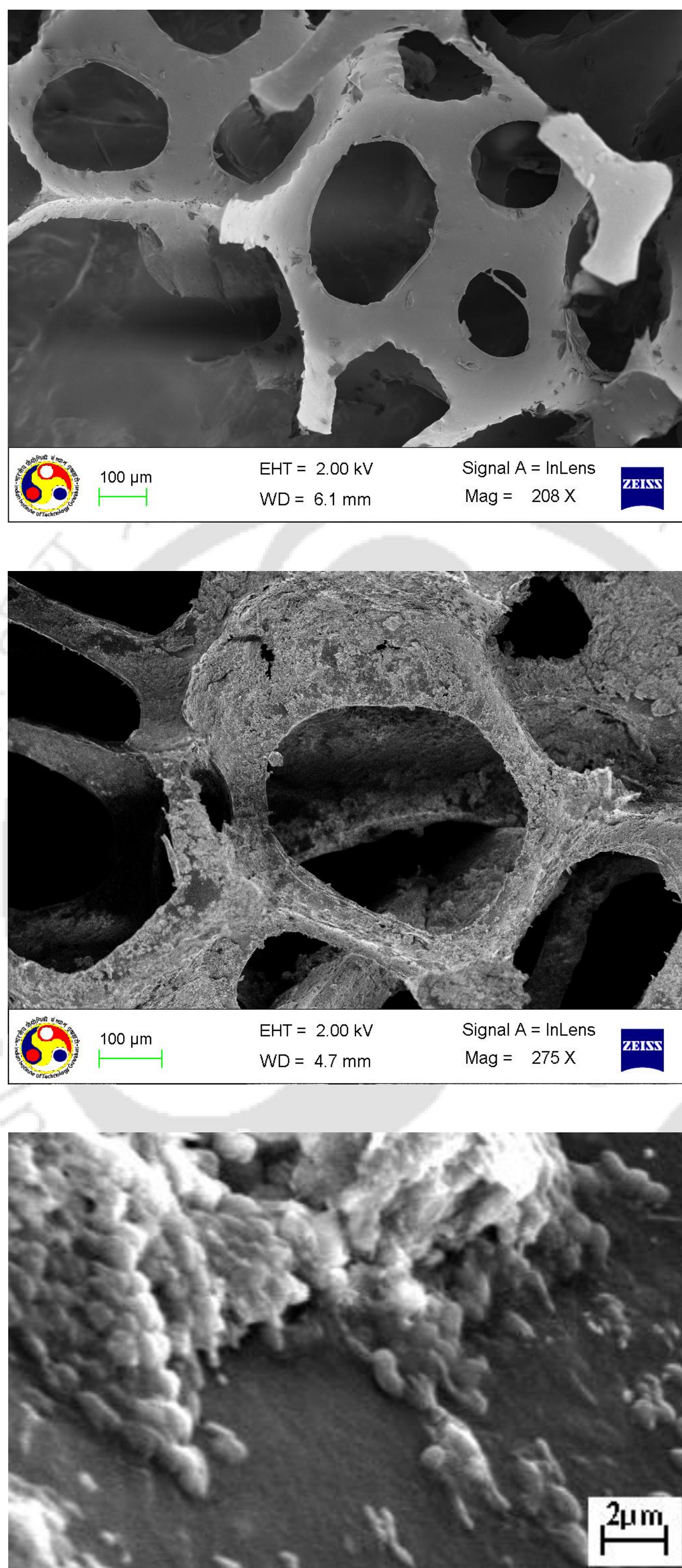

Figure 4.11 FESEM image of the PUF (a) structure of PUF before acclimation and (b) bacterial growth (c) more clear bacterial growth

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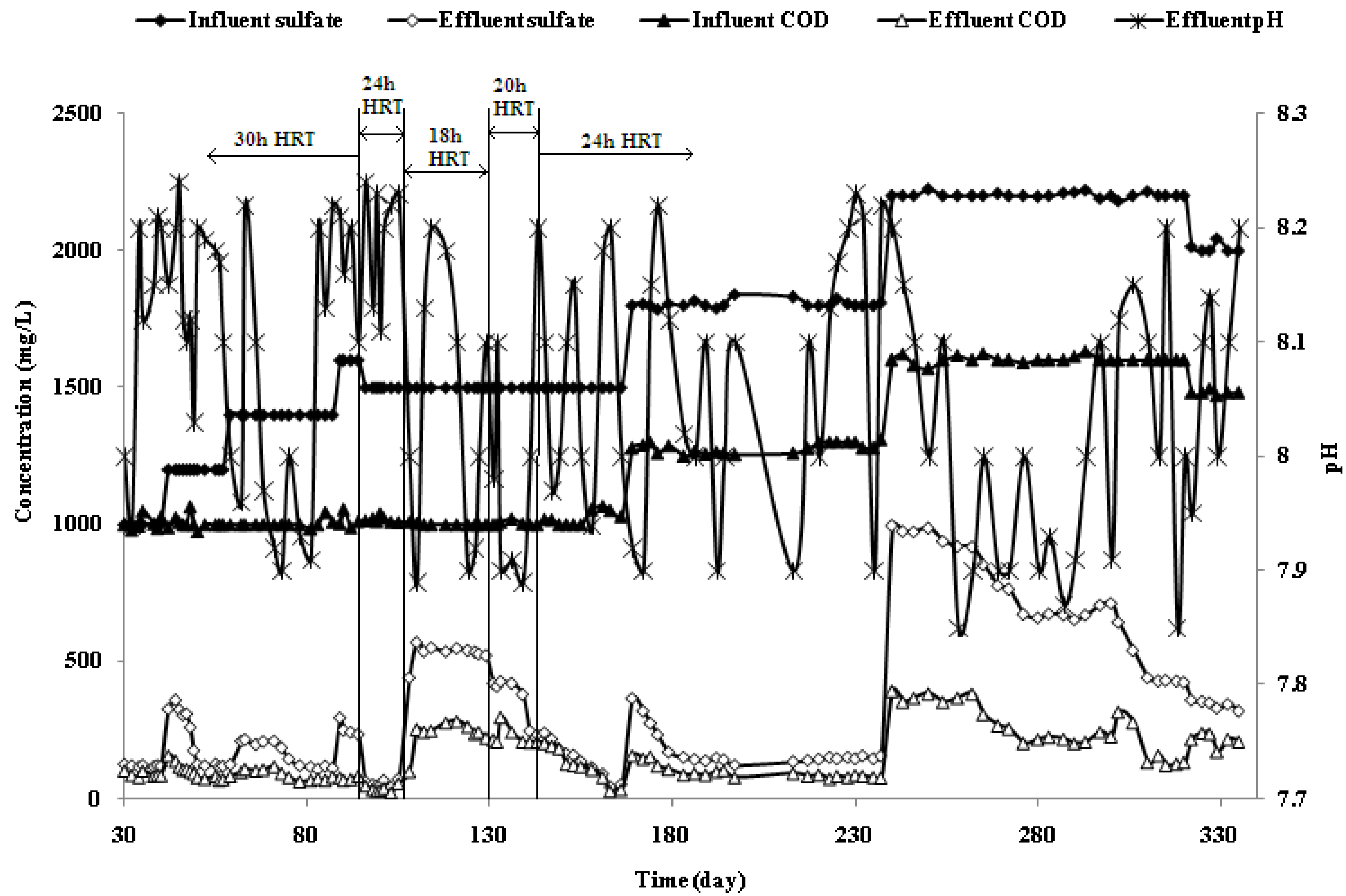

Figure 4.12 Overall performance of the PBR in Phase I

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4.3.2 pH variation and alkalinity and VFA generation in PBR

pH of the effluent was increased from influent pH of 7.0±0.2 due to generation of alkalinity during SO_4^{2-} reduction, as shown in the Figure 4.12. However, the effluent pH was kept between 7.8 and 8.2 by varying the bicarbonate dose between 0.5 and 0.8 g/L (Table 3.3). This value of pH was maintained to keep most of the sulfides in ionized form (HS^- and S^{2-}) without hampering the reactor performance. Neculita et al. (2007) reported that for effective SO_4^{2-} reduction to take place, pH needs to be maintained between 5–8 and redox potential (Eh) less than -100 mV. Outside this range, the rate of microbial SO_4^{2-} reduction generally declines. VFA formation was noticed but never exceeded 200 mg/L as acetate. As such no inhibitory effect of lactate on SRB growth kinetics has been reported. However, as reported by Reis et al.(1992) acetate in its undissociated form is known to inhibit SRB activity at low pH values (≤6). VFA was not a major problem in the present study as the reactor was operated at a pH ≥7. The DO in the effluent was found to below 10 ppb. It is reported that the DO requirement for microaerobic condition is around 100 to 200 ppb (Xu et al., 2012). In the present case, it is well below that level so micro aeration can be ruled out.

4.3.3 Effect of influent COD/SO_4^{2-} ratio

After startup, during the initial phases of reactor run, around 85 % SO_4^{2-} reduction was observed even if the COD/SO_4^{2-} ratio were high. The COD removal efficiency was slightly improved with the increase in SO_4^{2-} concentration from 1000 to 1500 mg/L. However, more than 90% removal of SO_4^{2-} and COD was obtained from day 75 onwards so long the influent COD/SO_4^{2-} ratio was more than or equal to 0.67. The reactor performance deteriorated (day 89–94) when influent SO_4^{2-} was increased to 1600 mg/L. Removal efficiency of the PBR at steady state condition at different COD/SO_4^{2-} ratio is shown in Figure 4.13.

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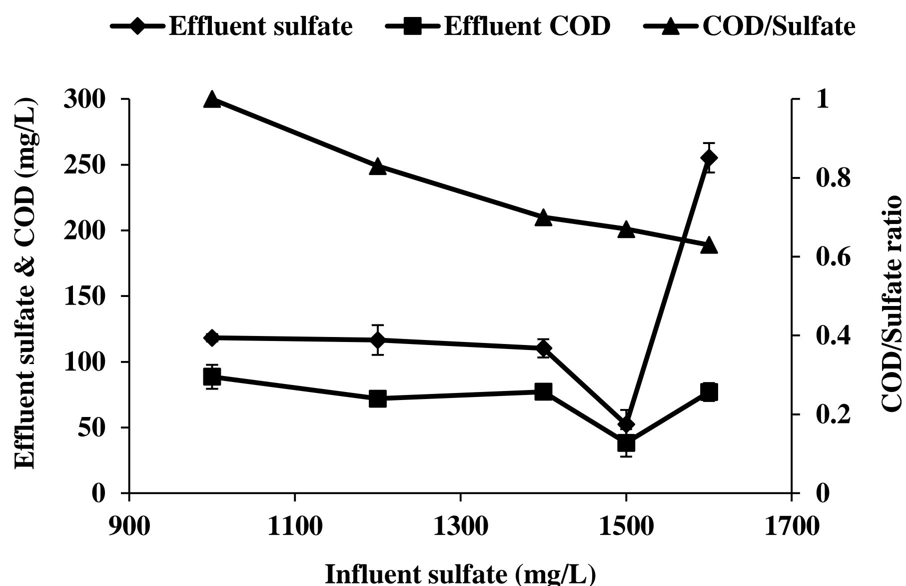

Figure 4.13 Steady state % removal efficiency at different initial SO_4^{2-} concentration and COD=1000 mg/L

During initial phases of reactor run at HRT = 30 h; COD = 1000 mg/L; SO_4^{2-}= 1000 mg/L and 1200 mg/L, profile sampling were done on day 34 and 50, respectively (Table 3.6). It was observed at sampling port 3 that SO_4^{2-} reduction on day 34 and 50, was 83.8% and 82.8%, whereas at sampling port 6, reduction efficiencies were 88.5% and 89.6%, respectively. Similarly, COD removal at port 4 was around 82% and 84.5%, whereas it was almost same (92%) at port 6 on day 34 and 50, respectively. Although, the SO_4^{2-} removal efficiency was better on day 50 when compared with day 34, but the major amount of sulfate reduction took place up to port 4 in both the cases. This suggested non abundance of SO_4^{2-} reducers beyond sampling port 4 even after 50 days of operating the reactor. However, COD reduction was significantly improved at port 6 suggesting the growth of anaerobes as the reactor operations went on. Percentage removal of SO_4^{2-} and COD along the height of the reactor at different COD/SO_4^{2-} ratio is shown in Figure 4.14. It can be clearly seen from Figure 4.14 that the performance of the PBR was improved irrespective of the sampling port of the reactor. Enhancement of SO_4^{2-} and COD reduction with time might be due to the growth of the SO_4^{2-} reducers along the height of the reactor. As compared to day 50

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(COD/SO_4^{2-} = 0.8), profile sampling on day 78 (COD/SO_4^{2-} = 0.7, influent SO_4^{2-} = 1400 mg/L) clearly shows the reduction taking up to the 5th port. Same trend was followed in day 89 as well as in day 99 (Influent SO_4^{2-} = 1600 and 1500 respectively) with significant reduction up to the 5th port. The decrease in reduction efficiency at 1600 mg/L influent SO_4^{2-} may be attributed to the non-availability of the required COD to the SRB along the reactor height. In an anaerobic system, the COD removal can take place through the methanogenic and the SO_4^{2-} reduction pathways. With the increase in the SO_4^{2-} concentration the COD was utilized by the SO_4^{2-} reducers for the reduction of SO_4^{2-} rather than being used by the methanogens under substrate non limiting conditions. In this study, the steady increase in COD removal is credited to the increase utilization for SO_4^{2-} reduction. Hilton and Archer (1988) also observed a higher COD removal in a SO_4^{2-} laden reactor in comparison to a methanogenic reactor when the sulfide toxicity was eliminated. Kaksonen et al. (2004) have showed that stoichiometric COD/SO_4^{2-} ratio of 0.67 were adequate to attain around 60% of SO_4^{2-} reduction with initial SO_4^{2-} concentration of 2000 mg/L. More than 90% SO_4^{2-} reduction was obtained at a COD/SO_4^{2-} ratio of 0.7 and 0.67 from initial SO_4^{2-} concentrations of 1400 and 1500 mg/L, respectively in the PBR at 30 h HRT. The consumption of almost 90% of COD indicates that the biofilm established was capable of utilizing the intermediates generated during lactate metabolism (Kousi et al., 2011). However, performance of the reactor was reduced at COD/SO_4^{2-} ratio less than 0.67. Therefore, all the subsequent experiments were carried out at an initial COD/SO_4^{2-} ratio between 0.67 and 0.7.

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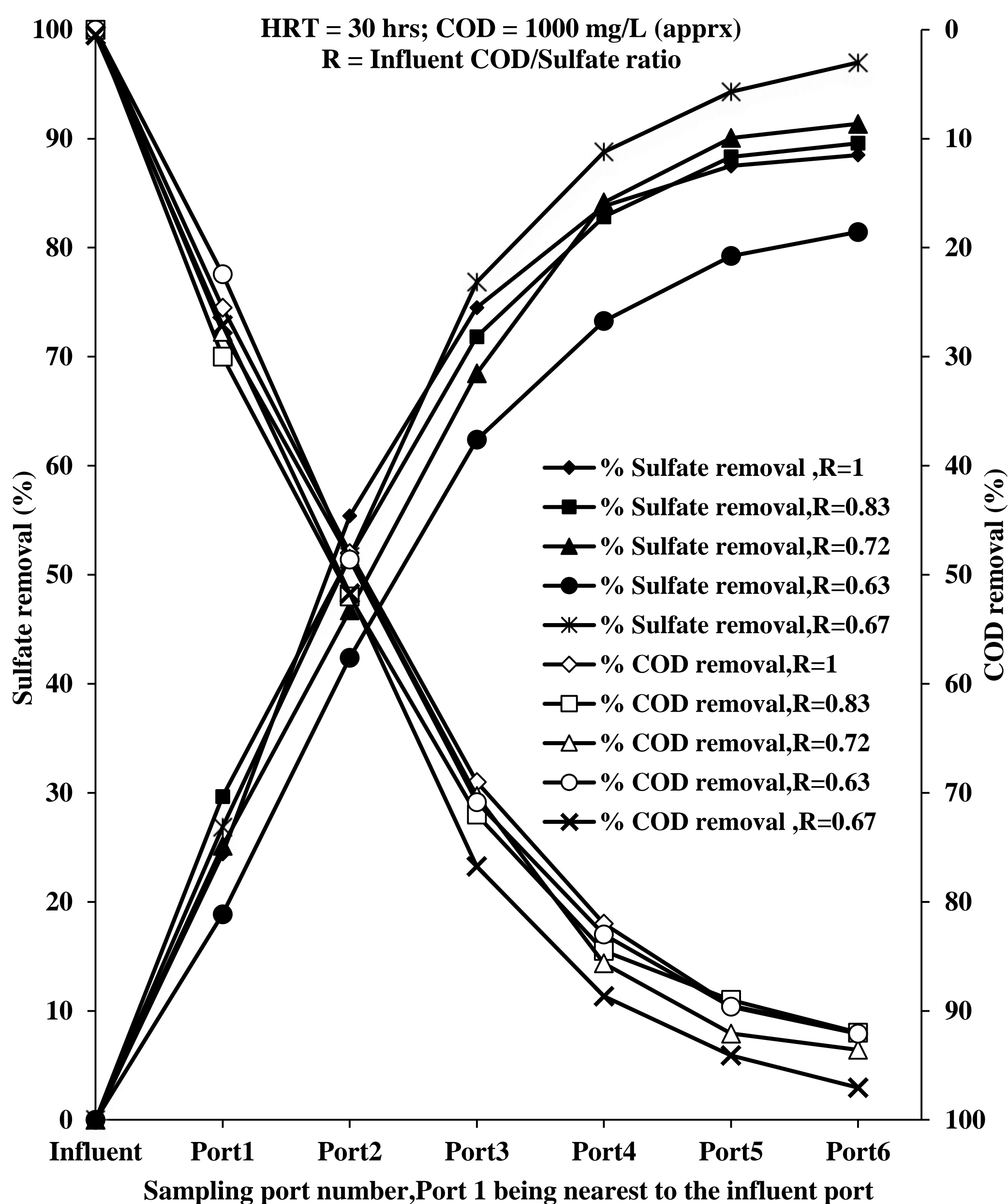

Figure 4.14 Removal efficiency of the PBR at different ports at 30 h HRT with different COD/SO_4^{2-} ratio

4.3.4 Effects of HRT

The removal efficiency at 30 h HRT was very high with around 96% SO_4^{2-} and 97% COD removal. Even when HRT was reduced to 24 h, the removal efficiency of the PBR remained unaffected. The HRT was then further reduced to 18 h after attaining

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the steady state at 24 h HRT with 1500 mg/L of initial SO_4^{2-}. However at 18 h HRT, progressive system failure was observed, indicated by increase in the effluent SO_4^{2-} concentration to around 548±13.7 mg SO_4^{2-}/L (~ 64% removal). During the 18 h HRT, effluent COD was also observed to decrease significantly to 263±17.9 mg/L (~ 73% removal). As no significant improvement in reactor performance was observed, the HRT was then increased to 20 h. Following no improvement in either SO_4^{2-} reduction or COD reduction, the HRT was further increased to 24 h. The reactor performance then improved steadily with around 97% SO_4^{2-} reduction. The SO_4^{2-} removal rate of 60.33 mg/L/h observed at 24 h HRT was the highest as compared to the removal rates at other HRT. Performance of the PBR at steady state conditions at different HRT is shown in Figure 4.15. Thus, the optimum HRT observed was 24 h and all the subsequent experiments were carried out in the PBR at an HRT of 24 h and COD/SO_4^{2-} ratio of 0.67-0.7.

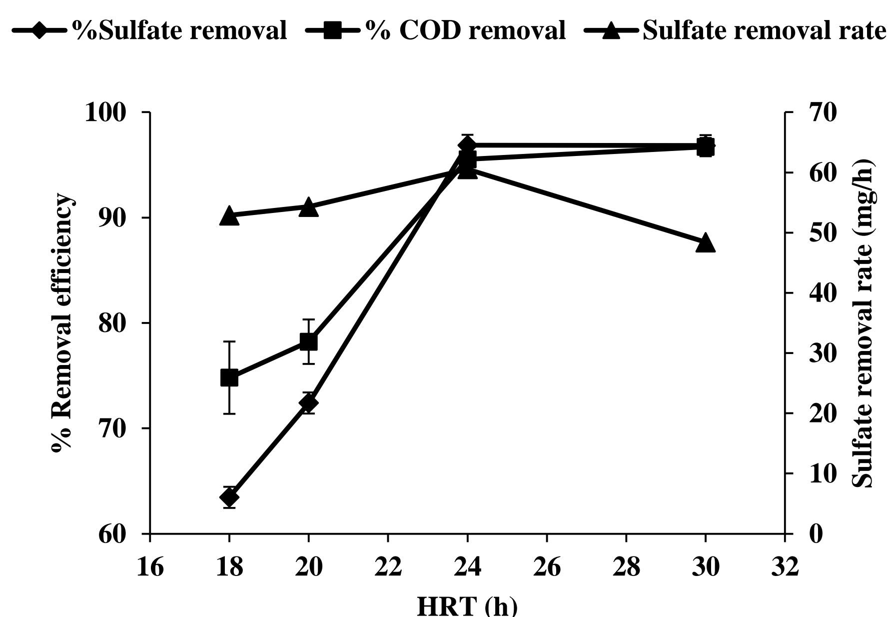

Figure 4.15 Steady state removal efficiency at different HRT. Initial SO_4^{2-} =1500mg/L

Percentage removal of SO_4^{2-} and COD along the height of the reactor at different HRT is shown in Figure 4.16. From the profile sampling, it could observed that significant removal of SO_4^{2-} took place till the 4th port at HRT of 30 and 24 h. However, in the

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case of 18 and 20 h HRT, performance of the PBR reduced drastically. When the reactor was once again operated at 24 h HRT, reactor performance once again improved to its initial level. Profile samples taken on day 163 shows that only 30 mg/L of COD and 42 mg/L of SO_4^{2-} were left in effluent samples (Table 3.6). During initial stages of reactor operation, almost 50% of the total SO_4^{2-} reduction has occurred by port 2. This might be due to non-attachment of the SRB biomass to the packing material in the reactor in the initial stages of reactor operation thereby leading to settling down of the biomass on the packing material with higher bacterial concentrations near the bottom of the reactor, which was also observed by El Bayoumy et al.(1999).

