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Sulfate inhibition effect on sulfate reducing bacteria

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Abstract

There is an increasing interest in the potential of bacterial sulfate reduction as an alternative method for sulfate removal from wastewater. Under anaerobic conditions, sulfate-reducing bacteria (SRB) utilize sulfate to oxidize organic compounds and generate sulfide (S2-). SRB were successfully isolated from sludge samples obtained from a local petroleum refinery, and used for sulfate removal. The effects of initial sulfate concentration, temperature and pH on the rate of bacterial growth and anaerobic sulfate removal were investigated and the optimum conditions were identified. The experimental data were used to determine the parameters of two proposed kinetic model, which take into consideration substrate inhibition effect.

Keywords: Sulfate Reducing Bacteria, Sulfate, Kinetic Model, Biotreatement, Inhibition

Introduction

High sulfate containing wastewaters are generated from various industrial activities including petroleum refineries. In some cases, the amount of sulfate in wastewater can reach concentrations as high as 4000 g m-3, which is drastically above the acceptable levels (500 gm-3) set by many environmental legislations including that of the UAE. Several problems arise from high sulfate concentrations in water such as corrosion of water transport systems and of concrete structure. To prevent corrosion, the pH is neutralized by addition of Ca(OH)2 resulting in the precipitation of CaSO4. This in turn may result in another problem, which is scaling that leads to loss in production and damage to equipment, as well as higher labor costs due to regular cleaning of the equipment. In addition, intake of water with high sulfate concentration can lead to illnesses like diarrhea. Therefore, the World Health Organization (WHO) recommended a limit of 250 gm-3 for Europe in 1961 (Visser et al. 2001). Moreover, any present sulfate in water can be reduced to hydrogen sulfide that corrodes copper, iron and carbon steel. In addition to these corrosion effects, hydrogen sulfide has a unique odor which is very harmful to the environment. In the United Arab Emirates, environmental legislation is strict towards the release of poor-quality water into the environment. Therefore, it is clear that sulfates must be removed from industrial aqueous effluents. In this work, the effectiveness and applicability of bioremoval of sulfate using SRB are investigated.

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Biotreatment using SRB

There is an increasing interest in the potential biotechnological applications of bacterial sulfate reduction as an alternative method for sulfate and heavy metal removal from environmental contamination (Chang et al. 2000; Elliott et al. 1998; Kim et al. 1999). Under anaerobic conditions, SRB oxidize simple organic compounds by utilizing sulfate as an electron acceptor and generate sulfide (S2-) and alkalinity. The produced sulfide can react with dissolved metals to form metal sulfide precipitates, since the solubilities of most toxic metal sulfides are generally very low (Kim et al. 1999).

SRB carry out sulfate reduction based on the reaction shown in Eq (1)

$$SO_4^{2-} + 8e^- + 4H_2O \rightarrow S^{2-} + 8OH^- \quad (1)$$

The electrons which are needed for the sulfate reduction are generated by the oxidation of a carbon and energy source. According to their ability to oxidize organic compounds into carbon dioxide, SRB are generally classified as complete or incomplete oxidizers. Lactate is the widely used carbon source by most SRB species (Postgate 1984).

Kinetics Studies

Several attempts to study the kinetics of anaerobic reduction of sulfate have been reported in literature. Kalyuzhnyi and Fedorovich

(1998) developed a structured mathematical model of competition between sulfate reduction and methanogenesis in anaerobic reactors. The model included multiple-reaction stoichiometry, microbial growth kinetics, conventional material balances for an ideally mixed reactor, liquid gas interactions, and liquid phase equilibrium chemistry. Moosa et al. (2002) examined the effects of initial sulfate concentration and its volumetric loading on the kinetics of reaction and activity of a mixed population of complete oxidizers SRB growing on acetate in a continuous bioreactor. Pirt equation (Eq 1) (Prit 1975) was adopted to relate the rate of substrate utilization (sulfate reduction in this case) to the rate of biomass formation,

$$r_s = \frac{dS}{dt} = \frac{1}{Y_{X/S}} \frac{dX}{dt} \qquad (2)$$

Where, r_s is the rate of substrate utilization (reduction rate of sulfate or utilization rate of acetate), $Y_{X/S}$ is the yield coefficient and X is the biomass concentration.