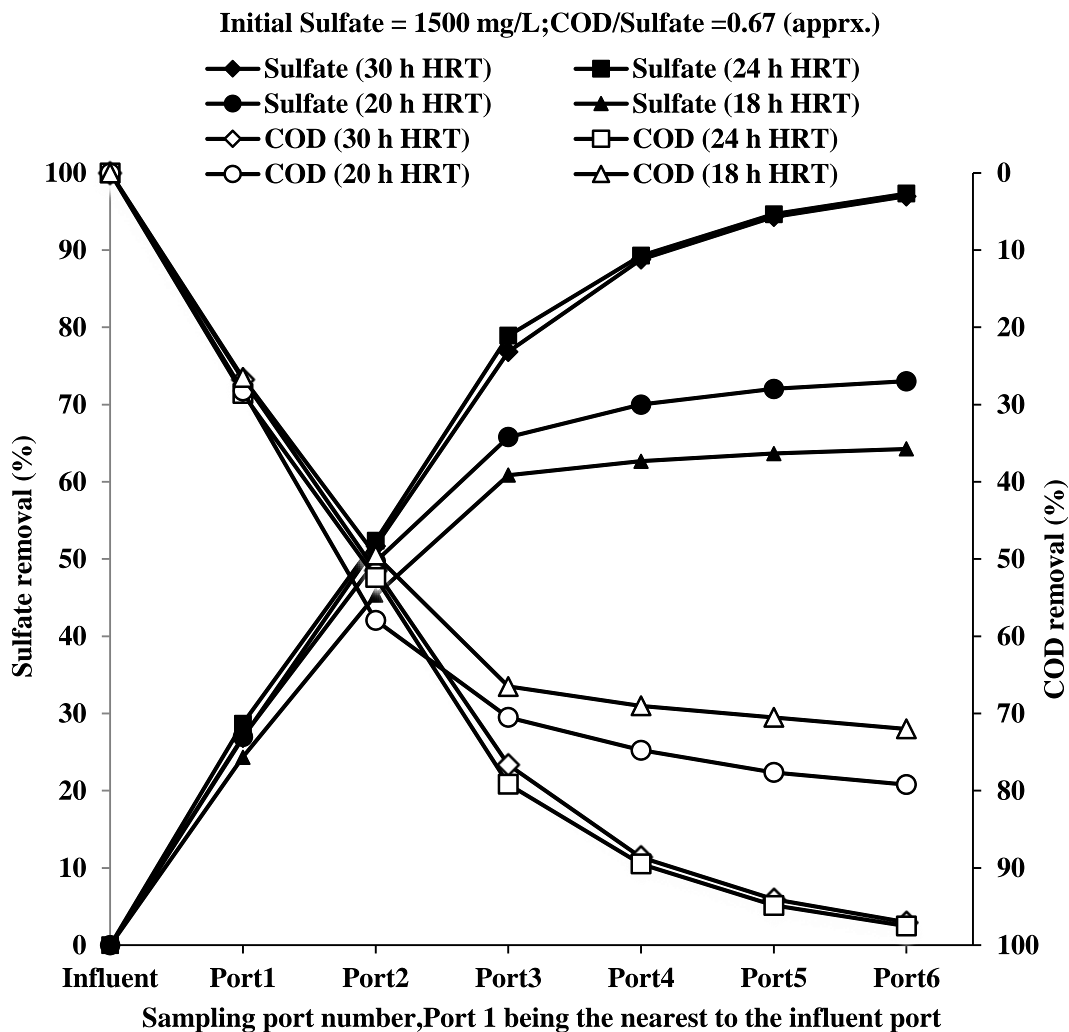

Figure 4.16 Removal efficiency of the PBR at different ports at 1500 influent SO_4^{2-} and different HRT

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4.3.5 Effects of initial SO_4^{2-} concentration

As mentioned in the previous section, the PBR could remove almost 97.3% SO_4^{2-} and 96.7% COD from 1500 mg/L of influent SO_4^{2-} operated at optimum operating conditions of COD/SO_4^{2-} ratio 0.67 and HRT of 24 h. After optimizing the COD/SO_4^{2-} ratio and HRT, the PBR performance was tested at higher influent SO_4^{2-} concentration (i.e. at higher SO_4^{2-} loading rates). At an 1800 mg/L of SO_4^{2-} in the influent around 90% SO_4^{2-} removal was achieved, leaving 180.5±11.5 mg/L of SO_4^{2-} in the effluent. However, the SO_4^{2-} reduction was reduced to about 70%, leaving 634.5±43.5 mg/L of SO_4^{2-} in the treated effluent, when the PBR was fed with 2200 mg/L of SO_4^{2-}. The COD removal was also reduced. The PBR could remove a maximum of 1.8 kg of SO_4^{2-}/day/m^3 of reactor volume at 24 h HRT. This is the maximum specific SO_4^{2-} loading rate that was reported at a minimum HRT (1 day) and COD/SO_4^{2-} ratio (0.70) at which any bioreactor was operated (Khanal and Huang, 2003; Sabumon, 2008; Thabet et al., 2009).

4.3.6 Profile sampling at higher SO_4^{2-} concentration

Sulfide generation increases along the height of the reactor in the overall profiling till SO_4^{2-} concentration of 1800 mg/L. However, at the initial SO_4^{2-} concentration of 2200 mg/L, there was a slight variation in the trend of sulfide generation. After a certain sulfide concentration, a decrease in concentration was observed in the upper sampling ports. It was found from the profile sampling that after the 3rd port, SO_4^{2-} reduction has also decreased. Furthermore, a yellowish white precipitate was seen to deposit in the reactor near sampling port 5 and 6. Anticipating the precipitate as elemental sulfur due to formation of other intermediates and reactions among them, sulfite and thiosulfate concentration was measured. Results of profile samples taken on day 262 (influent SO_4^{2-} = 2200 mg/L) is given in Figure 4.17.

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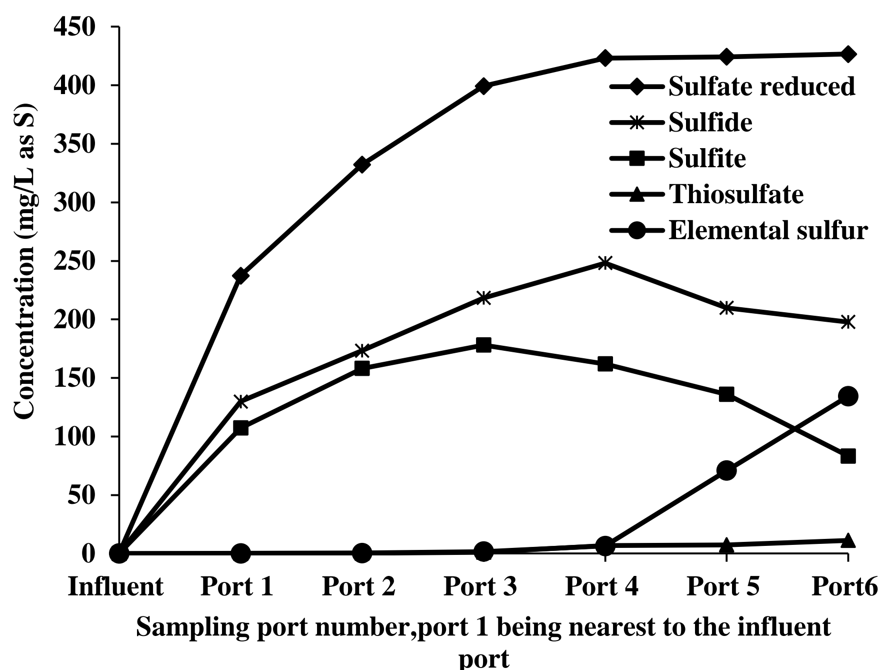

Figure 4.17 Profile sampling of PBR at 2200 mg/L on day 262

The heterogeneity in mass balance in the upper ports shows that in addition to thiosulfate, sulfite, and sulfide, formation of other sulfur species, most probably elemental sulfur, took place in the upper portion of the bioreactor. Energy diffraction X-ray (EDX) data (Figure 4.18) showing chemical composition of the precipitate in the PBR shows that sulfur content is 88% by weight (Table 4.2).

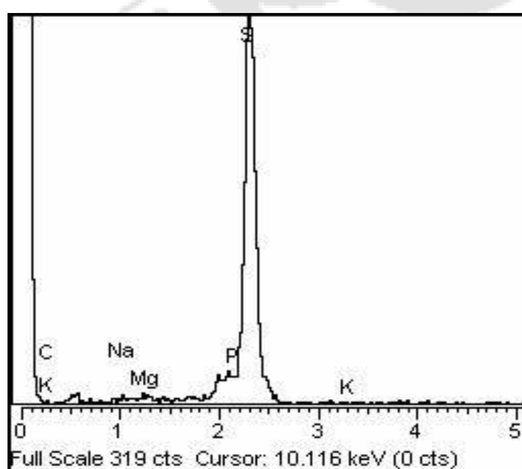

Figure 4.18 EDX spectra of precipitate

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Table 4.2 Chemical composition of precipitate

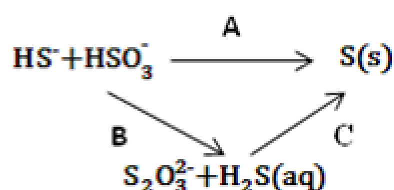
Element	Weight %
C K	8.03
Na K	0.27
Mg K	0.76
P K	2.91
S K	88.00
K K	0.04

Moreover, Gibbs free energy also supports the hypothesis of possibility of reaction between sulfite and sulfide species as shown in Eq. 4.8 generated in the upper ports, leading to the formation of elemental sulfur.

$$2HS^{-}+SO_3^{2-}+4H^{+}\rightarrow 3S^{0}+3H_2O \quad 4.8$$

$\Delta G^{o'}$(KJ/electron) = -22.36

The products in the reaction between sulfite and sulfide are complicated and very much dependent on the pH of the solution and the ratio of the two reactants (sulfide and sulfite) as well. In the higher ports of the reactor, as the sulfide concentration increases with pH, reactions between sulfite and sulfide takes place leading to the formation of thiosulfate and elemental sulfur, which was observed as yellow precipitates in the upper portion of the bioreactor. Siu and Jia (1999) proposed a reaction scheme having pathway ‘A’ representing all possible reactions leading to elemental sulfur without formation of thiosulfate. Reactions ‘B’ and ‘C’ in sequence offer another pathway of elemental sulfur formation where thiosulfate is produced as a stable intermediate.



This reaction explains the decrease in the sulfite as well as sulfide concentration in the upper ports as both have been converted to thiosulfate or elemental sulfur as shown in the pathways A, B, and C.

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4.4 Performance of the PBR in Phase II: Effect of phenol as co-substrate on sulfate reduction

The performance of the PBR was studied under two different conditions. In condition 1, both phenol and lactate were varied in concentration to maintain a constant COD/SO_4^{2-} ratio of 0.7. After analyzing the residual COD, phenol and SO_4^{2-} periodically, the reactor was then operated in another condition where the lactate COD was kept constant at 1480 mg/L and phenol was varied from 100 mg/L and increased upto 350 mg/L as earlier.

A) Reactor performance at constant COD/SO_4^{2-} ratio

The reactor was fed with SO_4^{2-} concentration of 2000 mg/L and COD of 1480 mg/L, thus giving COD/SO_4^{2-} ratio of 0.7. Phenol was increased stepwise from initial dose of 25 mg/L upto 350 mg/L with adjustment of lactate concentration. The PBR performance at constant COD/SO_4^{2-} is shown in Figure 4.19. In the initial stages of reactor run, the effluent SO_4^{2-} concentration for phenol dose of 25 to 50 mg/L remained in between 340 to 370 mg/L, thus giving SO_4^{2-} removal efficiency greater than 80%. However as the phenol dose was increased stepwise from 100 mg/L to 200 mg/L, a larger residual SO_4^{2-} was observed in the effluent showing a decline in sulfate removal efficiency. This might be due to the less abundance of phenol utilizing bacteria in the reactor and apparently more time for adaptation was required by the biomass to sustain the presence of phenol in the influent feed. When the phenol concentration was increased to 250 mg/L, the reactor was operated for a span of 59 days (day 133 to192) in order to provide sufficient time for acclimation of the phenol utilizing bacteria. Thus, SO_4^{2-} reduction improved with due course of time and reduction efficiency as high as 78% was obtained with 250 mg/L phenol. The reduction efficiency continued to remain in this range but with the addition of 350 mg/L, there was a sudden decrease. In this phase, the effluent SO_4^{2-} did not decrease to less than 1147± 48 mg/L, thus giving an efficiency of 40±2% even when the reactor was operated for more than one month. Phenol reduction was also observed to

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decrease from 100 % at 25 mg/L concentration to 72% at 150 mg/L. However, the efficiency increased slightly to 75% when the reactor was run with 200 mg/L phenol. In the same stage, SO_4^{2-} reduction has increased indicating utilization of phenol as a co-substrate. However, the phenol reduction efficiency dropped to 43% at 350 mg/L from 78% at 250 mg/L. This indicates that phenol at higher concentration becomes inhibitory and SO_4^{2-} removal efficiency decreases.

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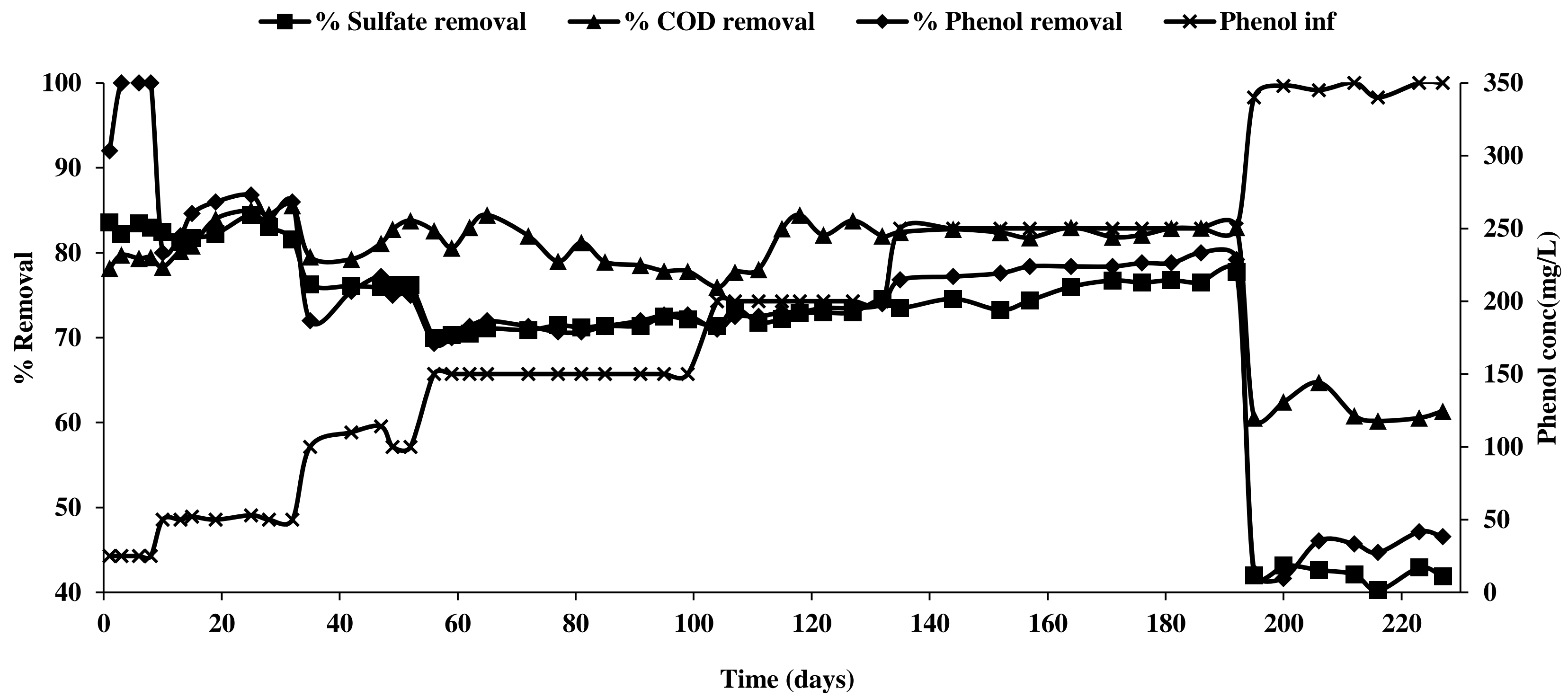

Figure 4.19 PBR performance with phenol addition at constant COD/SO_4^{2-} ratio

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B) Reactor performance at varying COD/SO_4^{2-} ratio

The PBR was operated at varying COD/SO_4^{2-} ratio by addition of varied phenol concentration starting from 100 mg/L while maintaining a constant lactate COD of 1480 mg/L for around 91 days as shown in Table 3.7. The PBR performance with varying COD/SO_4^{2-} ratio is shown in Figure 4.20. SO_4^{2-} reduction improved to 76±2 % in a span of 15 days when influent phenol concentration was lowered to 100 mg/L from day 228 after reactor operation with high phenol shock load of 350 mg/L. This showed that decrease in SO_4^{2-} removal efficiency, caused by increasing phenol concentration, was reversible. Similar observation was made by Tay et al. (2001), who studied phenol shock loading in UASB reactors and stated that reactors were able to recover fully when the shock loading of phenol was returned to the pre-shock level. The increase in phenol on day 193 caused a drop in the SO_4^{2-} as well as phenol removal, indicating that phenol had a toxic effect on those bacteria which could readily degrade lactate by reducing sulfate. When the phenol loading was lowered from 350 mg/L to 100 mg/L, it removal efficiency improved in less than 15 days. This is due to subsequent lower toxicity exerted by phenol on the microbial population. The reduction efficiency increased to 84% at 250 mg/L of influent phenol. This increase in reduction efficiency can be attributed to the acclimation of biomass for phenol utilization. The removal efficiency again decrease to around 54% again when the influent phenol concentration was increased to 350 mg/L. Phenol removal efficiency continued to be greater than 80% when concentration was maintained between 100 to 250 mg/L. The SO_4^{2-} and phenol removal efficiency as compared to that at constant COD/SO_4^{2-} ratio with the same phenol concentration improved slightly when the lactate COD was kept constant. The reason for this improvement can be attributed to the availability of easily degradable lactate carbon to the SRB for their metabolism and acclimation of biomass to phenol. However, at phenol concentration of 350 mg/L; phenol effluent was around 150±17 mg/L, thereby lowering the reduction efficiency to around 54%. Phenol toxicity to lactate/and phenol consuming microorganisms in the reactor resulted in decrease of full utilization of phenol which was utilized as a co-substrate.

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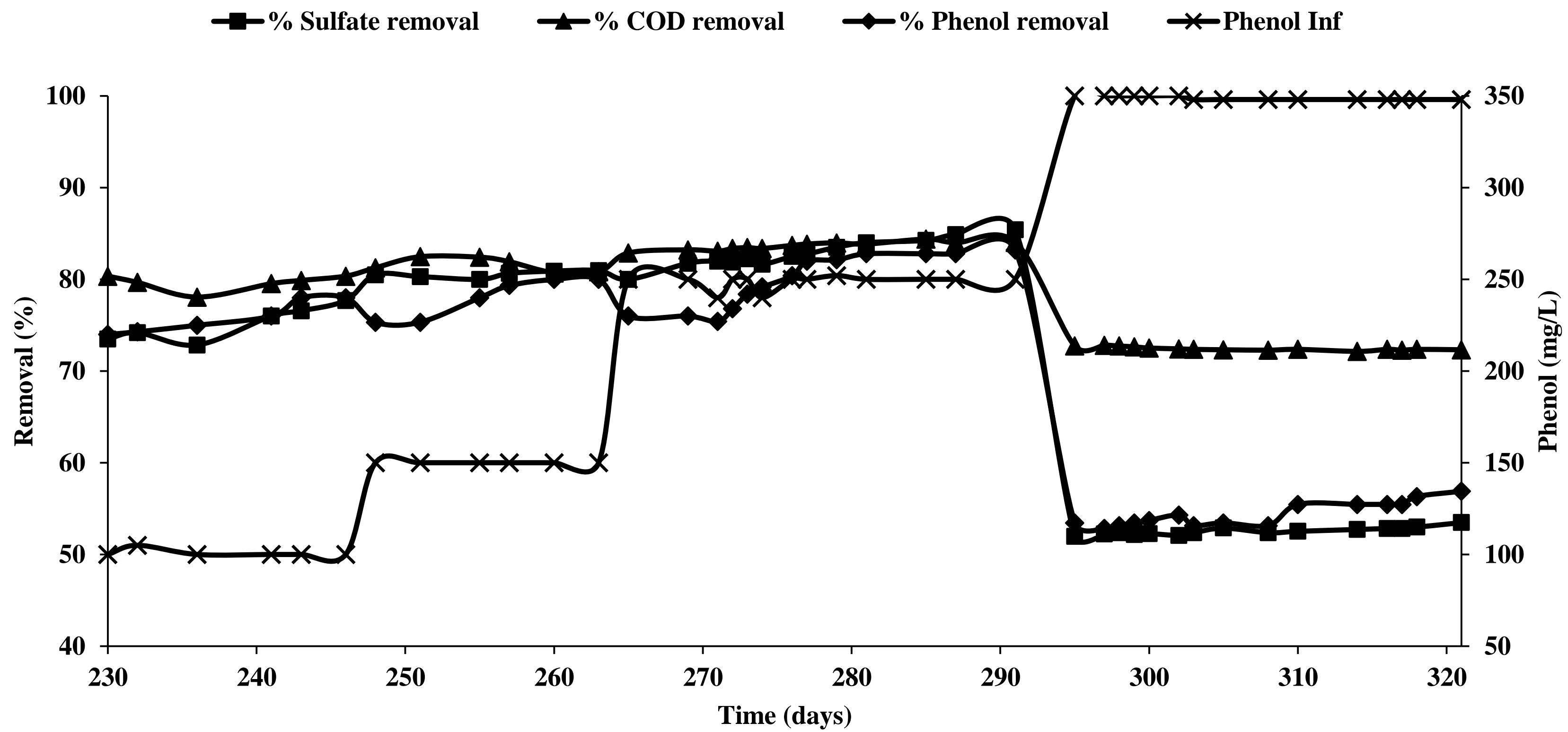

Figure 4.20 PBR performance at varying COD/SO_4^{2-} ratio

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4.4.1 Sampling profile of the PBR at constant and varying COD/SO_4^{2-} ratio

Sampling profile of the PBR at constant and varying COD/SO_4^{2-} ratio with phenol concentration of 250 and 350 mg/L clearly shows that SO_4^{2-} reduction takes place more efficiently when more lactate COD is available (Figure 4.21). The reason is due to the preference of lactate over phenol as the carbon source by the SRB for SO_4^{2-} reduction. However, at phenol concentration of 350 mg/L, even when sufficient lactate is available, the SO_4^{2-} removal efficiency reduced when compared with the removal efficiency at 250 mg/L phenol concentration. This observation shows that at phenol concentration of 350 mg/L, it starts to exhibit inhibitory effects to the SRB. Thus, it can be concluded that lactate COD and phenol concentration are the most important parameters which controls SO_4^{2-} removal efficiency in the PBR with phenol as a co-substrate.