The rate of biomass formation dX/dt was presented by Eq (3)

$$\frac{dX}{dt} = \mu X \qquad (3)$$

Where, μ is the specific growth rate. The decay coefficient k_d is not considered in Eq (3) as the death period was not encountered during the early growth phase considered. Moosa et al. (2002) fit their experimental data into different models, including Monod (Eq 4), Chen and Hashimoto (5) and Contois models Eq (6) (Chen and Hashimoto 1980).

$$\mu = \frac{\mu_m S}{K_S + S} \qquad (4)$$

$$\mu = \frac{\mu_m S}{K_S S_o + (1 - K_S) S} \qquad (5)$$

$$\mu = \frac{\mu_m S}{K_S X + S} \qquad (6)$$

Where, μ_m is the maximum specific growth rate, S_o is the initial substrate concentration and K_s is the half saturation constant. The values of various kinetic coefficients were determined by non-linear regression technique using various initial concentrations of sulfate. Among the tested models, the Contois expression (6) fitted the data with the highest accuracy. Moosa et al. (2002) reported that the increase in initial concentration of sulfate enhanced the reaction rate. The saturation constant K_s, was reported to increase linearly with initial sulfate concentration. However, the initial concentration of sulfate did not have a significant effect on maximum specific growth rate μ_m, decay coefficient k_d, or bacterial yield $Y_{X/S}$. On the other hand, for a given initial sulfate concentration, increasing the volumetric loading rate of sulfate led to a linear increase in volumetric reduction rate. Moosa et al. (2005) extended their previous work (Moosa et al. 2002) to incorporate the effect of temperature on the kinetics model. The effect of temperature on maximum specific growth rate and bacterial yield was found to be insignificant in the range of temperature considered (20 – 35 ^oC). However, the decay coefficient k_d, and apparent saturation constant $K`_S=(K_S S_O)$ were both temperature dependent. The increase of temperature resulted in decreased values of $K`_S$ and higher values for k_d.

In this work the effects of sulfate concentration, temperature, pH on the bacterial growth rate and the sulfate reduction rate by the locally isolated SRB will be evaluated. Previous studies (Moosa et al. 2002; Moosa et al. 2005) that were based on continuous operation, resulted in variable values of the kinetic parameters. However, the proposed kinetic study is based on a batch experiments and precise values of the parameters are determined.

Materials and Methods

Growth Media

Twenty gram of sludge sample, obtained from a local petroleum refinery, were dispensed into 100 ml of autoclaved pre-reduced bicarbonate buffered solution as described by Holowenko et al. (2000). Serial dilutions were prepared and 0.2 ml aliquots of each dilution were spread on the surface agar medium. The isolation was carried out in Postgate medium C (sPGC) (Postgate 1984). The medium consists of the following: NaCl (0.12M), $MgCl_2.6H_2O$ ($5.9x10^{-3}$ M) KH_2PO_4 ($3.6x10^{-3}$ M) NH_4Cl (0.019 M), Na_2SO_4 (0.032 M), $CaCl_2.2H_2O$, ($2.8x10^{-4}$ M), $MgSO_4.7H_2O$ ($1.2x10^{-4}$ M), $FeSO_4.7H_2O$ ($1.4x10^{-5}$ M), trisodium citrate ($1.1x10^{-3}$ M), sodium lactate (70% w/v, 0.077 M), yeast extract (1 g L^{-1}) and agar (20 g L^{-1}). The pH was finally set at 7.2.