Phenol is a potential inhibitor for microbial growth even at low concentration and may cause instability to biological treatment processes. However some bacteria are able to biodegrade phenol at low concentrations even though inhibition occurs at higher concentrations. The inhibitory potential of phenol to the bacteria is because of the hydrophobicity of the functional groups (Fang, 1997).

When highly inhibitory compounds such as phenol are added along with non-inhibitory co-substrates such as a glucose, acetate, and yeast extract, competition from microbes that can rapidly metabolize the easily biodegradable substrates in preference over the recalcitrant and inhibitory compounds occurs. The fast growing bacterial strains in the consortia that utilize the easily biodegradable substrates and not the inhibitory compounds will outgrow the slow growing bacterial strain capable of utilizing the inhibitory compound (Maszenan et al., 2011). It would be necessary to develop the consortia by directly exposing the seed sludge to the target inhibitory compound without providing any co-substrate.

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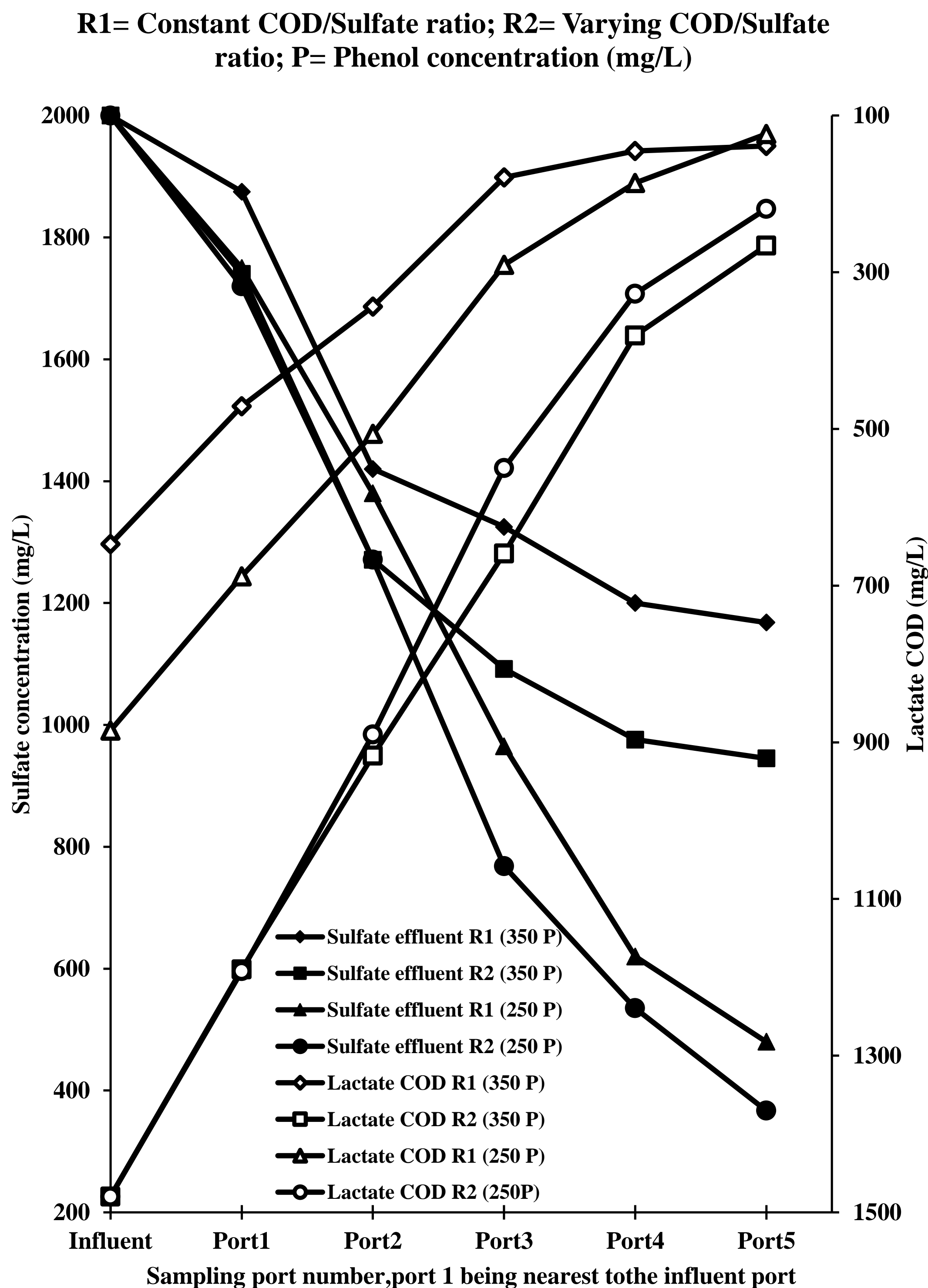

Figure 4.21 Sampling profile of the PBR at constant and varying COD/SO_4^{2-} ratio with phenol concentration of 250 and 350 mg/L

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4.4.2 Implementation of RSM for optimization

Response Surface Methodology (RSM) is an efficient statistical method which explores the relationship between responses and experimental variables to obtain more accurate combination of optimized conditions. The experimental observations (factors) are arranged using a Central Composite Design (CCD) approach with a lower and higher level around the center point. Based on the interactions between different parameters, the optimum value of response can be obtained using desirability function. The experiments conducted under different operating conditions as described before, indicated that lactate COD concentration and phenol are the two critical parameters which affect the process of SO_4^{2-} reduction. The maintenance of COD/SO_4^{2-} of at least 0.7 was also essential for achieving SO_4^{2-} reduction and was dependant on the presence of lactate COD and phenol at different concentrations. Hence to optimize these parameters for achieving maximum SO_4^{2-} reduction, RSM methodology was applied using Design Expert 9.0.

Experiments were carried out to obtain optimum values of lactate COD (mg/L) and phenol (mg/L) based on central composite design (CCD) methodology. Total number of experimental combination was formulated as $2k+2^k+n_0$, where k is the number of factors, and n_0 is the number of replicates at the center point (Araujo and Brereton, 1996). In the present study, a lactate concentration of 1300 mg/L and phenol of 250 mg/L were chosen as the center point (0). The experimental scheme and results are shown in Table 4.3.

A second order polynomial Eq. 4.9 obtained by application of the regression analysis on the experimental coded data for sulfate reduction (Y_1) was formulated as follows:

$$Y_1=82.7+4.37\,X_1-13.64\,X_2-2.80\,X_1^2-14.45\,X_2^2-0.62\,X_1X_2 \quad 4.9$$

Where,

Y_1=Sulfate Reduction (%)

X_1=Lactate COD (mg/L)

X_2= Phenol (mg/L)

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Table 4.3 CCD design for Case-1 and Case-2 with Un-coded, coded values and experimental responses

Run Order No.	Un-coded values		Coded values		Experimental observed Sulfate reduction efficiency(SRE)
	Lactate COD (mg/L)	Phenol conc.(mg/L)	Lactate COD (mg/L)	Phenol conc.(mg/L)	
1	1582.84	250	+α	0	86.2
2	1100	150	-1	-1	71.27
3	1300	108.579	0	-α	76.1
4	1500	150	+1	-1	81
5	1500	350	+1	+1	52.86
6	1300	250	0	0	82.7
7	1017.16	250	-α	0	73.5
8	1300	250	0	0	82.7
9	1100	350	-1	+1	45.6
10	1300	391.421	0	+α	37
11	1300	250	0	0	82.7
12	1300	250	0	0	82.7
13	1300	250	0	0	82.7

4.4.3 Regression analysis

The regression analysis gives an idea of the closeness of the model to the experimental observations. Table 4.4 depicts the regression analysis for the process output. The effect of p values (probability) higher than 0.05 are not significant at the 95% confidence level (Dey and Mukherjee, 2013). In other words, lower the p-value, higher is the significance of the term. In both the cases, the p values for model were very less (<0.0001) and hence it proved that the model was found to be highly significant in describing the sulfate reduction process. If the *F*- value is larger than the critical value from the *F* distribution it indicates that null hypothesis can be rejected whereas p-value determines the appropriateness of rejecting the null hypothesis.The Fisher's F-value for model terms i.e. 70.86 from regression analysis was found to be greater than F critical value ($F_{0.05}$ 5, 7=3.97) thereby suggesting that most of the variations could be expressed by the regression model equation. Moreover, lower p-value was observed in case of linear and quadratic terms of phenol suggesting that it

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is an important parameter which can affect the sulfate reduction in both the cases. In addition, Coefficient of co-relation i.e R^2 for quadratic model was found to be 0.9806 implying a high degree of correlation between the modeled and predicted results.

Table 4.4 Regression results for the experimental responses at different factor levels

Source	**DF**	**SS**	**F-Value**	**p-Value ($F>F_{0.05}$)**	**Significance**
Model	5	3101.62	70.86	0.0001	significant
X_1-Lactate	1	152.69	17.44	0.0042	significant
X_2-Phenol	1	1488.01	169.98	0.0001	significant
X_1* X_1	1	54.71	6.25	0.0410	significant
X_2* X_2	1	1453.42	166.03	0.0001	significant
X_1* X_2	1	1.53	0.17	0.6889	
Residual	7	61.28			
Lack of fit	3	61.28			
Pure Error	4	0.000			
Total	12	3162.90			

4.4.4 Contour and surface response plot

The 3D response surface and the 2D contour plots are the graphical representations of the regression equation. The main goal of response surface is determining the optimum value of the involved variables such that the response can be maximized. Figure 4.22(a) and (b) shows the interaction between various process variables while setting SO_4^{2-} reduction (%) as the target. The contour plots were found to be elliptical suggesting significant interactions between the process variables for achieving SO_4^{2-} reduction. Such elliptical contours are obtained when there is a good interaction between the variables involved (Muralidhar et al., 2001). In addition, the maximum value for SO_4^{2-} reduction was found to take place at the point of interaction between the major and minor axes of ellipse.

It can be clearly seen that the SO_4^{2-} reduction increased with the increase in phenol concentration above 200 mg/L and lactate COD above 1300 mg/L. The peak SO_4^{2-}

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reduction takes place around phenol concentration of 210 mg/L, after which SO_4^{2-} reduction continued to decline. This decline in reduction of SO_4^{2-} removal efficiency could be attributed to inhibitory effects of phenol to SO_4^{2-} reduction with increase in phenol concentration beyond a certain value.

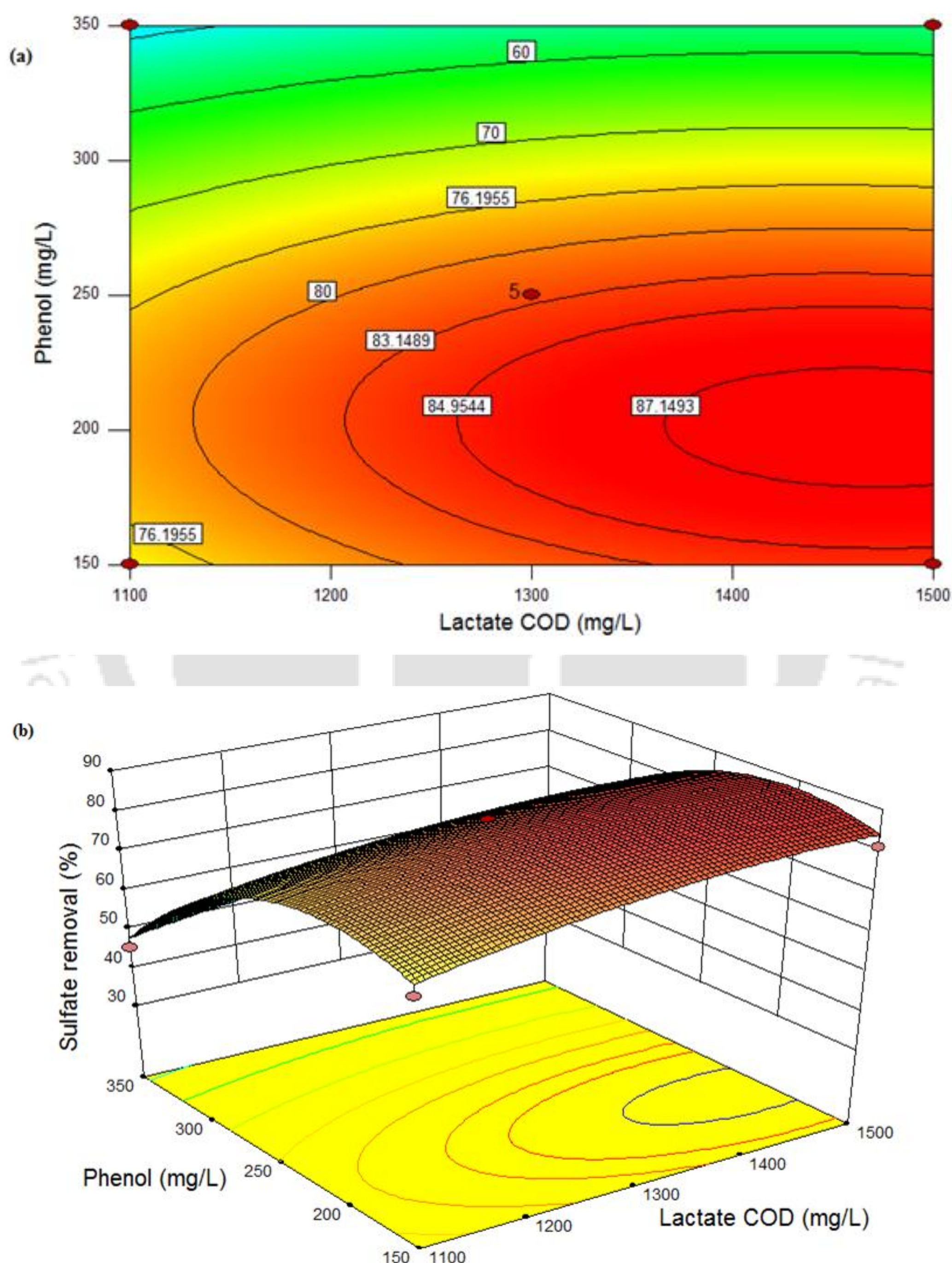

Figure 4.22(a) Contour Plot and (b) 3D Surface Response Plot showing the response of SO_4^{2-} reduction due to varied concentration of phenol and lactate COD

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4.4.5 Optimum lactate COD and phenol concentration

Numerical optimization using desirability function in Design Expert 9.0 has been used to compute the optimum values of lactate COD and phenol at which maximum SO_4^{2-} reduction can be achieved (Figure 4.23). The D-optimality index is used to determine the values of independent variables which results in a maximum response. This process includes the use of a desirability function. The composite desirability is obtained by combining the individual desirability functions of the individual parameters. The composite desirability (D) was found to be 1.000. A value of 1 for D represents an ideal case (Vera Candioti et al., 2014). The plot also reveals that phenol concentration of 210 mg/Land lactate concentration of 1400 mg/L could give the highest SO_4^{2-} reduction of 87.44%. This highest removal efficiency will occur at a COD/ SO_4^{2-} ratio of around 0.95.

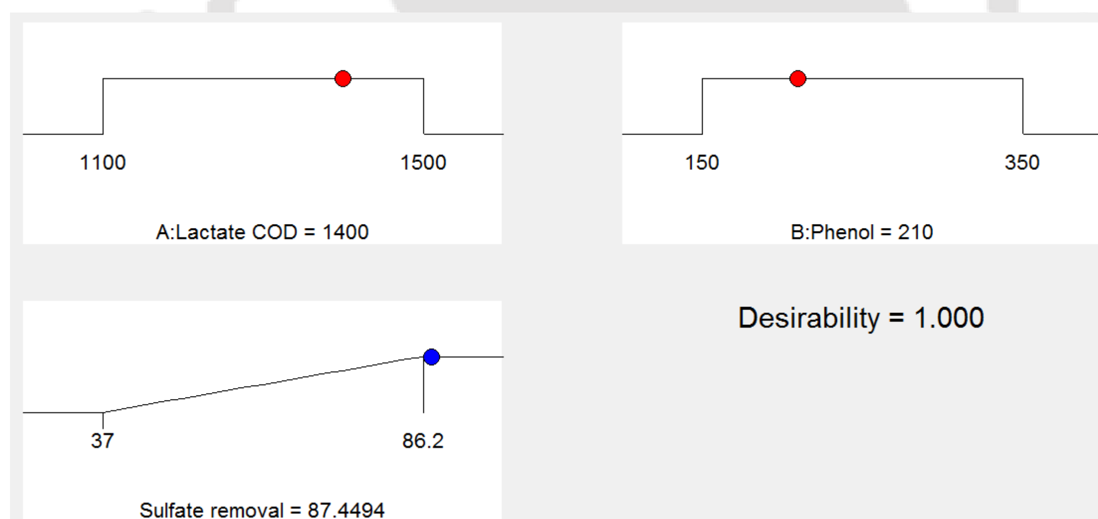

Figure 4.23 Desirability plot showing Maximum SO_4^{2-} reduction due to optimum amounts of phenol and lactate COD concentration (mg/L)

4.4.6 Evaluation of predicted values and model validation

The computed value of SO_4^{2-} reduced as per Eq.5.10 correlates with the experimental values (R^2 >0.9) as can be seen from Figure 4.24. This proves that the model fitting could explain the experimental results.

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The computed optimum conditions were lactate COD of 1400 mg/Land phenol concentration of 210 mg/L. In this condition, SO_4^{2-} removal observed from experiments was 86.4 % while the predicted SO_4^{2-} removal efficiency was 87.45 %, indicating reliable optimized result.

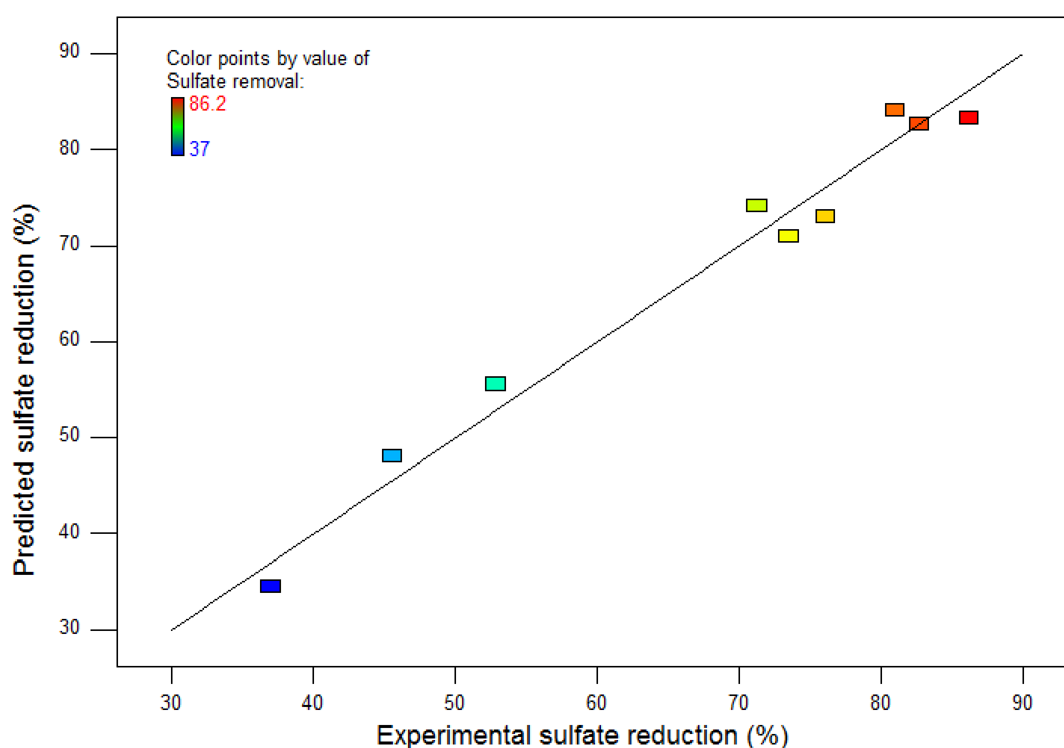

Figure 4.24 Assessment of the accuracy of the RSM using a plot of model SO_4^{2-} reduction and experimental sulfate reduction

4.5 **Performance of the PBR in Phase III: Effect of phenol as the sole carbon source**

When phenol was used as the sole carbon source, SO_4^{2-} and phenol removal of about 22% and 60% respectively was observed with an HRT of 24 h. The overall PBR performance with phenol as sole carbon source is shown in Figure 4.25. The mixed consortia in the PBR had become acclimatized to phenol and could utilize it as the carbon source for sulfate reduction when lactate was no longer provided. However, even after 34 days of operation the phenol removal efficiency has not improved. Therefore, the HRT was increased to 30 h from day 35 onwards. The SO_4^{2-} and phenol removal efficiency then improved to around 34 and 77%, respectively. Further

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improvement in reactor performance was observed with increase in HRT to 36 h. Therefore, the HRT was further increased to 48 h so as to get higher removal efficiency. However, the removal efficiency was almost similar to that at 36 h HRT, around 84% phenol and 39% SO_4^{2-} removal. HRT was brought down to 36 h in view of the no further improvement in performance with increase in HRT. In the next step, maintaining constant HRT of 36 h, the phenol concentration was increased stepwise from 350 to 450, and then to 550 and 750 mg/L. Almost 83 % of phenol removal was observed and SO_4^{2-} removal also increased to 63% when phenol concentration was increased from 350 to 450 mg/L. The reason for this could be attributed to the fact that mixed consortium had become acclimatized to phenol. However, with the increase in phenol concentration to 750 mg/L, both phenol as well as SO_4^{2-} removal efficiency dropped down to 40 and 36%, respectively.

The effluent sample at 350 mg/L phenol (day 72) was analyzed by LC-MS to know the intermediates and understand the degradation pathway. The spectra obtained shows the formation of 4-hydroxybenzaote, protocatechuate, and phenylphosphate (Figure 4.26) which confirm the anaerobic benzoate pathway proposed by Williams and Evans (1975) leading to the formation of acetyl CoA as the degradation product (Sridevi et al., 2012).

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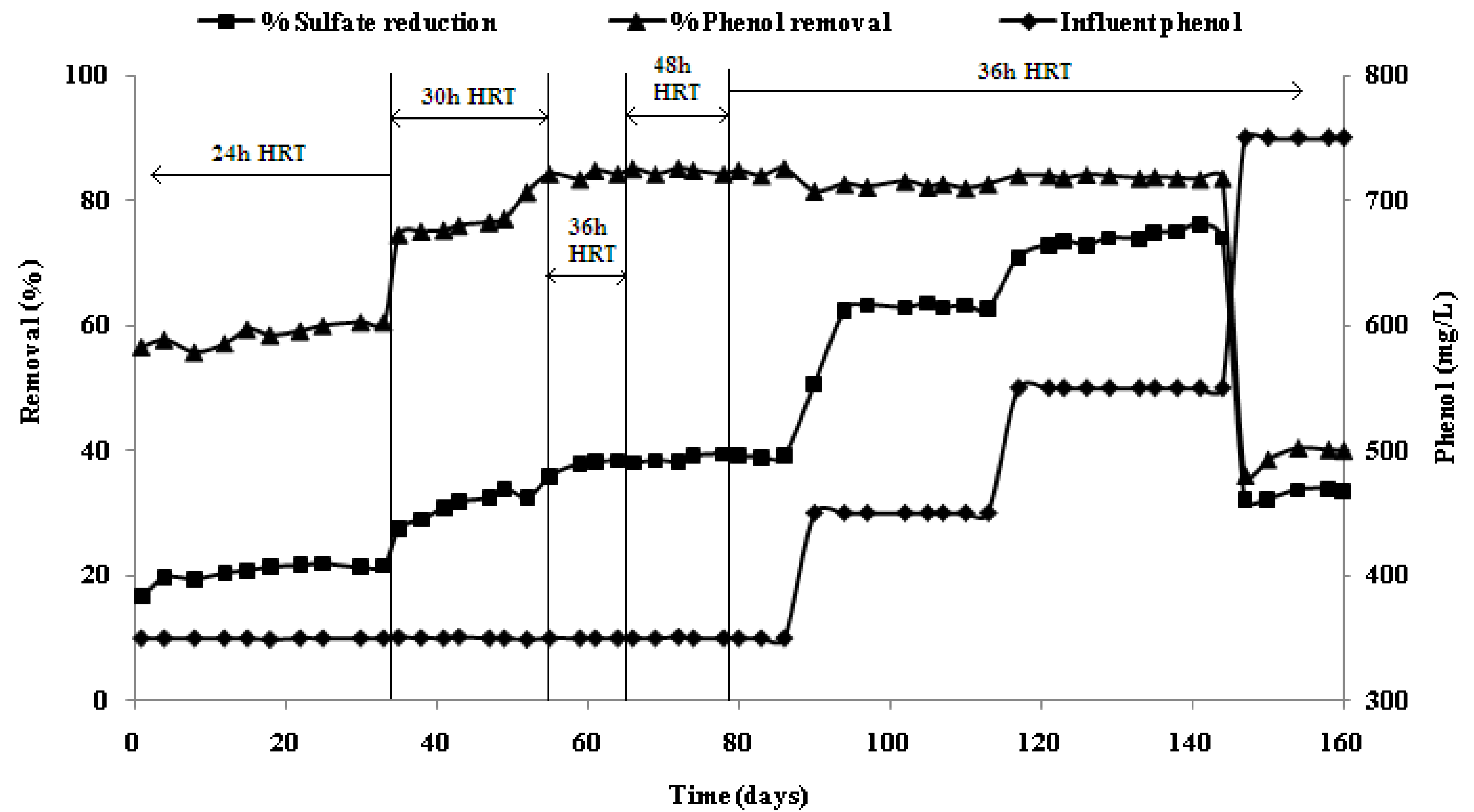

Figure 4.25 Overall PBR performance during the Phase III

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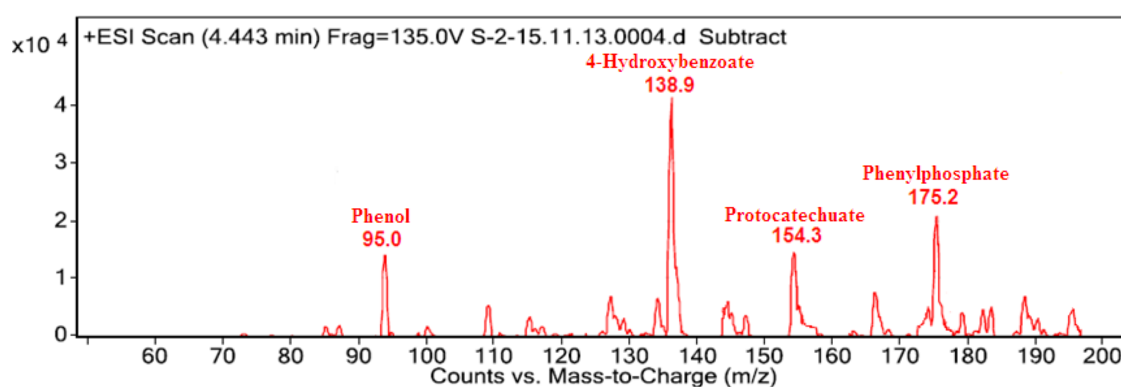

Figure 4.26 LC-MS spectra of intermediates obtained with phenol as sole carbon source

4.5.1 RSM implementation for optimization

After the reactor was run up to 750 mg/L phenol at a HRT of 36 h, experimental runs following RSM methodology were carried out to search for the optimum phenol concentration, and HRT at which maximum phenol and sulfate removal (%) can be achieved simultaneously. In these experiments, the response surface design was based on central composite design (CCD) where the factorial portion was a full factorial design (FFD) with all combinations of the two factors i.e phenol concentration and HRT at two levels (high, +1 and low,-1 levels), the centre points (coded level 0 or the basic level), which were the midpoint between the high and low levels. The coded levels with 0 for phenol and HRT were repeated six times and the axial or star points for which all but one factor was set at level 0 and the 1 factor was set at the outer value corresponding to an value of 1.414. The processing parameters involved in the study are shown in Table 4.5 while the full factorial design is represented in Table 4.6.

Table 4.5 Processing parameters involved in Central composite design (CCD)

Process variables	-α	-1	0	+1	+α	Variation interval
Phenol (mg/L)	267	350	550	750	834	200
HRT (h)	19	24	36	48	53	12

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Table 4.6 Experimental runs following a full factorial design

Run Order No.	Un-coded values		Coded values		Experimental observed Sulfate reduction efficiency	Experimental observed phenol removal efficiency
	Phenol conc. (mg/L)	HRT (h)	Phenol conc. (mg/L)	HRT (h)		
1	550	53	0	+α	80	87
2	550	36	0	0	76	84
3	550	19	0	-α	47	56
4	267	36	-α	0	34	85
5	550	36	0	0	76	84
6	834	36	+α	0	30	32
7	550	36	0	0	76	84
8	750	48	+1	+1	35	43
9	550	36	0	0	76	84
10	350	24	-1	-1	22	61
11	550	36	0	0	76	84
12	750	24	+1	-1	27	32
13	550	36	0	0	76	84
14	350	48	-1	+1	39	85

4.5.2 Regression analysis

The regression analysis demonstrated that the quadratic regression models for describing phenol and sulfate removal give a value of the determination coefficient (R^2) of 0.9552 and 0.984, respectively (Table 4.7). This suggests that the variation could be explained by the fitted quadratic model. The closer the value of R^2 to unity, the better the empirical models fits the actual data. On the other hand, the smaller the value of R^2 the less relevant the dependent variables in the model have in explaining the behaviour of variations (Xu et al., 2010).The p-values were used as a tool to check the significance of each coefficient, which in turn might indicate the pattern of the interactions between the variables. Through multiple regression analysis on the experimental data, the model for the predicted response for phenol and sulfate removal efficiency was expressed by the following quadratic polynomial Eqs. 4.10 and 4.11 using un-coded values.

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$$Y_1=-114.8+0.375\,X_1+5.90\,X_2-3.79X10^{-4}\;X_1^2-0.060\,X_2^2 -0.00135\,X_1X_2 \qquad 4.10$$

$$Y_2=-260.1+0.75X_1+6.51X_2-6.564X10^{-4}\;X_1^2-0.072X_2^2 -9.375X10^{-4}\,X_1X_2 \qquad 4.11$$

where,

Y_1= Phenol removal efficiency (%)
Y_2= Sulfate Reduction efficiency (%)
X_1= Phenol (mg/L)
X_2= HRT (h)

Table 4.7 shows the regression analysis for the process output. The regression equations for phenol removal and sulfate removal were highly significant, demonstrating that the degree of fit was better on the border of the variables taken to describe the process output.

Table 4.7 Regression analyses for phenol removal and sulfate reduction

Terms	**Phenol reduction (%)**			**Sulfate reduction (%)**		
	F-value	**p**	**Significance**	**F-value**	**p**	**Significance**
Regression	43.48	0.000	significant	15.41	0.001	significant
X_1-Phenol	33.58	0.000	significant	42.85	0.000	significant
X_2-HRT	28.38	0.001	significant	10.89	0.011	significant
X_1* X_1	65.92	0.000	significant	62.38	0.000	significant
X_2* X_2	21.7	0.002	significant	10.01	0.013	significant
X_1* X_2	1.64	0.236		0.25	0.632	
R^2			0.964			0.905

4.5.3 Contour and Surface response plot

Another way to predict the relationships between responses, factors, and interactions is to analyse the contour and surface response plot. Each plot represents a number of combinations of two test variables along with the effect on the response. In a contour plot, the response surface is viewed as a two dimensional plane where all points that have the same response are connected to produce contour lines of constant responses. The surface response plot on the other hand describes the interaction between two different variables on the response in space. These plots show how a response variable relates to two factors based on the model equation. They are useful for establishing a

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desirable response values and operating conditions. Figure 4.27(a) and Figure 4.28(a) depicts the contour plots for achieving maximum SO_4^{2-} and phenol removal, respectively. These show that the highest phenol removal and SO_4^{2-} reduction for higher phenol concentration could be achieved when a higher HRT was maintained. The surface response plot shown in Figure 4.27(b) and Figure 4.28(b) for achieving maximum SO_4^{2-} and phenol removal, respectively, further strengthens the results obtained in contour plots.The elliptical contour plot showed a significant interaction between the two variables (Wang and Lu, 2005).

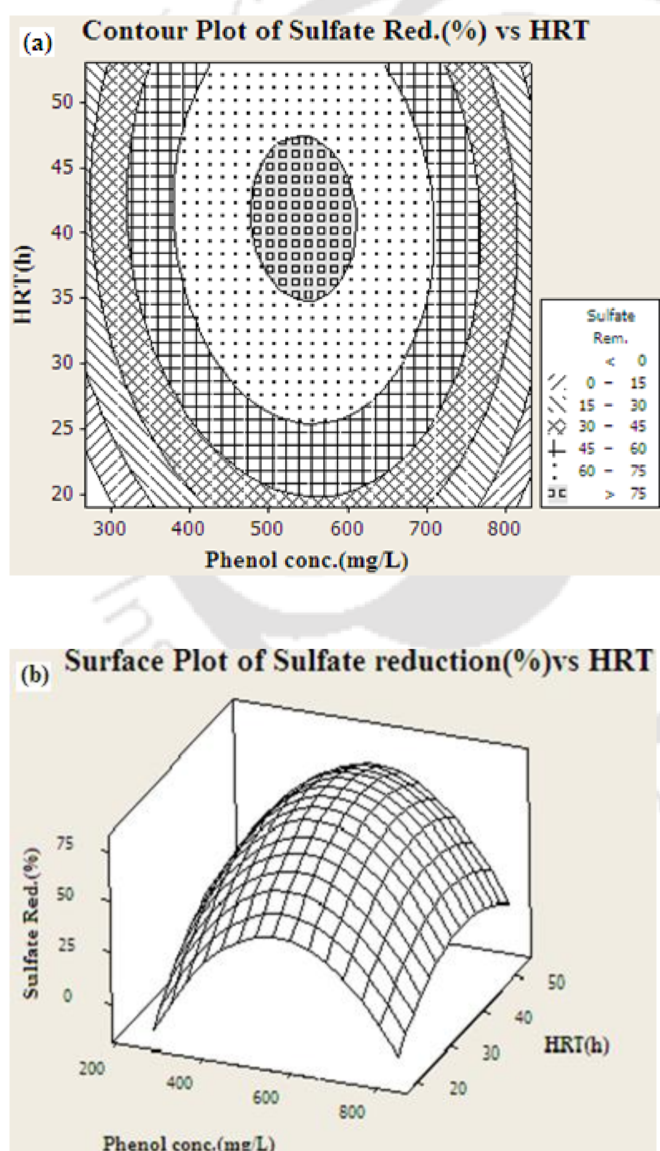

Figure 4.27(a) Contour Plot and (b) 3D Surface Response Plot showing the response of SO_4^{2-} reduction due to phenol concentration and HRT

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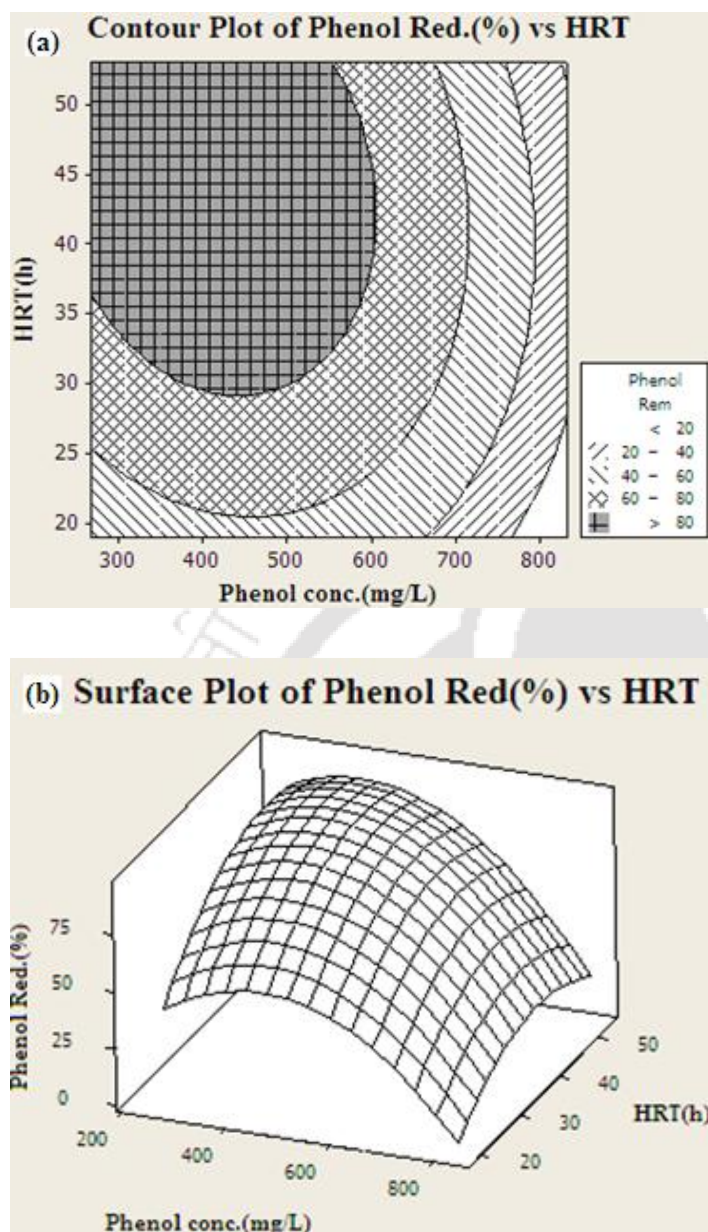

Figure 4.28(a) Contour Plot and (b) 3D Surface Response Plot showing the response of phenol removal due to phenol concentration and HRT

4.5.4 Optimum values for phenol removal and SO_4^{2-} reduction

Further response optimizer of MINITAB 15.0 was used to find the combination of optimum phenol concentration and HRT required for achieving maximum phenol removal and SO_4^{2-} reduction. The response optimizer takes desirability function into account and calculates individual desirability and composite desirability (weighted geometric mean of the individual desirabilities for the responses) into account. The

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optimizer yielded a value of 511.7 mg/L for phenol concentration and 42 h HRT, with corresponding maximum phenol removal of 90% and SO_4^{2-} reduction of 77.2%, as shown in Figure 4.29.

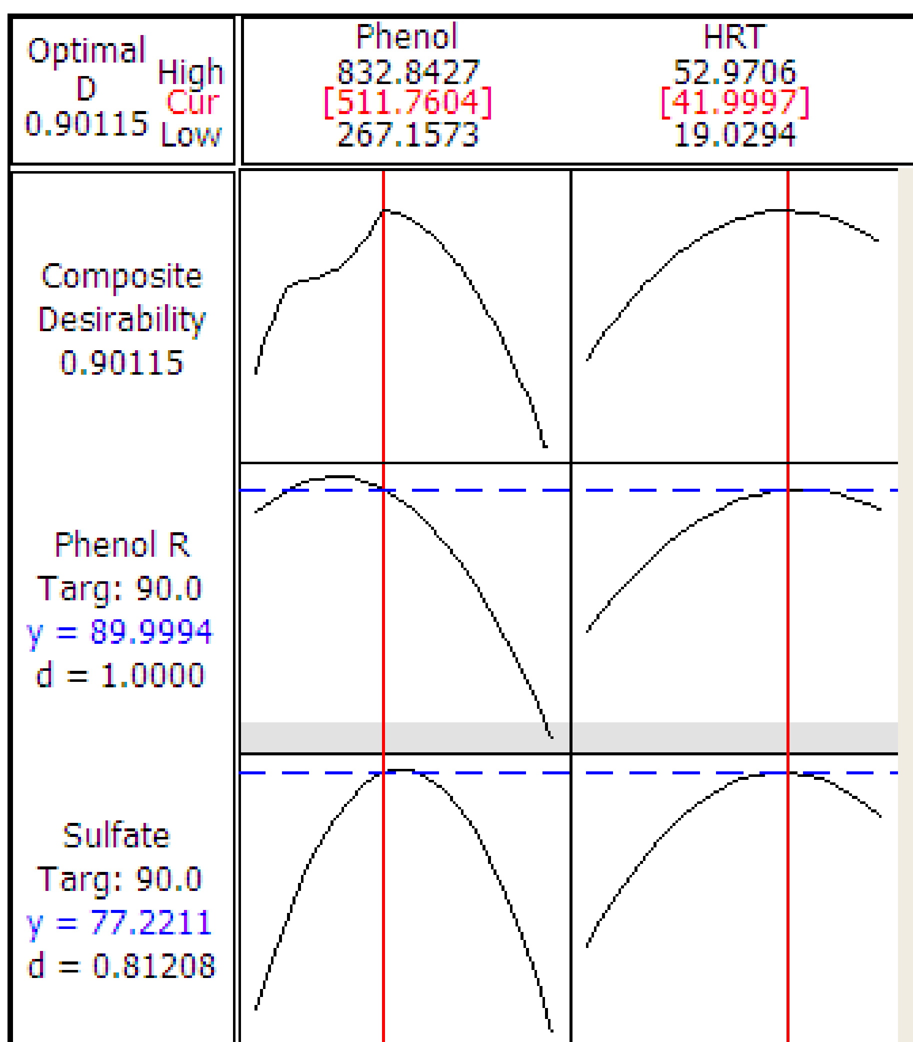

Figure 4.29 Response surface optimization for achieving maximum SO_4^{2-} reduction

4.5.5 Evaluation of predicted values and model validation

The graphical representation of the experimental degradation efficiency versus the predicted values is shown in Figure 4.30(a) and (b) for SO_4^{2-} and phenol removal, respectively. The value of R^2 between predicted values and experimental values were greater than 0.9, which shows that there is a good agreement between experimental data and predicted results. SO_4^{2-} and phenol removal of 75.5 % and 87% were

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achieved from initial concentration of SO_4^{2-} of 2000 mg/L, phenol of 512 mg/L and HRT of 42 h (optimum conditions as obtained by RSM) when phenol was used as the sole carbon source indicating reliable optimized results.