SRB Isolation

The plates were incubated at 30 ºC for 10 days under anaerobic conditions in a GasPak anaerobic jar (GasPak system; B&E ASEARLE Company) filled with carbon dioxide and hydrogen which was produced by using anaerogen sachets according to the instructions of the manufacturer. Preparation and inoculation of plates were carried out inside an environmental chamber which contained a mixture of gases (Nitrogen 87%, carbon dioxide 10%, and hydrogen 3%) in oxygen free environment. Preparation and inoculation of plates were carried out inside anaerobic controlled environmental chamber. After incubating for a week, several colonies of SRB were observed. The different bacteria were isolated and allowed to grow on separate plates and were found to be of the same type.

Scanning Electron Microscope Image Capturing

Isolated SRB Bacteria were collected from Agar plate and centrifuged at 1000 rpm for 5 minutes to get a pellet in a Eppendorf tube and fixed with Karnovsky fixative (2% Paraformaldehyde and 2.5% Glutaraldehyde) for 1 hour at room temperature then washed with 0.1 M phosphate buffer pH 7.2 for three times then post fixed with chilled 1% osmium tetroxide for one hour. The pellet was washed with distilled water three times for 5 minutes then dehydrated with ascending series of ethanol from 30% to 100%. After critical point drying (Polaron CPD Bell Brook Business Park. Bolton Close Uckfield. East Sussex TN22 IQZ England), the pellet was resuspended in 100 % ethanol and then fixed on a Carbon tabed 0.5 Aluminium stub. Then stubs were fixed in the Polaron Sputter Coater Vacuum chamber and were sputtered with gold Au/Pd target for 5 minutes, at 20 mA (Polaron sputter Coater Bell brook Business Park.Bolton Close Uckfield. East Sussex TN22 IQZ England). Then bacteria were studied under the Scanning Electron Microscope (ESEM Philip-XL30, Neatherland) and pictures were taken at different magnification. Figures 1 and 2 show different magnifying levels of the isolated SRB. The isolated bacteria was verified to be SRB, as it was grown on a selective media and share the same properties with common SRBs, such as being motile, stain gram negative and have a rod shape (Bacilli). The morphology of the isolated bacteria suggests that it is of *desulfovibrio* genus (Stetter et al. 1993). The genus was further confirmed from the effect of temperature as explained in section 5.2.

Kinetics Study

Various concentrations of sulfate from Na_2SO_4, in the range of 500 to 4000 gm^{-3}, were added to a series of 100 ml of the growth media

placed in 120 ml bottles. The bottles were flushed with nitrogen gas for five minutes to ensure anaerobic conditions, and then sealed and autoclaved for 30 minutes at 121°C. After the bottles are allowed to reach the desired temperature in a temperature controlled incubator, equal amounts of isolated SRB culture suspension are inoculated into each bottle. Aliquots are withdrawn at suitable time intervals inside an oxygen free environmental chamber and their absorption was measured at a wavelength of 600 nm (Monod 1949) using spectrophotometer (WPR, lightwave, S2000 UV/V, UK).

Results and Discussion

Effect of Sulfate Concentration

The results in Figure 3 show the effect of initial sulfate concentration, in the range of 500 to 4000 gm^{-3}, on the SRB growth at 35 ^{o}C and pH 7. The experiment at initial concentration of 2500 gm^{-3} was repeated in duplicates, and the results shown are the averages. The experiment at initial sulfate concentration of 2500 gm^{-3} was done twice, and the values shown in Figure 3 are the averages. The small error bars represents the standard deviation between the two experiment repetitions and clearly demonstrate the reproducibility of the results. The solid lines in the figure are connections between the experimental data, shown to highlight the trend. It can be seen from Figure 3 that the lag phase (acclimation period), which is the time required for a bacterial cell to acclimatize to new environment and begin metabolizing lasted for 500 minutes. Once nutrients can be utilized the growth rate moves from 0 to reach a specific growth rate, μ. The solid lines in the figure are connections between the experimental data, shown to highlight the trend.