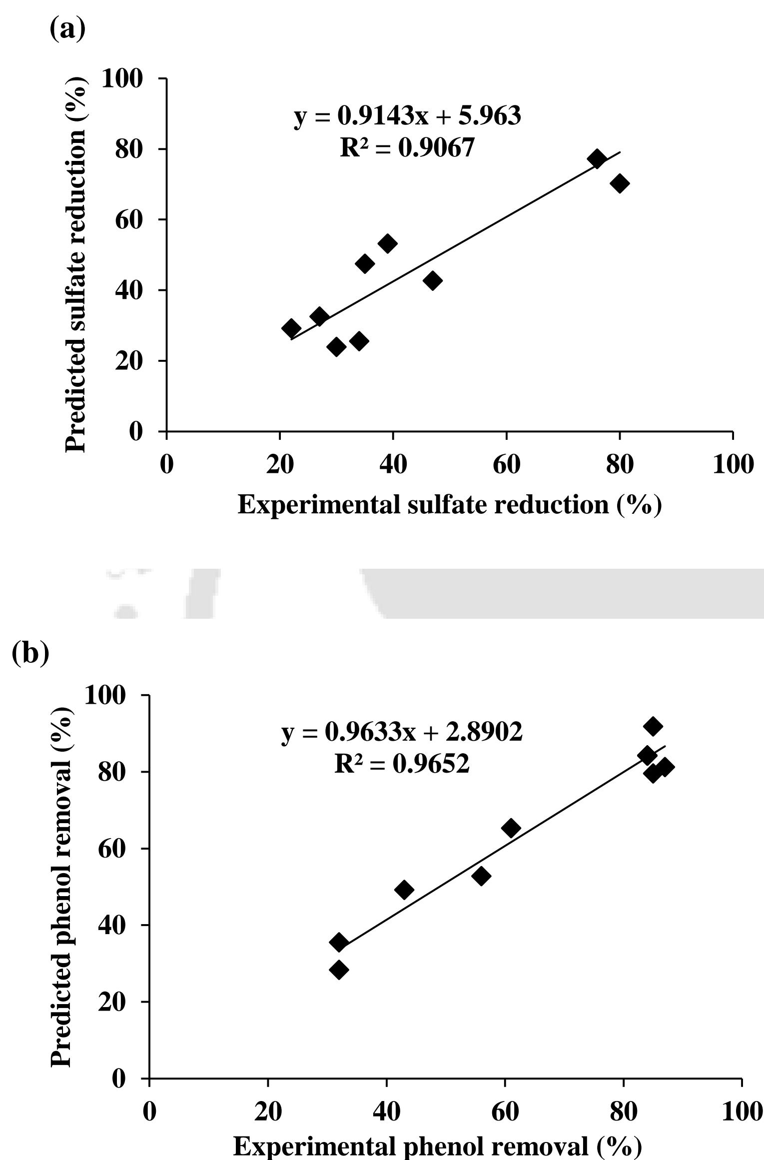

Figure 4.30 Linear plot of predicted versus experimental values of (a) SO_4^{2-} reduction and (b) phenol removal in the optimization study

4.6 Performance of PBR in Phase IV: Shock loading condition

Variation in the influent and effluent concentration of SO_4^{2-} and COD in the PBR at different SO_4^{2-} shock loadings is shown in Figure 4.31. The overall PBR performance

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is given in Table 4.8. During the first shock loading (3000 mg/L for 10 days), there was no drastic effect of the shock on SO_4^{2-} and COD removal. However, during the shock loading, SO_4^{2-} concentration in effluent increased to 568 mg/L, as shown in Figure 4.32. On resuming the normal sulfate loading (2200 mg/L), it took about 5 days for SO_4^{2-} concentration in effluent to go down to its normal level. During the second shock loading of 5000 mg/L for 14 days at the HRT of 24, 36 and 48 h, percentage removal of sulfate were 47.8, 53.6 and 56.5%, respectively, with corresponding COD removal of 79.3, 84.3 and 85.3 %, respectively. It took about 5 days to stabilize the reactor performance after resumption of normal sulfate loading of 2200 mg/L sulfate at 24 h HRT. On administration of the third shock load (7000 mg/L of sulfate) the SO_4^{2-} concentration in the effluent reached 4880, 4530, 4420 mg/L with corresponding COD removal of 52.2, 56.1, and 61.4 %, respectively. Once the shock loadings were stopped and normal SO_4^{2-} loading was resumed, the reactor took almost 10 days to come back to normal performance level.

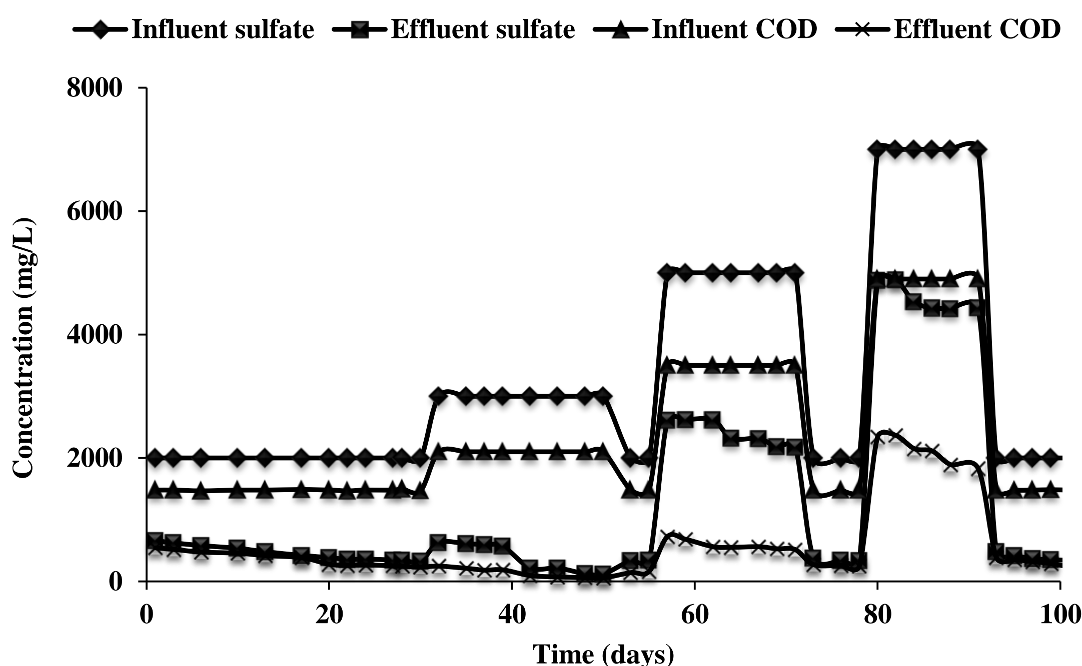

Figure 4.31 Variation of sulfate concentration and COD through the different conditions of the PBR.

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Table 4.8 Overall PBR performance at different sulfate concentration and HRT

Influent sulfate (mg/L)	HRT (h)	Sulfate loading rate (mg/L/h)	Sulfate reduction rate (mg/L/h)	% Sulfate reduction	% COD removal
3000	24	125	101.3	81.1	91.2
	36	83.3	77.3	92.8	96.7
	48	62.5	60	96	97.4
5000	24	208.3	99.58	47.8	79.3
	36	138.9	74.4	53.6	84.3
	48	104.2	58.8	56.5	85.3
7000	24	291.7	88.3	30.3	52.2
	36	194.4	68.6	35.3	56.1
	48	145.8	53.75	36.9	61.4

The steady state profiles of SO_4^{2-} reduction, SO_4^{2-} reduction rate (SRR) as a function of SO_4^{2-} loading rate (SLR), and HRT for the different feed SO_4^{2-} concentrations of 3000, 5000, and 7000 mg/L are shown in Figure 4.32, Figure 4.33, and Figure 4.34, respectively.

The steady-state profiles at a feed SO_4^{2-} concentration of 3000 mg/L shows that SRR increased linearly from 60 to 101.3 mg/L/h with an increase in the volumetric SO_4^{2-} loading rate from 62.5 to 125 mg/L/h. The corresponding COD removal rate across this range of HRT also increased from 42.43 to 79.62 mg/L/h. Similarly, at a feed SO_4^{2-} concentration of 5000 mg/L, SRR increased linearly from 58.8 to 99.5 mg/L/h with increase in the volumetric SO_4^{2-} loading rate from 104.2 to 208.3 mg/L/h. Baskaran and Nemati (2006) also observed a decrease in SO_4^{2-} conversion from 100% to 58% as the SO_4^{2-} volumetric loading rate was increased from 0.001 to 2.85 g/L/h in case of a lactate-fed chemostat culture receiving a feed SO_4^{2-} concentration of 1000 mg/L.

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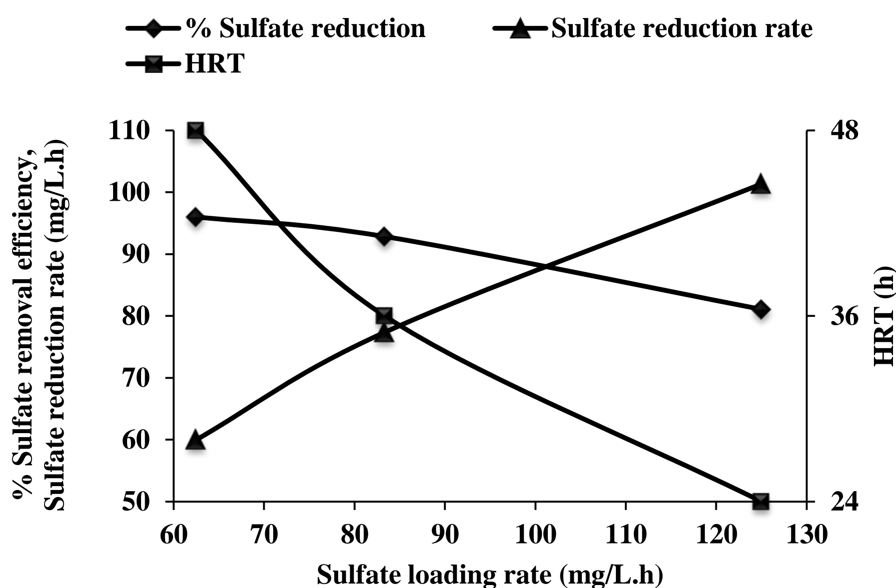

Figure 4.32 Steady state profiles at feed sulfate concentration of 3000 mg/L

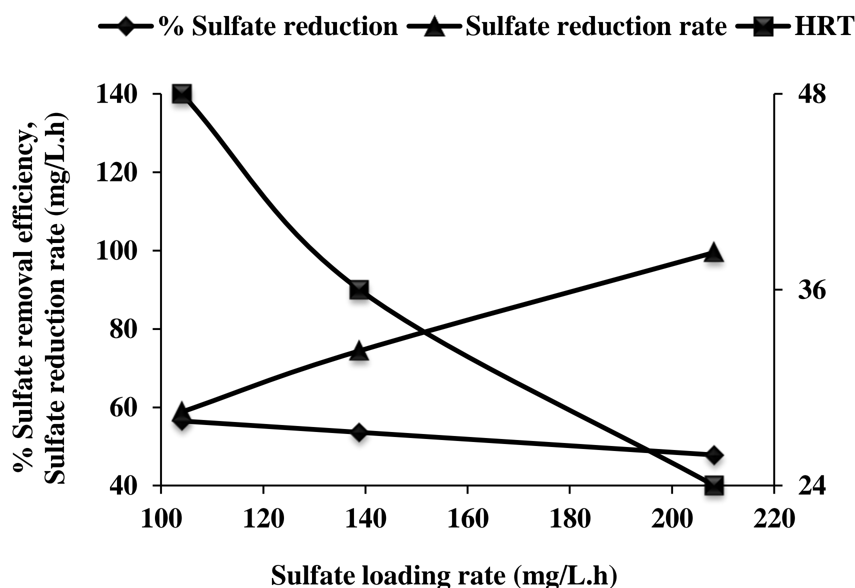

Figure 4.33 Steady state profiles at feed sulfate concentration of 5000 mg/L

Substrate inhibition was significant at a 7000 mg/L feed SO_4^{2-} concentration as reflected by the low SO_4^{2-} conversion of 36.8% observed at the lowest volumetric loading rate of 145.8 mg/L/h at HRT of 48 h. Nevertheless, this relatively low SO_4^{2-}

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conversion translated into a significant removal of 53.75 mg/L/h SO_4^{2-}. The volumetric SO_4^{2-} reduction rate increased linearly across the range of HRT studied, with the maximum value of 88.6 mg/L/h recorded at the residence time of 24h. The corresponding SO_4^{2-} removal efficiency was 30.28%.

The maximum SRR was reduced from 101.3 mg/L/h at a feed SO_4^{2-} concentration of 3000 mg/L to 53.75 mg/L/h at a feed SO_4^{2-} concentration of 7000 mg/L as shown in Figure 4.35. This result is in agreement with the result observed by Mohanty et al. (2000) which showed that SO_4^{2-} reduction rate decreased with increase in feed SO_4^{2-} concentration from 1.3 to 3.6 g/L in a batch study on account of SO_4^{2-} toxicity. In immobilized cell-systems fed with lactate, increasing feed SO_4^{2-} concentrations, in the range 1000-5000 mg/L, negatively influenced the reactor performance as measured by the volumetric SO_4^{2-} reduction rate. This could also be attributed to sulfide toxicity (Baskaran and Nemati, 2006).

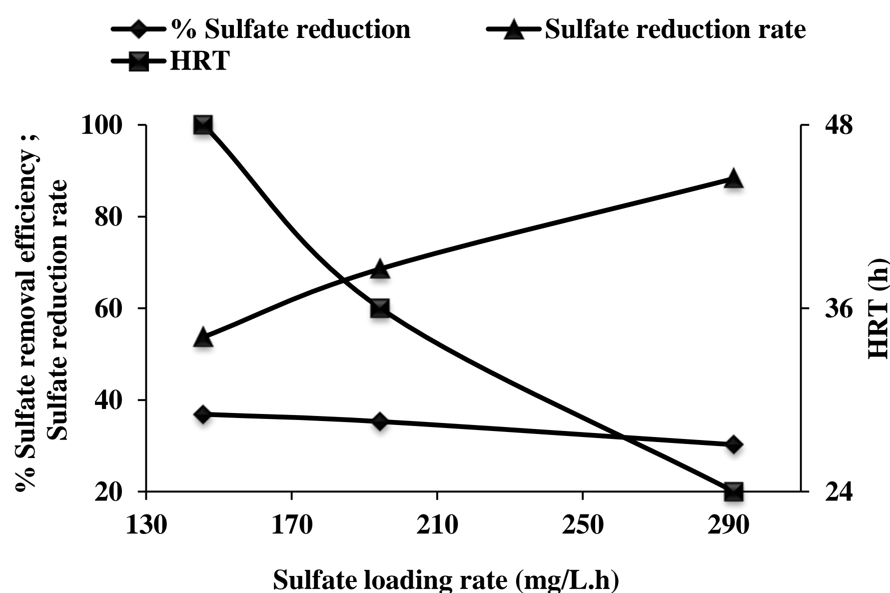

Figure 4.34 Steady state profiles at feed sulfate concentration of 7000 mg/L

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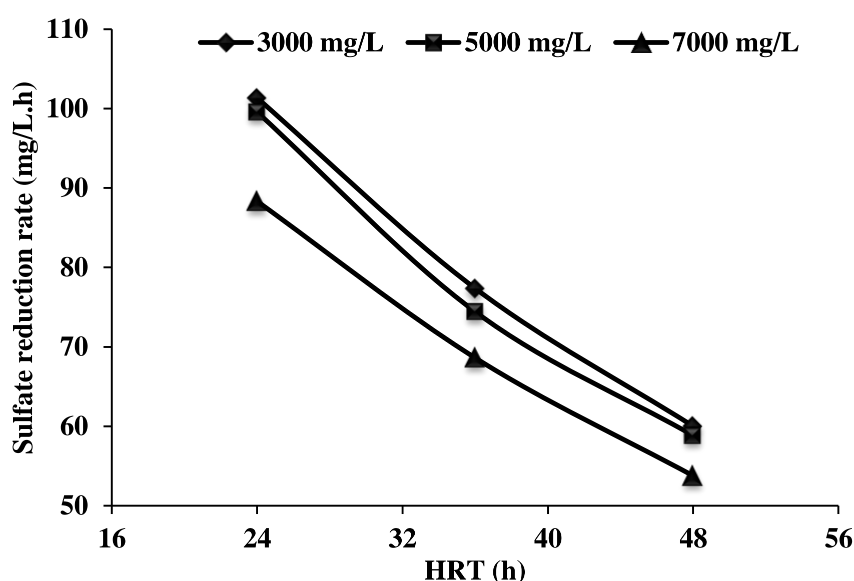

Figure 4.35 Effect of feed sulfate concentration and HRT on SO_4^{2-} reduction

Contrary to the observations reported in immobilized cell systems discussed above, in case of suspended-cell systems it was observed that SRR increased with increasing feed SO_4^{2-} concentration in the range of 1000 to 10000 mg/L (Moosa et al., 2002).

According to White and Gadd (1996), the inhibitory effect of residual SO_4^{2-} on the BSR in a lactate-fed sulphidogenic system was mainly due to its effect on the operating pH and redox potential. An increasing concentration of residual SO_4^{2-} increases the redox potential and reduces the operating pH. An increased redox potential would favour the growth of non-SRB lactate utilizers in the mixed consortia used since SRB are known to thrive at low negative redox potentials (Postgate, 1984; White and Gadd, 1996). Decreased SO_4^{2-} reduction with increase in SO_4^{2-} concentration as a result of increase toxicity of SO_4^{2-} ion towards SRB was reported by Mohanty et al. (2000). Baskaran and Nemati (2006) also reported that initial concentration of SO_4^{2-} in the feed influenced the SRB activity and reduction rates lowered with the increase in SO_4^{2-} concentration from 1 to 5 g/L. In the current study,

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residual SO_4^{2-} was recorded in the PBR across the operating conditions studied which might have restricted the activity of the SRB at high feed sulfate concentrations.

In acetate-fed sulphidogenic reactors utilizing a mixed consortium of SRB, 50% growth rate inhibition has been observed at HS^- concentrations between 0.4 and 1.04 g /L at the pH range of 7.2 and 8.5. The concentrations of sulfide detected (590-750 mg/L) in the PBR indicated that the SRB growth rate was also inhibited by sulfide which fall within the range at which 50% sulfide inhibition occurs as reported by O'Flaherty et al.(1998).

The present study showed that up to 3.5 times increase in the normal input SO_4^{2-} concentration in the form of SO_4^{2-} shock loading did not affect the PBR performance irreversibly and had enough potential to handle shock loading conditions. With the increase in SO_4^{2-} concentration, the volumetric reduction rate started to decline. An increase in feed SO_4^{2-} concentration has a negative influence on the SO_4^{2-} conversion. The maximum SO_4^{2-} conversion achieved at each feed SO_4^{2-} concentration was at the highest HRT. It was evident that SO_4^{2-} removal decreased with increase in loading rate of SO_4^{2-} as the SO_4^{2-} concentration increased.

4.7 **Performance of the microaerobic reactor**

The sulfide oxidation taking place in the microaerobic reactor was expected to be a combination of chemical and biological oxidation as per Eq. 4.12.

$$H_2S\ (aq) + 0.5\ O_2\ (g) \rightarrow H_2O\ (aq) + S(s) \qquad 4.12$$

ΔG° = -267.1kJ/M

The sulfide formed as a result of sulfate reduction can be chemically oxidized to elemental sulfur as per Eq.4.12 in the presence of air. However, the main products of biological sulfide oxidation are elemental sulfur and sulfate according to Buisman et al. (1989) and Visser et al.(1997). These reactions as given in Eqs. 4.13 and 4.14 are

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independent of the pH within the range of 6.5-9.0 (Sabumon, 2008). These reactions are faster when compared with chemical oxidation of sulfide.

$$HS^{-}(aq) \rightarrow S^{0}(\text{Membrane bound}) \leftrightarrow S^{0}(s) \quad 4.13$$

$$S^{0}(\text{Membrane bound}) \leftrightarrow SO_3^{2-}(aq) \leftrightarrow SO_4^{2-} \quad 4.14$$

4.7.1 Batch study on sulfide oxidation

The batch study was performed in order to determine the contribution of chemical and biological oxidation. From the batch study, it was found that only around 11% of the sulfide was oxidized in the chemical oxidation study while the biotic oxidation gave more that 67% sulfide removal in the 30 h HRT suggesting biotic fraction to be major contribution for sulfide oxidation (Figure 4.36). Photographs of changes in the reactor content with time for chemical and biotic study is shown in Figure 4.37 and Figure 4.38, respectively. Visible elemental sulfur formation was also observed in the biotic study, as shown in Figure 4.38.