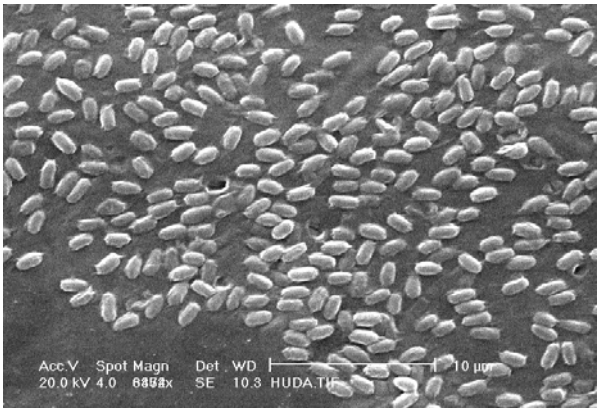

Figure 1: Scanning Electron Microscopic image (1) of the isolated SRB

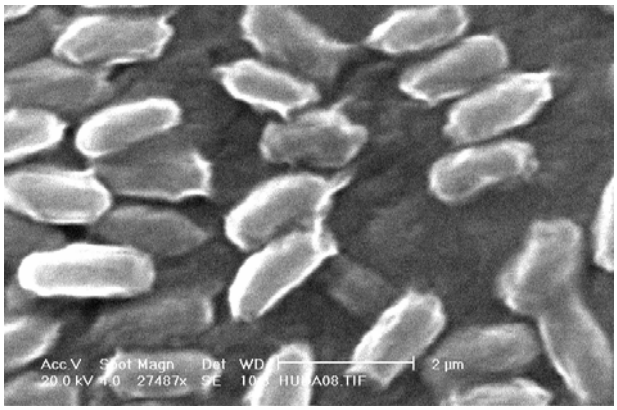

Figure 2: Scanning Electron Microscopic image (2) of the isolated SRB

In order to determine the biomass concentration in the withdrawn samples, a calibration curve was prepared by finding the absorption of several suspensions of known biomass concentrations that are determined by the parallel dilution analysis. Several dilutions of suspensions were determined in the range of 10^{-6} – 10^{-12}, where duplicate plates have been used for each dilution. The amount of bacteria was determined by microscopic counting technique using a transmitted light microscope (ZEISS, Germany), connected to KS300 Kontron Electronic software. The weight of the biomass was then determined from the average molecular weight of bacteria, which is 113 $gmol^{-1}$ (Widdel 1988). A calibration curve was then drawn between the biomass concentrations and the corresponding absorption. The biomass concentration in each withdrawn sample was then determined by comparing its absorption to the biomass concentration in the calibration curve.

To complete the kinetic studies, the concentrations of sulfate in the withdrawn samples were also determined. SulfaVer 4 sulfate reagent was added to 10 ml sample and the absorption at 450 nm was measured using spectrophotometer (Hach DR 5000, Germany) to determine the sulfate concentration.

By ignoring the death coefficient in Eq (3) as it is not encountered during the early growth phase considered, the specific growth rate, μ, was determined for each case, from the slope of the line of the log(X) vs time, and the results are shown in Figure 4. The specific growth rate μ, increased linearly from 0.0079 min^{-1} at 500 gm^{-3} to 0.0083 min^{-1} at 2500 gm^{-3}, and then dropped to 0.0063 min^{-1} at 4000 gm^{-3}. The results clearly show the presence of substrate inhibition, which was not considered in the previous works (Moosa et al. 2002; Moosa et al. 2005). Since the optimum growth rate was

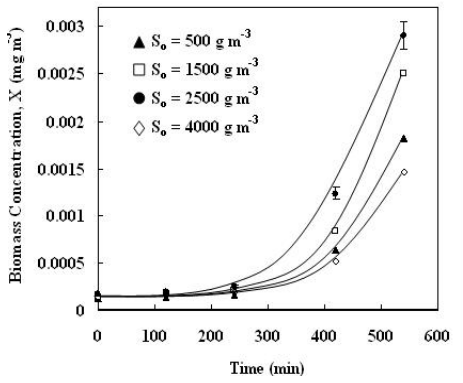

Figure 3: Effect of initial sulfate concentration of the SRB growth at 35 ^{o}C and pH 7

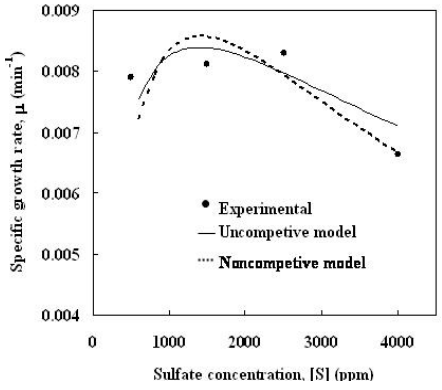

Figure 4: Effect of sulfate concentration of specific growth rate, μ, at 35 ^{o}C and pH 7

at sulfate concentration of 2500 gm^{-3}, all subsequent experiments have taken place using this concentration.

The growth period, considered in this work, was short enough to ensure that the sulfate concentration does not change dramatically, in addition to ignoring the death coefficient in Eq (3) as explained earlier. The concentration of sulfate was determined with time, and as seen from Figure 5, during the growth time considered (i.e, up to 540 min) the drop in sulfate concentration did not exceed 4.1% at the highest growth rate at 2500 gm^{-3}. It was until 1000 minutes, when considerable drop in sulfate concentration started to be significant. Figure 5 shows the drop in sulfate concentration for at highest two growth rates. It has also been determined for other initial sulfate concentrations; however their results are not shown in Figure 5 to avoid congestion. The experiment at initial sulfate concentration of 2500 gm^{-3} was done twice, and the values shown in Figure 5 are the averages. The small error bars represents the standard deviation between the two experiment repetitions and clearly demonstrate the reproducibility of the results. The solid lines in the figure are connections between the experimental data, shown to highlight the trend. Figure 5 shows that the rates of drop in sulfate concentration at initial concentration of 2500 gm^{-3} were higher than that at 1500 gm^{-3}. This is expected as the SRB growths at the 2500 gm^{-3} was the highest. A comparison between the results in Figures 3 and 5 shows that during the lag phase, the drop in sulfate concentration was low. However, as soon as the growth rate starts to increase and enters the exponential stage, the sulfate concentration starts to drop.

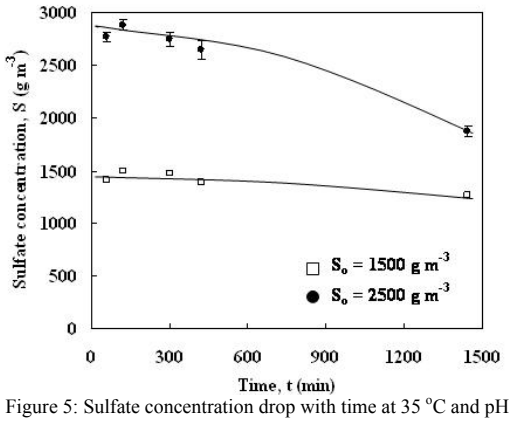

Figure 5: Sulfate concentration drop with time at 35 ^oC and pH 7

5.2. *Effect of Temperature*

The results in Figures 6 and 7 show the effect of temperature on the SRB growth rate and the sulfate drop rate, respectively, at initial sulfate concentration of 2500 gm^{-3} and pH of 7. The solid lines shown in the figure are connections between the experimental data, shown to highlight the trend. As expected, the fastest drop in sulfate concentration was observed at 35 ^oC. The drop in sulfate concentration at 20 ^oC was higher than that at 50 ^oC, however, they were almost in the same magnitude, contrary to the SRB growth at 20 ^oC which was much higher than that at 50 ^oC. This can be due to the increase in the maintenance coefficient with temperature, where all the substrate utilized for the survival of the bacteria, and less amount are utilized for the growth.