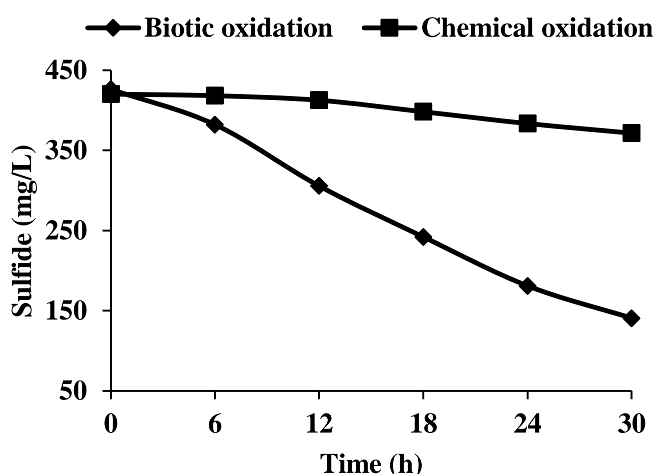

Figure 4.36 Sulfide oxidation profile in the chemical and biotic batch study

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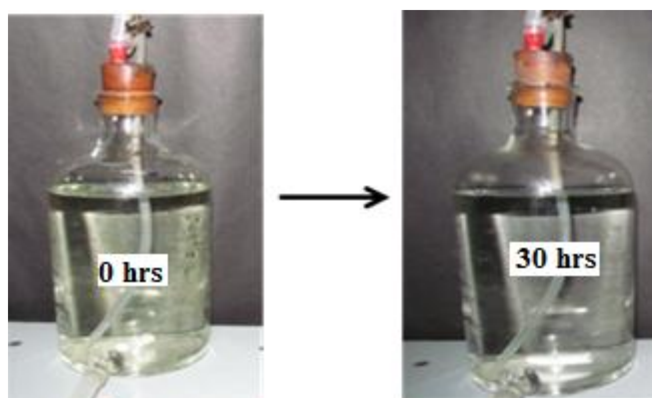

Figure 4.37 Photograph of changes in the chemical oxidation study

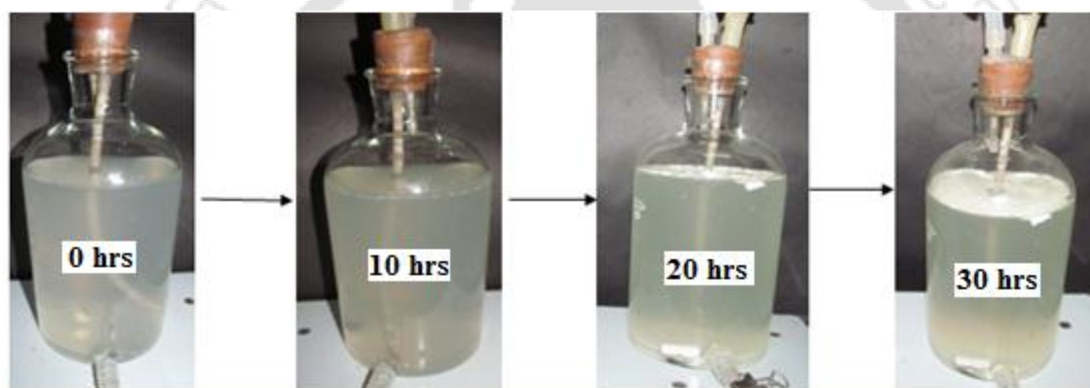

Figure 4.38 Photographs of changes in the biotic oxidation study

4.7.2 Performance of microaerobic reactor with different air supply rate for 5 hours

Microaerobic study was carried out with phenol as the sole carbon source, when phenol concentration was 350 mg/L at 24 h HRT. Approximately 22% reduction in SO_4^{2-} concentration was observed from an initial SO_4^{2-} concentration of 2000 mg/L, generating around 130 mg/L dissolved sulfide. Different airflow rate of 5 ml/min,10 ml/min, and 15 ml/min, respectively were supplied for 5 h to the microaerobic reactor, which was already filled up to study the sulfide oxidation to elemental sulfur.

Maximum sulfide removal and visible elemental sulfur formation with increase in turbidity at airflow rate of 5 ml/min, 10 ml/min and 15 ml/min was noticed at DO range of 80-120 µg/L at different time interval as shown in Figure 4.39, Figure 4.40 and Figure 4.41 respectively. A whitish yellow color sulfur formation (Figure 4.42

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and Figure 4.43) was observed in the microaerobic reactor which could be due to formation of sulfur as per reactions of sulfide oxidation described above in Eqs 4.12 and 4.13. With increase in air supply rate to 10 ml/min and 15 ml/min, the time required to reached the DO range of 80-120 μg/L was less as compared to that of the lower air flow rate of 5 ml/min. Maximum sulfide removal and corresponding elemental sulfur formation started at around 180 minutes and continued until the last minutes of the 5 hours of study as the DO was still under the optimum range when the air supply was given at the rate of 5 ml/min (Figure 4.39). However an early shift in the sulfide removal and elemental sulfur formation could be noticed as the air supply was increased to 10 ml/min and 15 ml/min within a short duration where the optimum DO range was maintained as shown in Figure 4.40 and Figure 4.41 respectively.

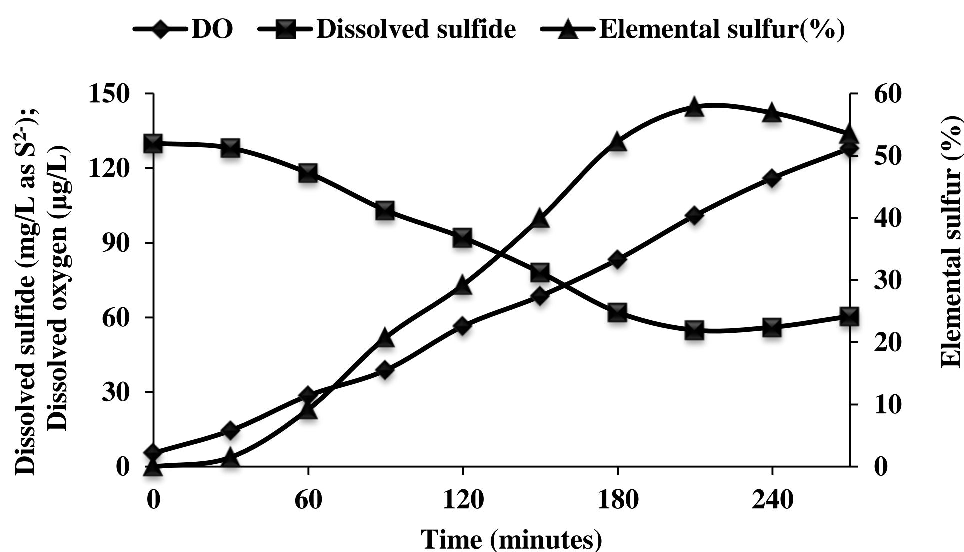

Figure 4.39 Time profile of DO, dissolved sulfide and elemental sulfur (%) when airflow rate was 5 ml/min

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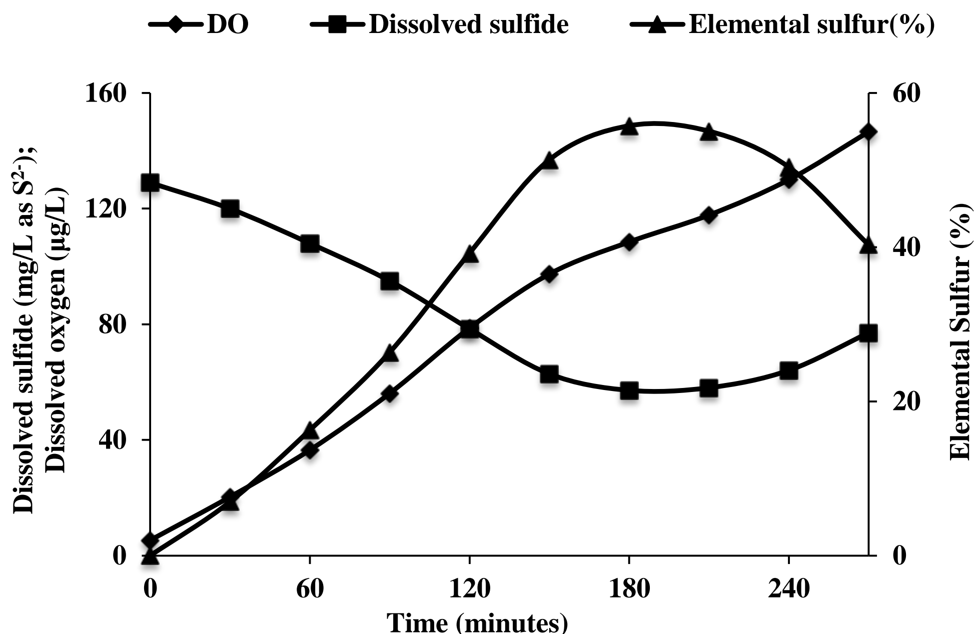

Figure 4.40 Time profile of DO, dissolved sulfide and elemental sulfur (%) when airflow rate was 10 ml/min

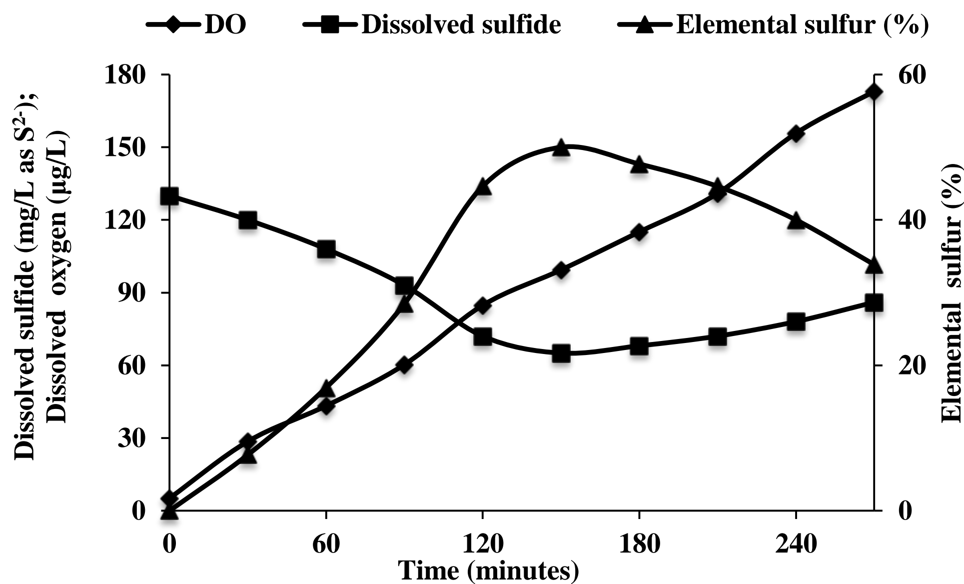

Figure 4.41 Time profile of DO, dissolved sulfide and elemental sulfur (%) when airflow rate was15 ml/min

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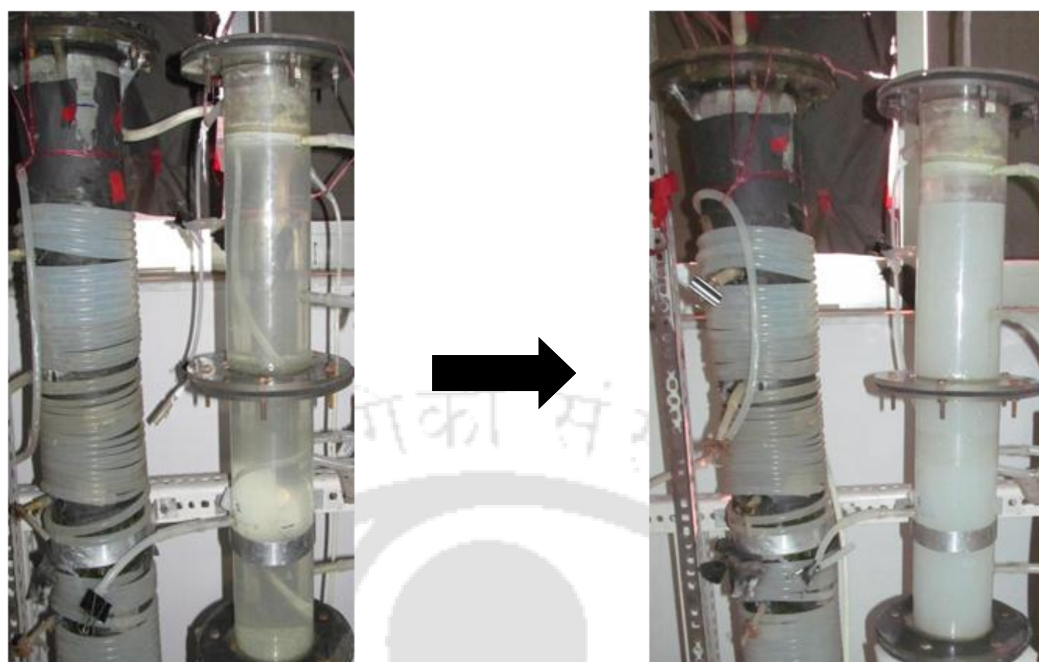

Figure 4.42 Photographs of microaerobic reactor before and after air supply at an airflow of 5 ml/min

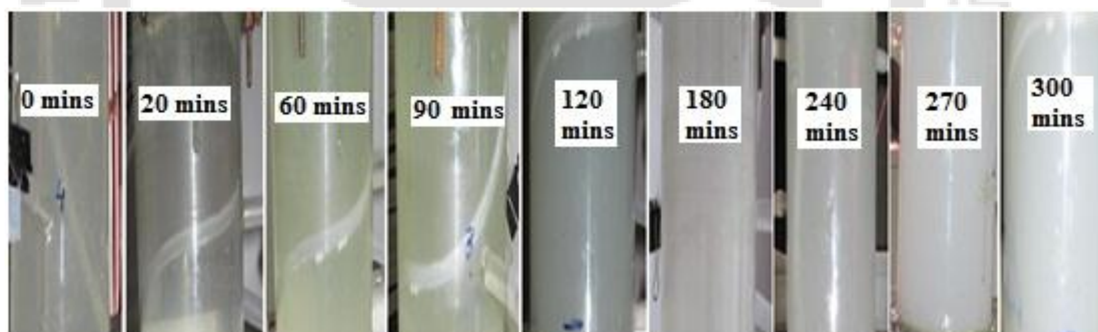

Figure 4.43 Photographs of microaerobic reactor as observed during the air supply at an airflow of 5 ml/min

4.7.3 Continuous study of microaerobic reactor with different airflow rates

When different airflow rate was provided to the microaerobic reactor, different range of dissolved oxygen was recorded. The performance of the microaerobic reactor at different airflow rate is shown in Figure 4.44. In the minimum airflow of 1 ml/min, the DO range of 40-60 μg/L was achieved along with only about 5% sulfide oxidation. The maximum conversion of sulfide to elemental sulfur was observed at a DO range of 70-100 μg/L when the airflow was increased to 2 ml/min. With the

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increase in DO level to 160-180 μg/L at airflow of 6 ml/min, the sulfide conversion was reduced to about 39 % as compared to 70% at DO range of 70-100 μg/L. With further increase in DO to 250-280 μg/L at 10 ml/min airflow, the effluent sulfate concentration started to increase which shows that the sulfide is oxidized to sulfate when more oxygen is available.

The sulfide conversion to elemental sulfur was also studied at different HRT. From Figure 4.45, it can be seen that the microaerobic reactor has four sampling ports which gives four different HRT with the increase in reactor height. In the earlier study at different airflow rate, sampling was done from the uppermost port of the reactor which gives an HRT of 20 h. In this study the airflow rate was maintained constant throughout at 2 ml/min which gave the optimum result while sampling was done from the different ports starting from the upper port to the lower port stepwise. The corresponding HRT which was calculated along the reactor heights were 20 h, 17 h, 8.5 h and 3 h respectively starting from top to bottom port (Figure 3.6). The sulfide conversion to elemental sulfur was almost constant at the HRT of 20 h and 17 h while it started to decrease to 58% at 8.5 h and at 3 h it gave a minimum conversion of 21%. The minimum HRT required for achieving sulfide oxidation to elemental sulfur in the given conditions was thus observed to be 17 h.

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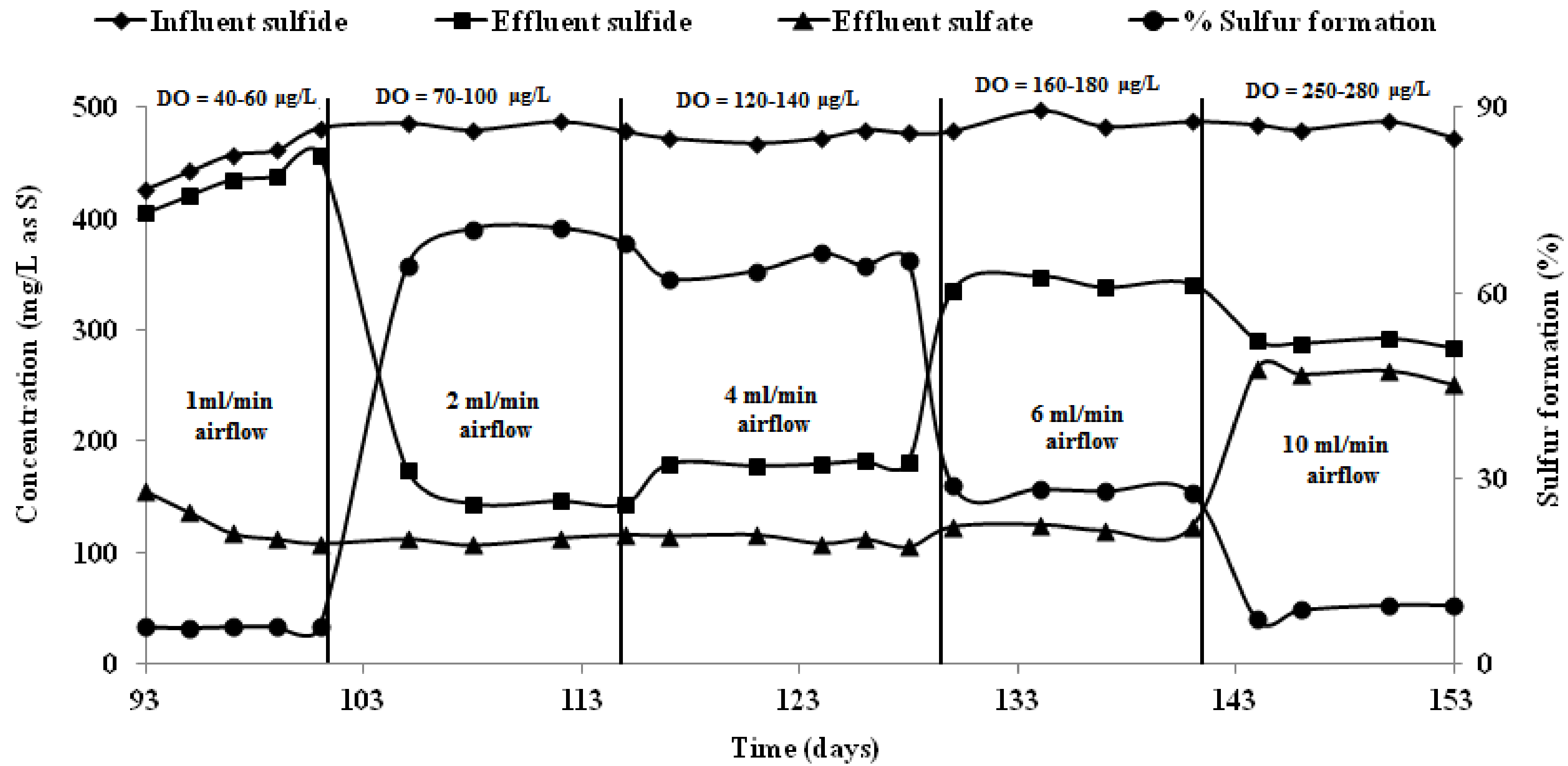

Figure 4.44 Formation of elemental sulfur (%) at different DO levels with continuous air supply

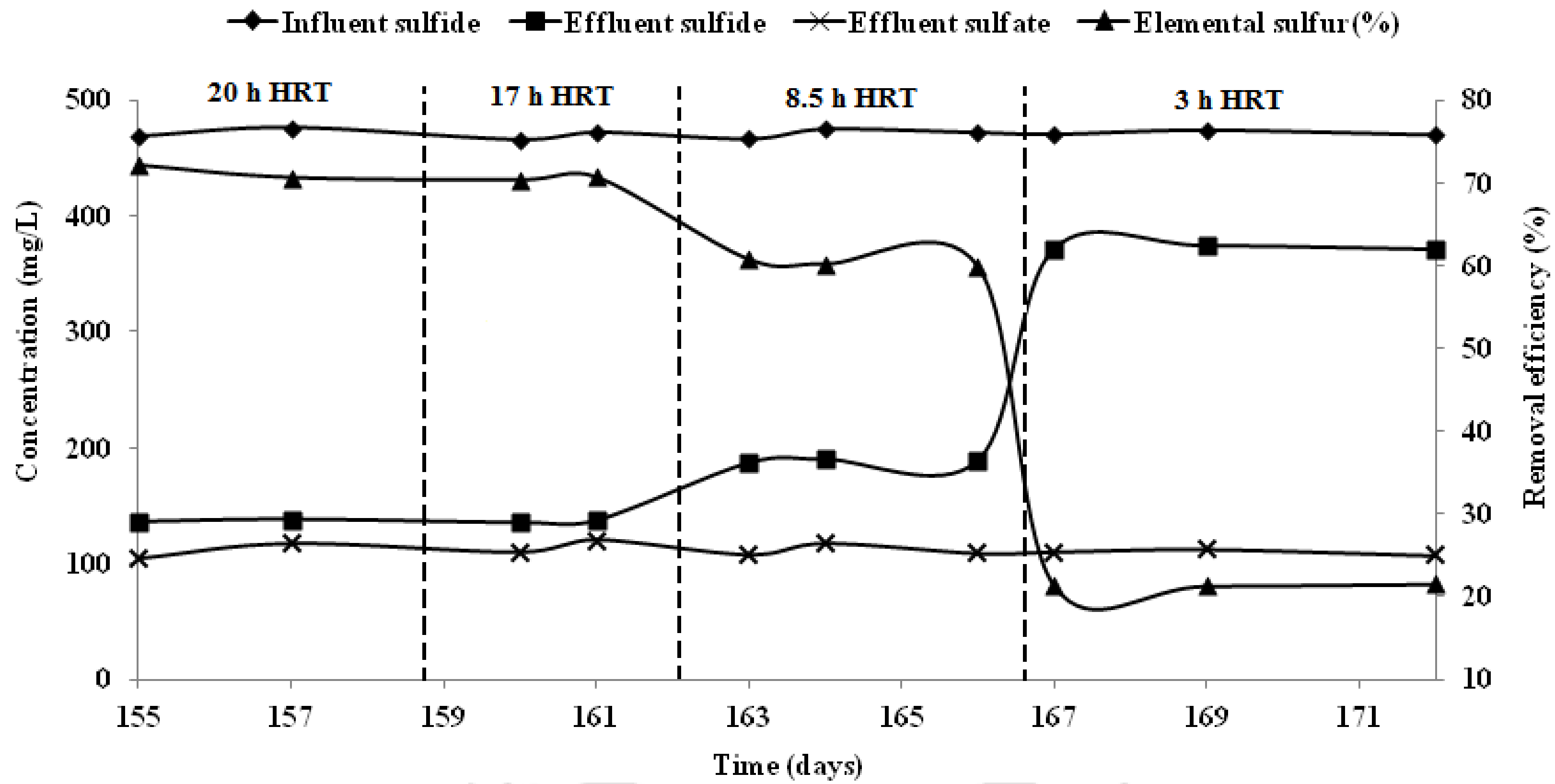

Figure 4.45 Formation of elemental sulfur (%) at different HRT with continuous air supply

The sulfur precipitate settled at the bottom of the reactor when the air supply was stop in case of the microaerobic study, as shown Figure 4.46 along with the SEM image and EDX spectra showing the sulfur peak (Figure 4.47).