The specific growth rate, μ, was determined for each case, from the slope of the line of the log(X) vs time, and the results are shown in Figure 8. The specific growth rate μ, increased linearly from 0.0052 min^{-1} at 500 gm^{-3} to 0.0083 min^{-1} at 2500 gm^{-3}, and then dropped to 0.00068 min^{-1} at 4000 gm^{-3}. These results agree with those of Moosa (2002) who employed a mixed culture containing sulfate reducers in a batch experiments. It was reported that SRB growth rate increased with increasing the reaction temperature from 20 to 35°C. Further increase of temperature to 40 °C led to inactivity of the bacteria. Since SRB grow at temperatures less that 40°C, they are classified as mesophiles. It is worth mentioning that a number of thermophilic SRB strains are available, such as some species from the *Archaeoglobus* and *Thermodesulforhabdus*, genera (Fortin et al. 1996). The fact that the SRB isolated in this work has optimum temperature at 35^oC further confirms that it belongs to the *desulfovibrio* genus as shown by Widdle (1998).

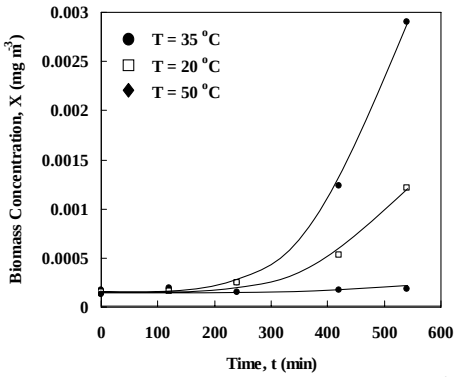

Figure 6: Effect of temperature on the SRB growth at 2500 gm^{-3} and pH 7

Effect of pH

The results in Figures 9 and 10 show the effect of pH on the SRB growth rate and the sulfate drop rate, respectively, at 2500 gm^{-3} initial sulfate concentration and 35 ^oC. To avoid congestion, Figure 10 shows the results at only three pH values. As expected, the fastest drop in sulfate concentration was observed at pH 7.

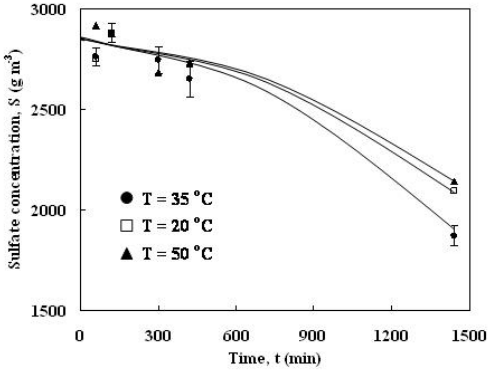

Figure 7: Effect of temperature on the sulfate drop at 2500 gm^{-3} and pH 7

The specific growth rate, μ, was determined for each case, from the slope of the line of the log(X) vs time, and the results are shown in Figure 11, which shows that the growth in the basic media (pH of 8 and 10) was high, which resulted in the significant drop in sulfate concentration shown in Figure 10. Whereas, in acidic media (pH 6 and 4) the growth was negligible that resulted in a negligible drop in sulfate concentration. The results found in the work agree with those of Fortin et al. (1996), who found that growth was not evident at pH lower than 7.