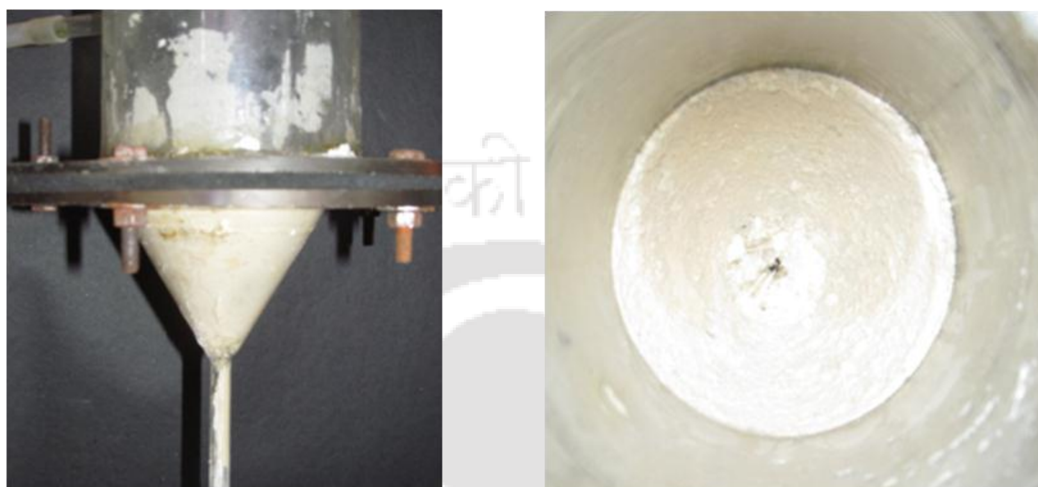

Figure 4.46 Sulfur deposited on walls and bottom of microaerobic reactor

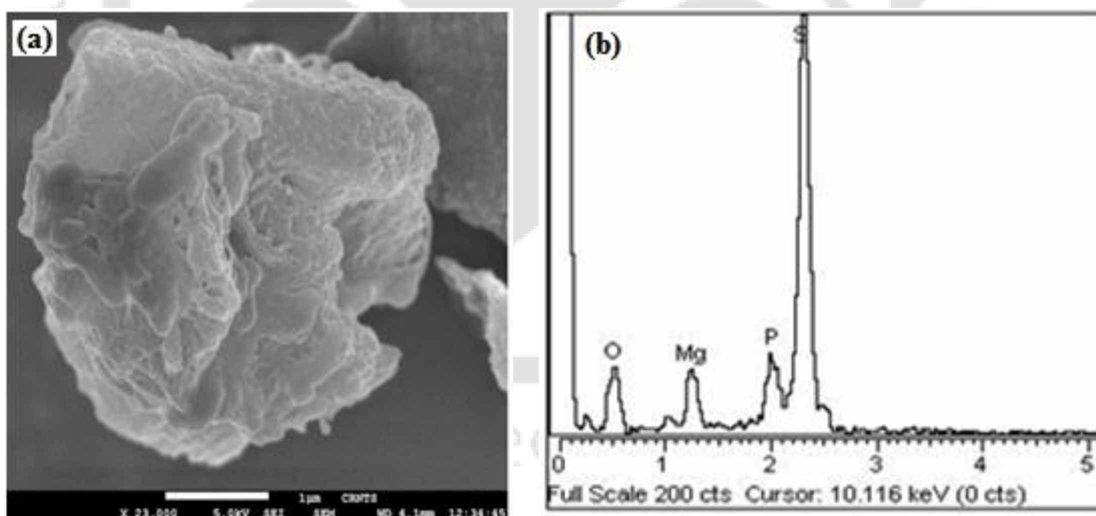

Figure 4.47 (a) SEM micrograph of precipitate and (b) EDX of the deposited precipitate

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4.7.4 Separation of sulfur particles through sedimentation

About 2L of the reactor effluent from the microaerobic reactor was collected in a measuring cylinder, sealed at the top with parafilm to prevent further oxidation with atmospheric oxygen, and then allowed to settle. It was observed that within 6 hours the sulfur precipitate settled at the bottom due to gravity sedimentation, which could then be easily separated for further use (Figure 4.48). Photographs of the settling profile of precipitate observed during the sedimentation study is shown in Figure 4.49.

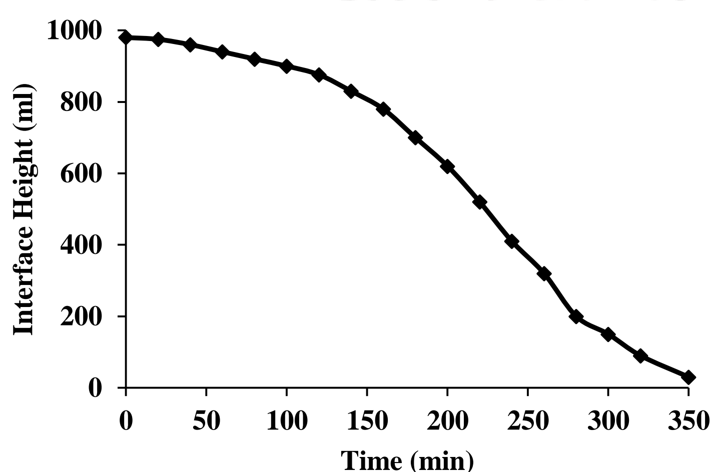

Figure 4.48 Settling profile of the precipitate with time

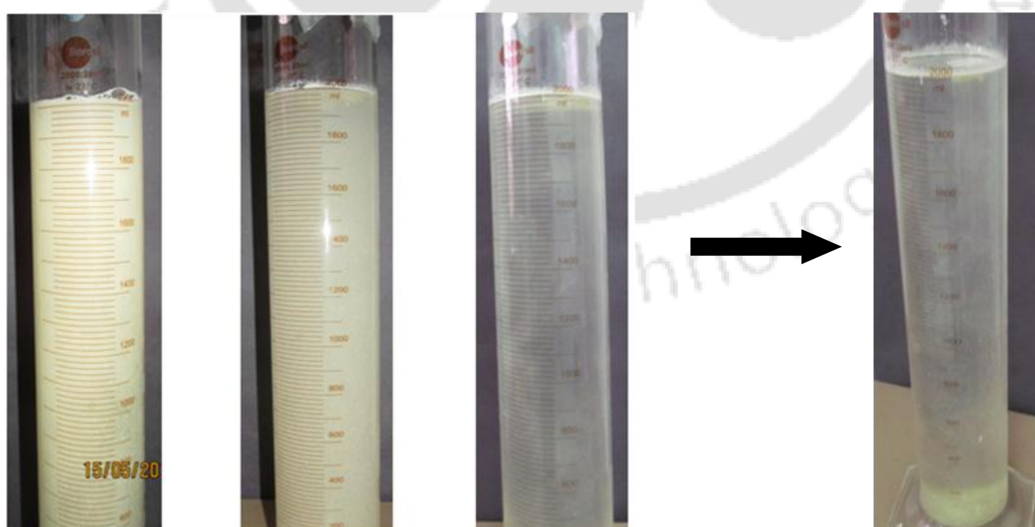

Figure 4.49 Photographs of the settling profile of precipitate during the sedimentation study

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4.7.5 Centrifugation

The effluent collected from the microaerobic reactor after the visible formation of elemental sulfur was collected in 15 ml centrifuge tubes and the tubes were centrifuged at different rotation speed, expressed in terms of 'g'. After centrifugation, the turbidity of the supernatant was measured and compared for the different rotational speeds. It was observed that the turbidity of the supernatant remain constant at 2500 g and above, suggesting that the precipitates could be separated out from the effluent after centrifugation at a rotational speed of 2500 g or above within 5 minutes.

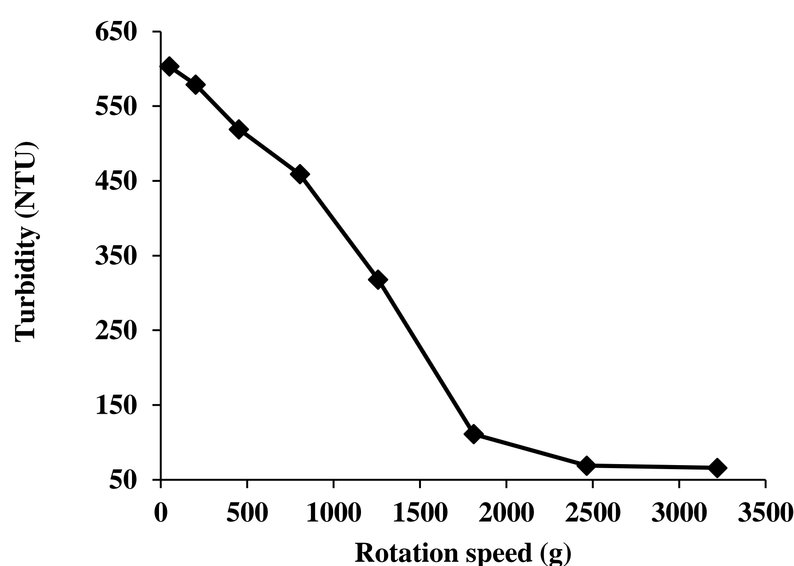

Figure 4.50 Settling profile of the precipitate at different rotation speed

4.8 **Bacterial community study results**

4.8.1 Bacterial community from batch reactor R0

Morphological characterization of the mixed consortium performed using FESEM revealed that the cells were of various sizes and shapes, from small rods to large rods (Figure 4.51). Most of the rods were between 1.336 × 0.308 µm to 0.858 × 0.328 µm in size. The predominant strain was isolated from the enriched mixed consortium and designated as strain r3. Partial 16S rDNA sequencing result showed that strain r3 had 1525 base pairs (bp). The sequence was submitted to the Genbank with JN600615.1 as accession number of the strain r3. Gene analysis by online BLAST tool indicated

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that the isolate contains sequences that are specific to the members of the γ subdivision of the family *Proteobacteria*. The phylogenetic tree shown in Figure 4.52was prepared using neighbor joining method based on near-full-length 16S rDNA gene sequences recovered from the isolated strain and other sequences obtained from the Genbank database (Genbank accession number has been indicated with the generic name in the tree). The high bootstrap support of the tree derived from the 16S rDNA analysis demonstrated that strain r3 is a typical member of the *Pseudomonas aeruginosa* and has the closest relation (99%) to *Pseudomonas aeruginosa strain* RI-1(JQ773431.1).

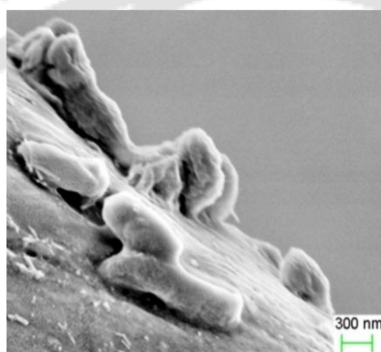

Figure 4.51 FESEM of the mixed culture obtained from batch study

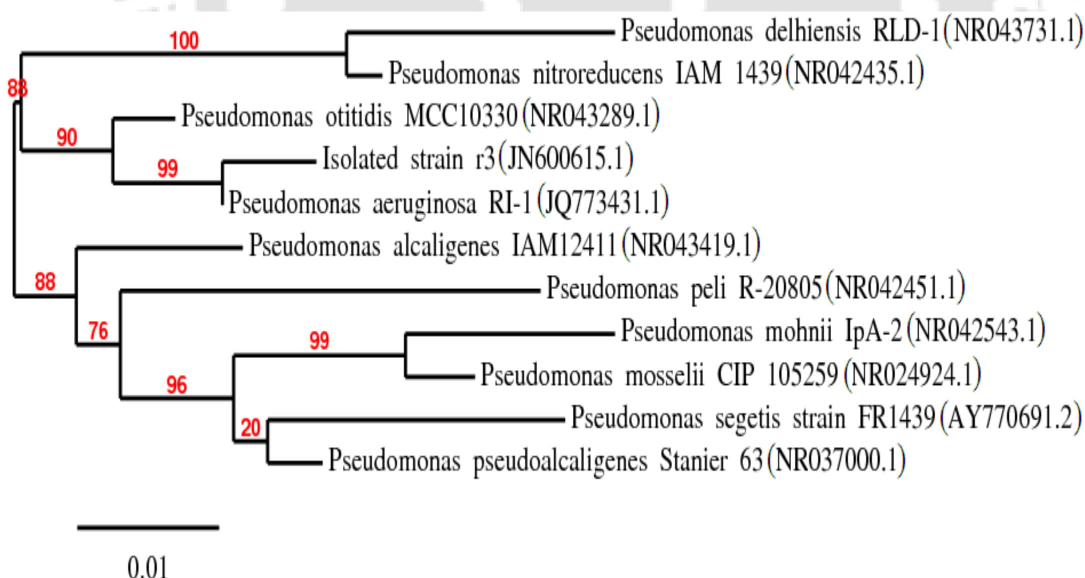

Figure 4.52 Phylogenetic relationships between the isolated strain r3 (JN600615.1) and other related strains based upon the analysis of aligned regions of 16s rDNA gene sequences

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4.8.2 Isolation and identification of microbial community in the PBR

Morphological characterization of the mixed consortium performed using FESEM revealed that the cells were of various sizes and shapes, from small rods to large rods (Figure 4.53).Most of the rods were between 1.43 × 0.3 μm to 0.8 × 0.35 μm in size.

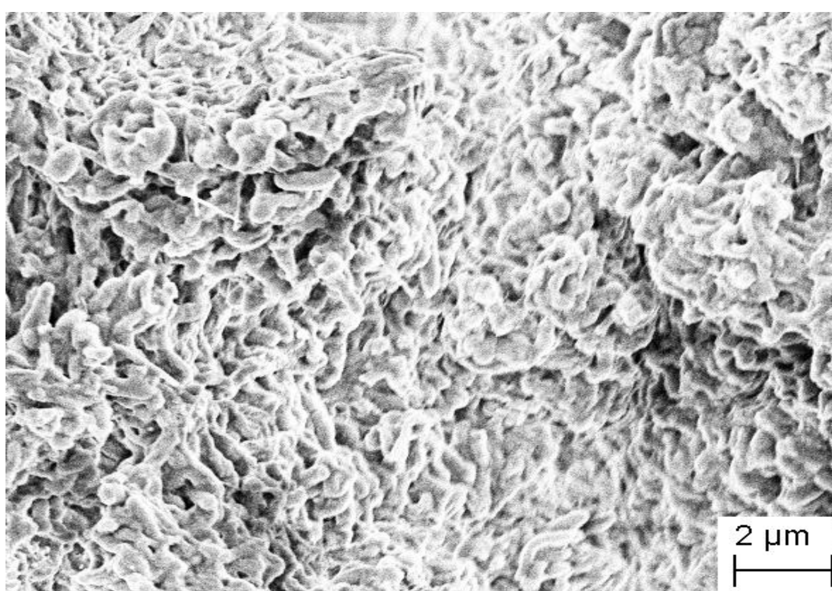

Figure 4.53 FESEM of the mixed microbial culture obtained from PBR

The partial 16S rDNA sequencing of these three colonies PB2, PB7 and PB8 gave results with 834, 700 and 450 base pairs, respectively. The sequences for PB2, PB7 and PB8 have been submitted to the Gene Bank database with the accession number KC967284.1, KF294163.1 and KF542908.1, respectively. Gene analysis by online BLAST tool indicated that the isolate PB2 and PB8 contain sequences that are specific to the members of the γ subdivision while PB7 contains sequences that are specific to the members of the δ subdivision of the *Proteobacteria* phylum. Gene analysis by online BLAST tool indicated that the strain PB2 belongs to the member of the genus *Pseudomonas* sp., PB7 belong to the *Desulfovibrio* sp and PB8 belong to the *Citrobacter* sp.

Photographs and FESEM images of PB2 is shown in Figure 4.54. The phylogenetic tree of PB2 as shown in Figure 4.55 was prepared using neighbor joining method based on near-full-length 16S rDNA gene sequences recovered from the isolated strain PB2 and other sequences obtained from the Genbank database (Genbank

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accession number has been indicated with the generic name in the tree). *Pseudomonas* sp. even though a facultative aerobe, when cultivated under strictly anaerobic conditions has been reported to be capable of reducing sulfate more intensively than under aerobic conditions (Kliushnikova TM et al., 1992).

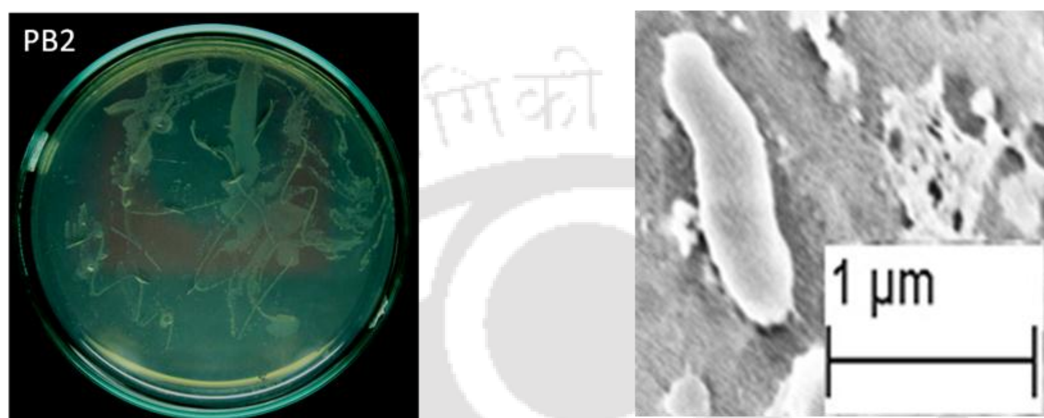

Figure 4.54 Photograph and FESEM image of PB2 strain

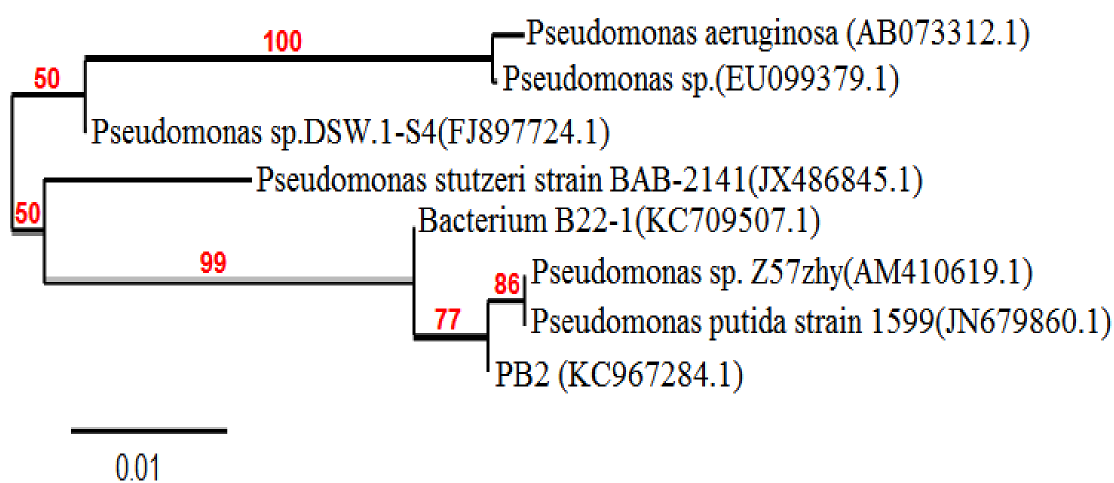

Figure 4.55 Phylogenetic relationships between the isolated strain PB2 (KC967284.1) and other related strains based upon the analysis of aligned regions of 16s rDNA gene sequences

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Figure 4.56 depicts the photograph and FESEM image of the strain PB7 while the phylogenetic tree prepared for this strain is given in Figure 4.57. The typical SRB *Desulfovibrio* sp. is capable of anaerobic growth in lactate and sulfate media with concomitant dissimilatory reduction of the sulfate to sulfide and has been classified as true dissimilatory sulfate-reducing bacteria (Postgate, 1984).

Figure 4.56 Photograph and FESEM image of PB7 strain

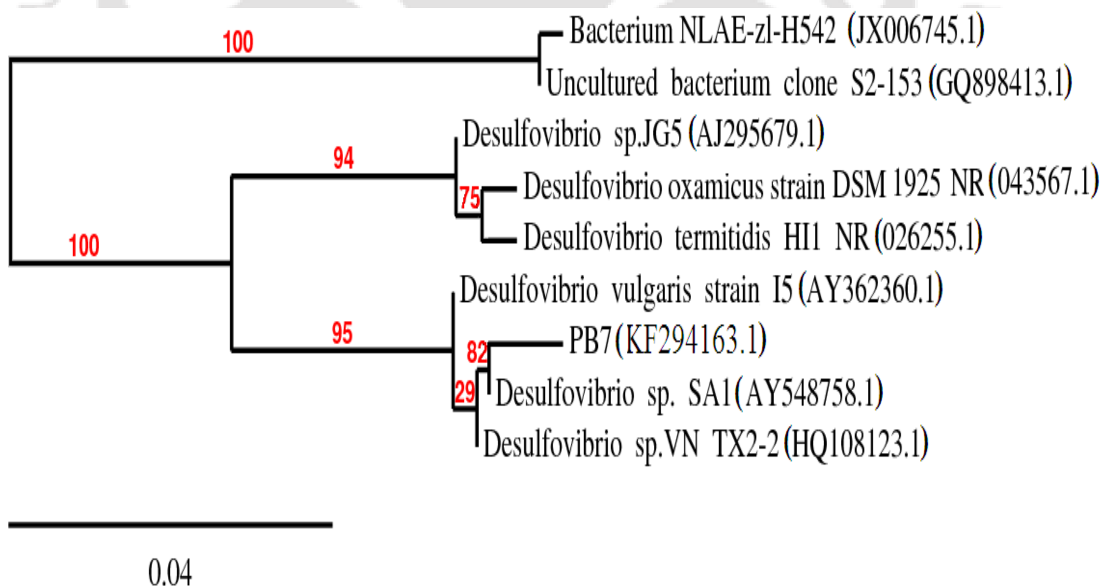

Figure 4.57 Phylogenetic relationships between the isolated strain PB7 (KF294163.1) and other related strains based upon the analysis of aligned regions of 16s rDNA gene sequences

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The photograph and FESEM image of strain PB8 is shown in Figure 4.58 while the phylogenetic tree is given in Figure 4.59. Isolation of *Citrobacter* sp. with sulfate reducing capacity from sludge of a sulfate-reducing up-flow anaerobic sludge bed (UASB) reactor for treating high concentration sulfate wastewater (Yang et al., 2010) and from sediments of mining areas (Qiu et al., 2009) have been reported.