The pH plays an important role, when sulfate is reduced to sulfide. The sulfide can be present in different forms like H_2S, HS^- and S^{2-}. The state of sulfide solely depends on the pH of the environment. At a pH of 7.0 most of the sulfide concentration is in the hydrogen

sulfide form (Perry and Green 1984). At low pH the produced hydrogen sulfide exists in undissociated form and as the pH increases it dissociates into HS^- and S^{2-}. There were different explanations for the inhibitory effect of sulfide. One explanation assumes irreversible inhibition by absorbed of sulfide into the cells, resulting in destruction of their proteins (Postgate 1984). On the other hand, another explanation assumes reversible inhibition of sulfide, which results in the cells regaining their activity once all sulfate are removed (Reis et al. 1992).

It has been shown that at pH less than 7.0, undissociated H_2S is the dominant inhibitor. Whereas, at pH above 7.0 the total sulfide is responsible for the inhibitory effect (O'Flaherty and Colleran 1998). Generally, 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. As the pH increases, less concentration of undissociated H_2S is needed to inhibit the growth.

Where K_I is the inhibition constant.

As mentioned earlier, within the growth period considered the sulfate concentration does not change dramatically, as shown in Figure 5. Hence, the substrate concentration S, in Eqs (7) and (8) was replaced with the initial substrate concentration S_o. The experimental results in Figure 4 were used in M.S. Excel spread sheet solver to determine the kinetic parameters found in Eqs (7) and (8). A nonlinear multiple regression technique was developed to determine the kinetic parameters that results in the minimum objective function (Eq 9) that compares the measured growth rate with that predicted by the proposed kinetic equations. A constraint is imposed on the values of the parameters to be always positive.

$$OF = \sum \left(\mu_{pred} - \mu_{expt}\right)^2 \qquad (9)$$

The model equations (Eq 7 and 8), with the evaluated kinetic parameters, are presented in Eqs (10) and (11). The effectiveness of the equations in predicting the experimental data is shown in Figure 4 and evaluated from the standard deviation value shown in the equations.