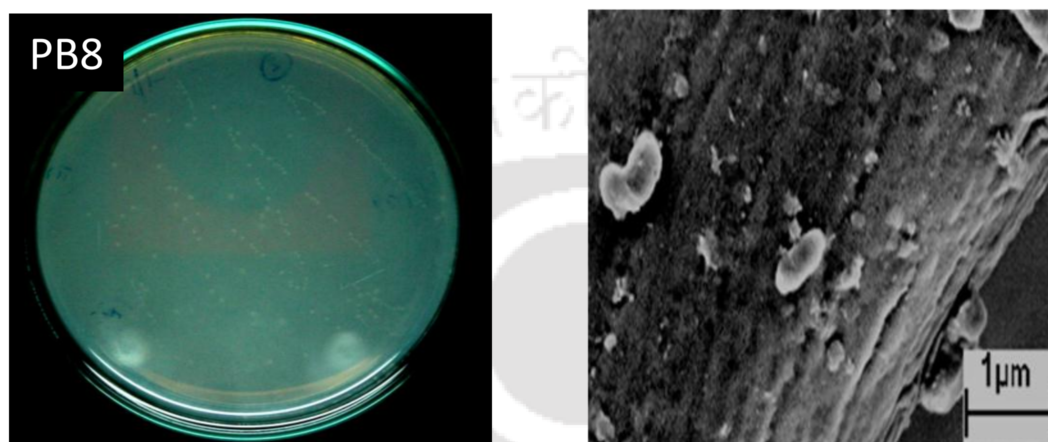

Figure 4.58 Photograph and FESEM image of PB8 strain

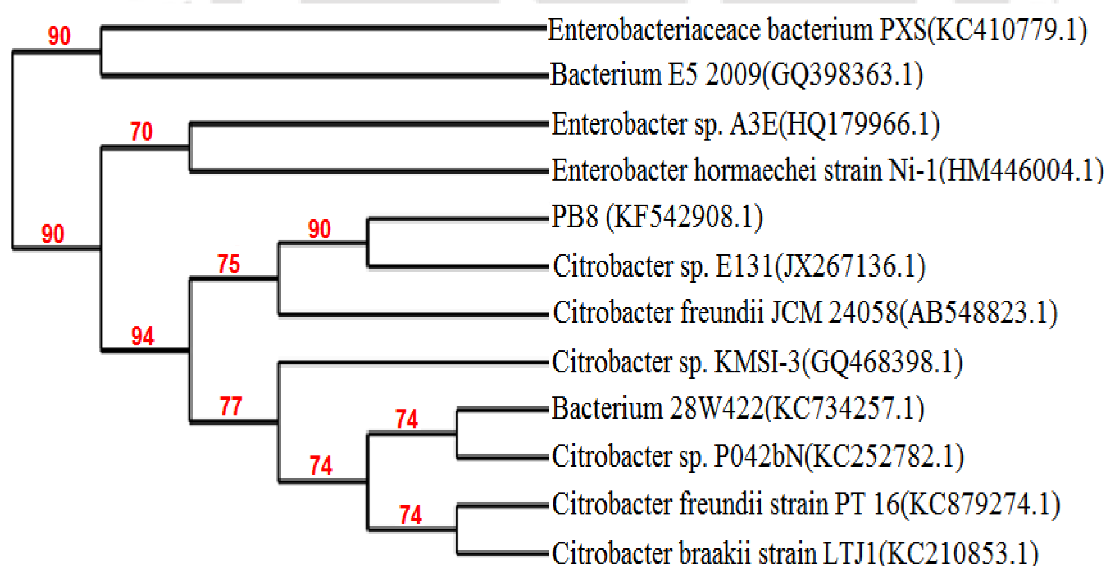

Figure 4.59 Phylogenetic relationships between the isolated strain PB8 (KF542908.1) and other related strains based upon the analysis of aligned regions of 16s rDNA gene sequences

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4.8.3 T-RFLP analysis

The result of a T-RFLP profiling is a graph called Electropherograms which is an intensity plot representation of an electrophoresis experiment (gel or capillary). In an Electropherograms, the X-axis marks the sizes of the fragments while the Y axis marks the fluorescence intensity of each fragment. Thus, what appears on an electrophoresis gel as a band appears as a peak on the Electropherograms whose integral is its total fluorescence. In a T–RFLP profile, each peak assumingly corresponds to one genetic variant in the original sample while its height or area corresponds to its relative abundance in the specific community. T-RFs having size less than 40 bases were eliminated from the analysis as they might result from primer-dimers. The data obtained from the T-RFLP analysis were compared with databases available at http://mica.ibest.uidaho.edu/pat.php to identify the probable species present.

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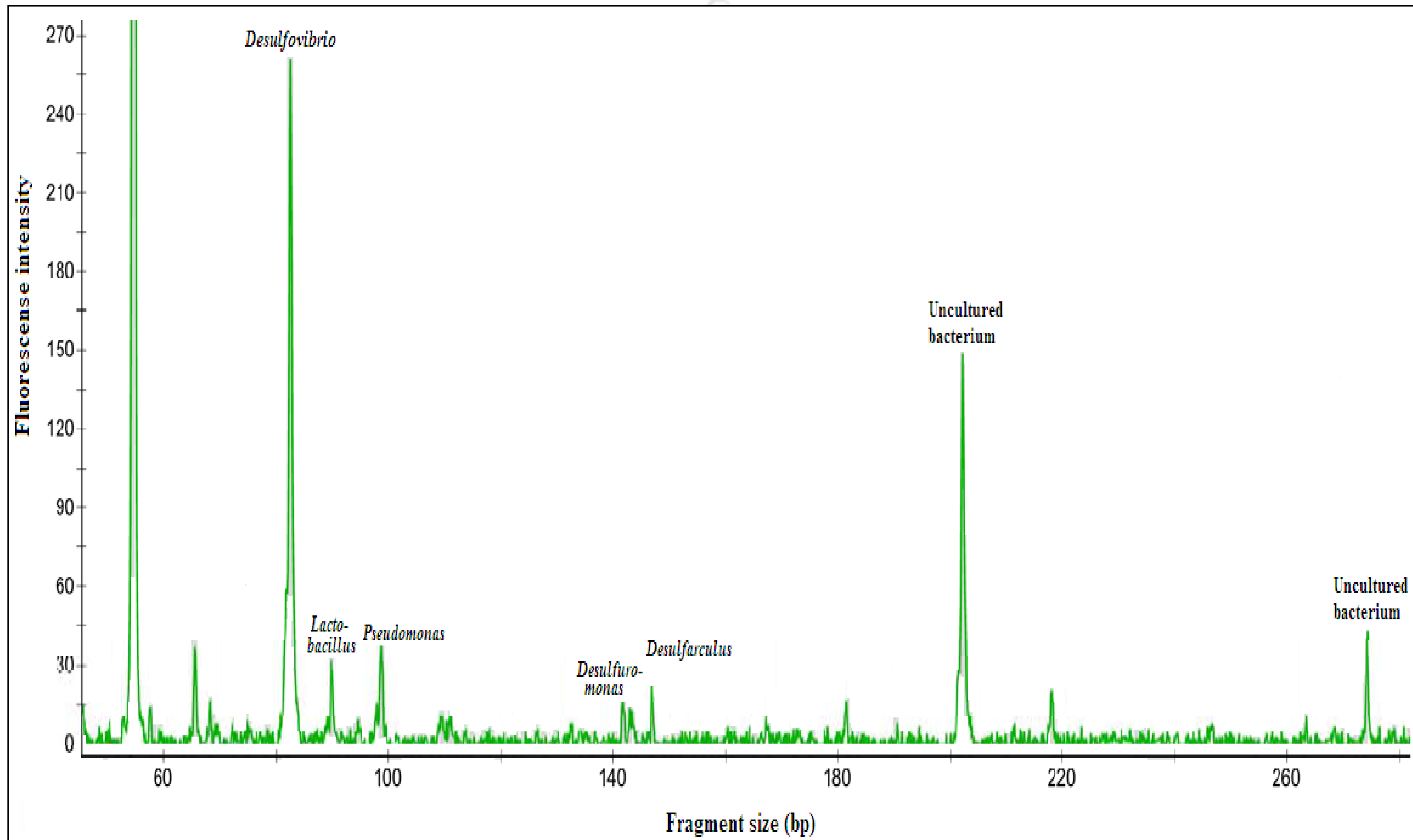

Figure 4.60 Electropherograms when lactate was the sole source of carbon in the PBR.

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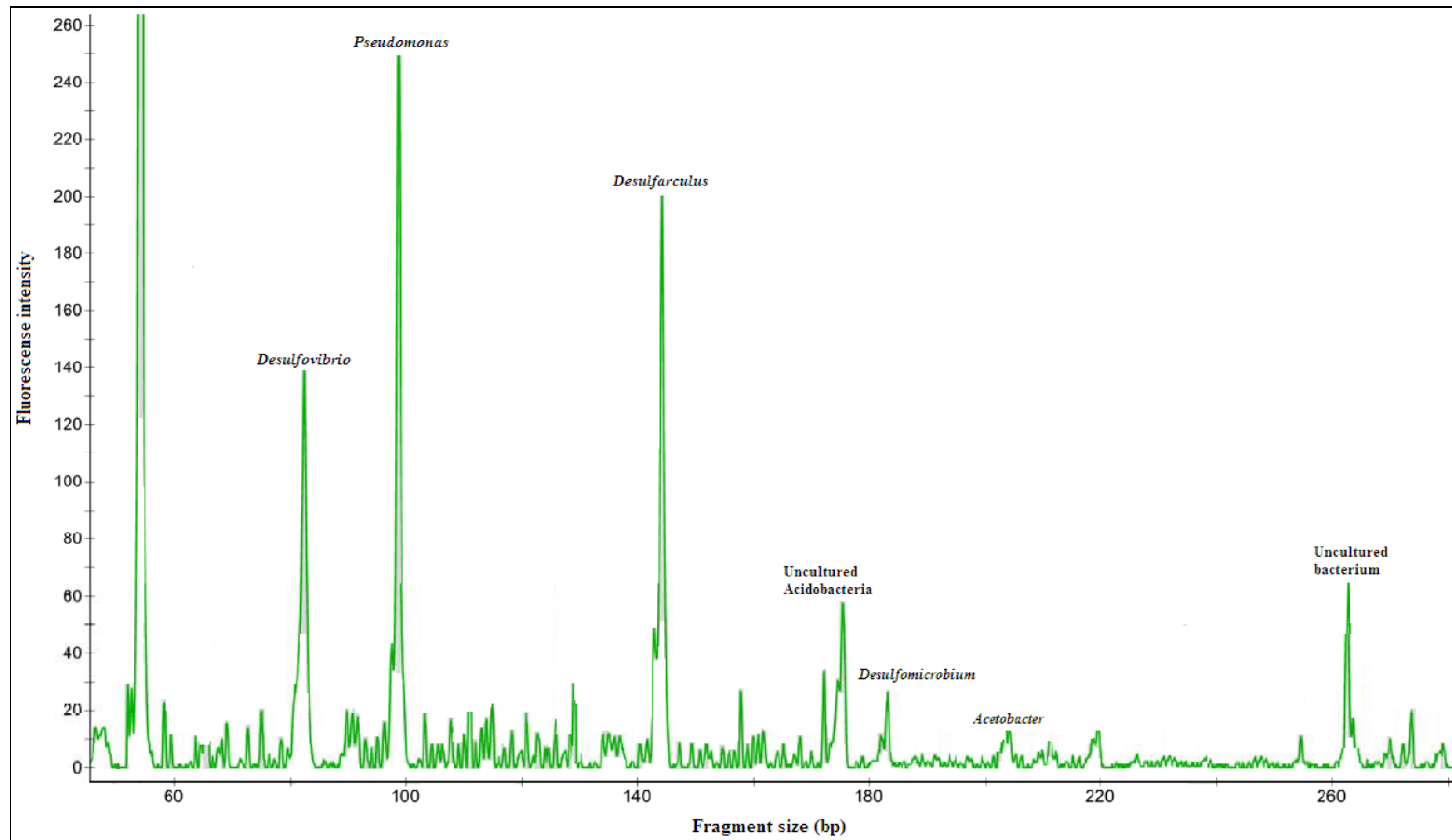

Figure 4.61 Electropherograms when phenol was the sole source of carbon in the PBR.

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The Electropherograms obtained when lactate and phenol were used as the sole carbon source in the PBR are given in Figure 4.60 and Figure 4.61, respectively. When lactate was the sole carbon source, presence of *Desulfovibrio* sp. with the highest intensity could be noted along with other species such as *Lactobacillus*, *Pseudomonas*, *Desulfuromonas,* and *Desulfarculus*.

Pseudomonas sp. gave the highest peak intensity when phenol was utilized as the sole carbon source. *Desulfovibrio* and *Desulfarculus* were also observed along with *Desulfomicrobium* and *Acetobacter*.

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Chapter 5

CONCLUSION AND FUTURE SCOPE OF WORK

5.1 Conclusions

The indigenous mixed microbial culture, isolated from the treatment plant, was highly effective in reducing SO_4^{2-} from synthetic wastewater. The phylogenetic analysis of the mixed consortia revealed *Pseudomonas aeruginosa* as the predominant strain which is reported in detail for the first time for its involvement in SO_4^{2-} reduction. The optimum condition of the mixed culture for SO_4^{2-} reduction was observed to be 30°C and pH 7.0.

Among the various carbon sources tested in the study, lactate showed maximum biodegradation of SO_4^{2-}. The performance of reactor fed with lactate as carbon source (R3) showed a much better efficiency for SO_4^{2-} reduction as compared with reactors R1 and R2, which used dextrose as the carbon source. Almost complete SO_4^{2-} removal was observed even when the COD/SO_4^{2-} was reduced to 0.8 thereby indicating that lactate was effectively utilized by the mixed consortia as the carbon source.

The effects of HRT, COD/SO_4^{2-} ratio and influent SO_4^{2-} concentration on SO_4^{2-} reduction were studied in a continuous PBR. The results indicates that more than 90% of SO_4^{2-} reduction took place upto a specific loading rate of 1800 mg/L, when operated at COD/SO_4^{2-} ratio of 0.67 and HRT of 24 h. Yellowish precipitate deposited in the upper portion of the PBR when it was operated with 2200 mg/L SO_4^{2-} was found to be predominantly elemental sulfur. The most probable reason for elemental sulfur formation may be due to the reaction between sulfide and sulfite in the upper ports, leading to the formation of thiosulfate and sulfur. The PBR was therefore found to be highly suitable for the treatment of SO_4^{2-} rich wastewater discharged from industries owing to its higher sulfate reduction efficiency. Bacterial strains such as

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Pseudomonas species, *Desulfovibrio* species and *Citrobacter* species were isolated from the PBR which have been already reported to have SO_4^{2-} reducing capacity.

The performance of PBR on SO_4^{2-} reduction with phenol addition was studied. It was clearly observed that SO_4^{2-} reduction was dependent on two factors, i.e. phenol and lactate COD concentration. At 350 mg/L phenol concentration, SO_4^{2-} reduction lowered drastically even when sufficient lactate COD was available, which suggests that it started to have inhibitory effects on the SRB. RSM was performed to optimize the system with the target to obtain maximum SO_4^{2-} reduction with the simultaneous removal of phenol in the process. SO_4^{2-} removal of 86.4 % (from initial 2000 mg/L) was observed in the PBR with the optimum lactate COD of 1400 mg/L and phenol concentration of 210 mg/L using RSM. Similar result was also obtained experimentally when phenol was added as a co-substrate thereby validating the RSM analysis.

The performance of PBR on SO_4^{2-} reduction with phenol as sole source of carbon was studied. Phenol was utilized effectively as the sole carbon source for SO_4^{2-} reduction till 550 mg/L of phenol concentration. However, with the increase in phenol concentration to 750 mg/L, both SO_4^{2-} and phenol removal decreased drastically which could be attributed to the inhibitory effect of phenol to SRB as well as PUB at higher concentrations of phenol. RSM was also performed to optimize the system with the target to obtain maximum SO_4^{2-} reduction with the simultaneous removal of phenol in the process. SO_4^{2-} and phenol removal efficiency of 75.5 % and 87% were achieved from initial SO_4^{2-} of 2000 mg/L, phenol of 512 mg/L and HRT of 42 h (optimum conditions as obtained by RSM) when phenol was used as the sole carbon source. The significant outcome of this observation is that phenol could be utilized as a sole carbon source, irrespective of its toxicity effect on SO_4^{2-} reduction.

Shock loading studies showed that up to 3.5 times increase in the normal input sulfate concentration (2000 mg/L) in the form of sulfate shock loading did not affect the PBR

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performance irreversibly. With the increase in sulfate concentration, the volumetric reduction rate started to decline. An increase in feed sulfate concentration has a negative influence on the sulfate conversion. The maximum sulfate conversion achieved at each feed sulfate concentration was at the highest HRT. It was also evident that sulfate removal decreases with increasing loading rate as the feed sulfate concentration was increased.

Optimum DO level for elemental sulfur formation in the microaerobic reactor was observed in the range of 70-100 µg/L with a minimum HRT of 17 h. The elemental sulfur can be easily separated from the reactor effluents by gravity sedimentation and centrifugation for further use.

Overall, the flow through bioreactor system is found to be effective in reduction of sulfate, utilizing lactate and/or phenol as carbon source. The bioreactor system is also able to regain its original performance level even after sulfate shock loadings and could therefore efficiently check the formation of hydrogen sulfide and convert ionic sulfides into elemental sulfur under microaerobic condition.

5.2 Future scope of work

The present study has indicated the ability of the bioreactor system to satisfactorily reduced sulfate rich wastewater but the study will require further investigation in the following areas:

1) Detailed study of the microbial community involved in each of the different operating conditions
2) Studies on the effects of air/oxygen flow rates on sizes and settling behavior of elemental sulfur particles in microaerobic reactor
3) Studies on performance of mixed microbial consortia on sulfate reduction to elemental sulfur in a single stage flow through bioreactor system
4) Laboratory and pilot plant scale studies on the treatment of sulfate bearing real industrial wastewater.
5) Laboratory and pilot plant scale studies on the treatment of sulfate as well as phenol bearing real industrial wastewater

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RESEARCH PUBLICATIONS

Published in journals

- **Brahmacharimayum, B.** and Ghosh, P.K. (2014). "Sulfate bioreduction and elemental sulfur formation in a packed bed reactor" Journal of Environmental Chemical Engineering, 2, 1287-1293.
- **Brahmacharimayum, B**. and Ghosh, P.K. (2012). "A study on efficiency of five different carbon sources on sulfate reduction." Journal of Environmental Research and Development, Vol. 7, No. 1, pp. 416-420.

Conferences/ workshop

- **Brahmacharimayum, B.,** and P. K. Ghosh. (2014). Sulfate Reduction in a Fixed Film Anaerobic Reactor under Shock Loading Conditions. 6th*International Conference on Chemical, Biological and Environmental Engineering,* Paris, France, September 15-16, 2014.
- Mohanty, M. P., **Brahmacharimayum, B**., and Ghosh, P.K. (2014). Simultaneous bioreduction of sulfate and nitrate in batch shake flasks. *National Conference on Sustainable Development of Environmental Systems,* Guwahati, India, June 20-21, 2014.
- **Brahmacharimayum, B.**, Mohanty, M. P., and Ghosh, P.K. (2014). A review on the intermediates, pathways and enzymes utilized in dissimilatory sulfate reduction. *International Conference on Harnessing Natural Resources for Sustainable Development-Global Trend*, Guwahati, India, January 29-30,2014.
- Laskar, M., Mohanty, M. P., **Brahmacharimayum, B**. and Ghosh, P.K. (2013). Kinetics of sulfate bioreduction and sulfide oxidation by mixed microbial consortia. *International Congress of Environmental research (ICER-2013)*, Aurangabad, India, December 19-21, 2013.

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- **Brahmacharimayum B.** and Ghosh, P. K. (2012). Biological sulfate reduction. *World Congress on Biotechnology*, Hyderabad, India, May 4-6, 2012.
- **Brahmacharimayum B.** and Ghosh, P. K. (2012). Bioreactors for sulfate reduction. *International Conference on Environmentally Sustainable Urban Ecosystems,* held at IIT Guwahati, India, February 24-26, 2012.
- **Brahmacharimayum B**. and Ghosh, P. K. (2012). Acid mine drainage (AMD) treatment to reduce sulfate into elemental sulfur by microaerobic process. *National Conference on Forest, Environment and Climate change: Issues and Challenges",* Bilaspur, India, January 30-31, 2012.
- **Brahmacharimayum B.** and Ghosh, P. K. (2011). A study on efficiency of five different carbon sources on Sulfate Reduction *International Congress of Environmental Research "ICER-2011"*, Surat, India, December 15-17, 2011.

Papers under review

- **Brahmacharimayum, B.,** Mohanty, M., and Ghosh, P.K. "Intermediates and end products of sulfate bio-reduction: a review". Critical Reviews in Environmental Science and Technology.
- **Brahmacharimayum, B.** and Ghosh, P.K. "Effects of different environmental and operating conditions on sulfate bioreduction in shake flasks by mixed bacterial culture predominantly *Pseudomonas aeruginosa". Desalination.

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