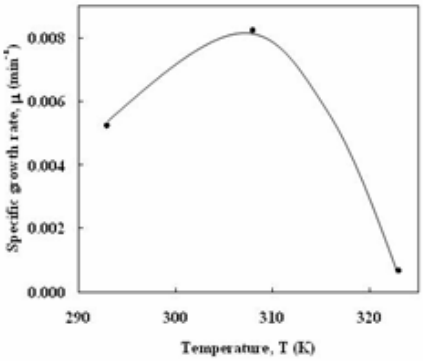

Figure 8: Effect of temperature on the growth rate, μ, at 2500 gm^{-3} and pH 7

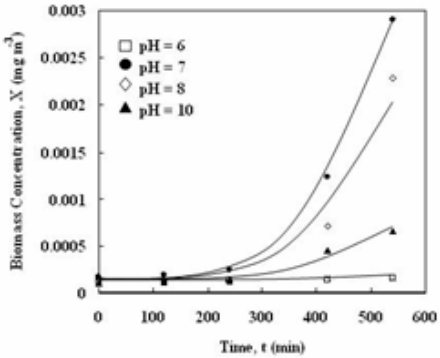

Figure 9: Effect of pH on the SRB growth at 35°C and 2500 gm^{-3}

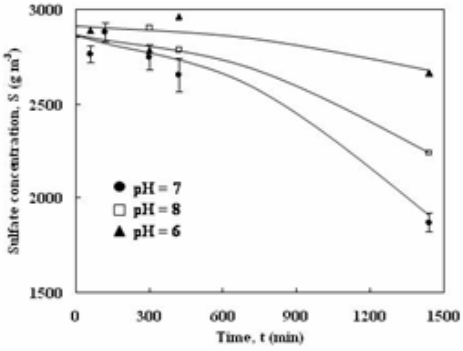

Figure 10: Effect of pH on the sulfate drop at 35 °C and 2500 gm^{-3}

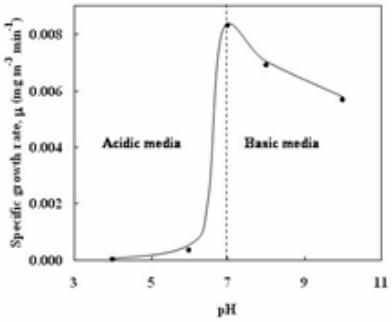

Figure 11: Effect of pH on the growth rate, μ, at 2500 gm^{-3} and 35 °C

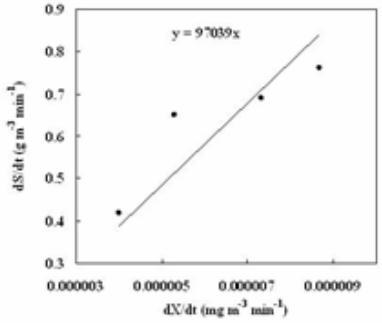

Figure 12: The linear relationship between dS/dt and dX/dt

Determination of Kinetic Parameters

The experimental results presented in Figures 3 and 4 have shown substrate (sulfate) inhibition effect on the growth of SRB. This phenomenon was not attended to any of the previous models proposed to describe the SRB growth (Moosa et al. 2002; Moosa et al. 2005).This is because previous works were done in region of sulfate concentrations prior to onset of inhibition. In this study, the effectiveness of uncompetitive and noncompetitive inhibition models (Eqs 7 and 8) to predict the experimental data is tested.

$$\mu = \frac{\mu_m S}{K_S + S + \frac{S^2}{K_I}} \qquad (7) \qquad \mu = \frac{\mu_m S}{\left(K_S + S\right)\cdot\left(1 + \frac{S}{K_I}\right)} \qquad (8)$$

$$\mu = \frac{0.012S}{300 + S + \frac{S^2}{6500}} \pm 2.4\times10^{-4} \qquad (10)$$

$$\mu = \frac{0.025S}{\left(100 + S\right)\cdot\left(1 + \frac{S}{200}\right)} \pm 4.7\times10^{-4} \qquad (11)$$

It can be seen that both models were able to predict the experimental results fairly well. However, the uncompetitive model (Eq 10) is slightly more accurate than the noncompetitive model (Eq 11).

Unlike the fixed values of the kinetic parameters determined in this work, Moosa et al. (2002) reported variable values of μ_m and K_S with initial sulfate concentrations. The change of μ_m with initial sulfate concentration was minor, increasing from 9.7×10^{-4} at 1000 g m^{-3} to 10.1×10^{-4} min^{-1} at 10,000 gm^{-3}. The change in the value of K_S was more acute, changing from 27 gm^{-3} at 1000 gm^{-3} to 125 gm^{-3} at 10,000 gm^{-3} (Moosa et al. 2002). Comparing these values to the ones found in this work, using the two inhibition models, shows that the values of K_S are in the same order of magnitude, whereas the values of μ_m found in this work are around twenty times higher than those found by Moosa et al. (2002). On the other hand, substrate inhibition was ignored in the work of Moosa et al. (2002) and hence, no value for K_I was reported.

The results in Figures 3 and 5 were used to determine the rate of bacterial growth, dX/dt and the rate of substrate depletion, dS/dt, respectively, in the exponential growth region. The linear relationship between the two rates, shown in Figure 12 was used to determine the yield coefficient $Y_{X/S}$ found in Eq (2). The value of the yield coefficient was found to be 0.01 $mg_{SRB}g_{Sulfate}^{-1}$.

Conclusions

SRB was successfully isolated from petroleum refinery wastewater sludge, and used in the removal of sulfate. The effects of initial sulfate concentration, temperature and pH on the rate of bacterial growth and sulfate removal were tested. It was found that the sulfate has an inhibition effect on the bacterial growth, with a maximum growth taking place at 2500 gm^{-3}. The optimum temperature and pH were found to be 35 ^oC and 7, respectively. Two kinetic models that take into consideration the inhibition effect by the substrate were used to describe the system. The uncompetitive inhibition model was found to predict the experimental data slightly better than the noncompetitive model.

Acknowledgments

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