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Study on Growth Influencing Factors and Desulfurization Performance of *Sulfate Reducing Bacteria* Based on the Response Surface Methodology

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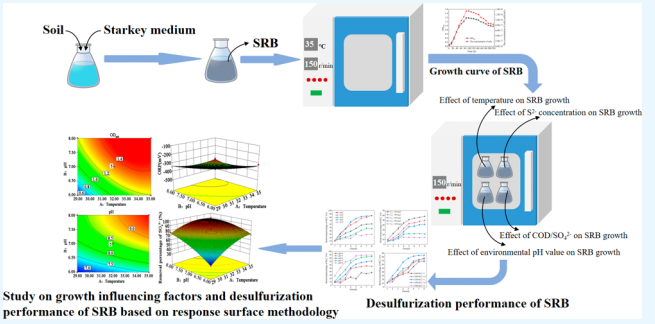
ABSTRACT: *Sulfate reducing bacteria* (SRB) can simultaneously and efficiently remove SO_4^{2-} and heavy metal ions from acid mine drainage (AMD). Environmental factors have a great influence on AMD treated by SRB metabolic reducing sulfate. Providing a suitable growth environment can improve the effect of SRB on AMD. In this paper, the wet soil around the tailings reservoir was used as seed mud to enrich SRB. Based on the single factor experiment method and the response surface methodology (RSM), the effects of temperature, environmental pH value, S^{2-} concentration, and COD/SO_4^{2-} on the growth of SRB were analyzed. The effects of environmental factors such as temperature and pH on the desulfurization performance of SRB were investigated. The results showed that the growth curve of SRB was "S" type. SRB was in the logarithmic phase when cultured for 14−86 h, with high activity and vigorous growth metabolism. When the temperature is 32∼35 °C, the activity of SRB is the highest. With the gradual increase of the S^{2-} concentration in the culture system, SRB activity will be inhibited and even lead to SRB cell death. The environmental pH value that SRB can tolerate is 5∼8, and when the environmental pH value is 7∼8, the SRB activity is the strongest. The chemical oxygen demand (COD)/SO_4^{2-} that is most suitable for SRB growth is 2. The optimal growth conditions of SRB obtained from RSM were as follows: culture temperature at 34.74 °C, initial pH being 8.00, and initial COD/SO_4^{2-} being 1.98. Under these conditions, the OD_{600} value was 1.45, the pH value was 9.37, the oxidation reduction potential (ORP) value was −399 mV, and the removal percentage of SO_4^{2-} was 88.74%. The results of RSM showed that the effects of culture temperature, environmental pH, and COD/SO_4^{2-} on the desulfurization performance of SRB were extremely significant. The order of affecting the removal of SO_4^{2-} by SRB was environmental pH > temperature > COD/SO_4^{2-}.



1. INTRODUCTION

Acid mine drainage (AMD) is one of the most serious environmental problems faced by the mining industry. The pH of such wastewater is usually acidic and rich in sulfate ions, iron ions, and toxic metal ions of certain concentrations (Cu^{2+}, Zn^{2+}, Pb^{2+}, etc.).[1,2] Cu^{2+}, Zn^{2+}, Pb^{2+}, and other heavy metal ions in AMD are easy to be absorbed into human health through the food chain.[3] The microbial method with sulfate reducing bacteria (SRB) as the dominant strain is a promising AMD treatment technology.[4] It has the advantages of high efficiency, low energy consumption, and environmental friendliness, which has attracted the attention of researchers.[5,6]

SRB is ubiquitous in the natural environment. It can use sodium lactate, ethanol, H_2, and other electron donors[7] to reduce SO_4^{2-} under anaerobic (or anoxic) conditions, produce sulfide (including S^{2-}, HS^-, and H_2S) to precipitate heavy metals, and produce alkali substances to improve pH.[8,9] SRB is very important in the biogeochemical cycle of the sulfur and microbial desulfurization process. Muhammad et al.[10] found that SRB could remove SO_4^{2-} and heavy metal ions synchronously. Among them, the fixation rate of iron, copper, lead, and other heavy metal ions is 87∼100%.[10] The removal percentages of Mn^{2+} and Pb^{2+} by SRB separated by Miao et al.[11] were 93 and 90%, respectively. Sahinkaya and Gungor[9] showed that SRB could remove 90% of SO_4^{2-} in wastewater with an initial SO_4^{2-} concentration of 2000 mg/L and COD/SO_4^{2-} = 0.75. The SRB separated by Torbaghan and Torghabeh[12] can remove 85% iron and 78% SO_4^{2-},

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respectively. Le Pape et al.[13] showed that SRB can remove As, Zn, and Fe from wastewater. SRB has good removal effect on SO_4^{2-} and heavy metals in AMD. Especially, the environment suitable for SRB growth can improve the metabolic activity of SRB. There are many factors affecting SRB metabolism, such as temperature, environmental pH, S^{2-} concentration, and COD/SO_4^{2-}. It is reported that the growth of SRB is greatly affected by environmental pH and temperature.[14] Fang et al.[15] showed that SRB flora will die when the temperature is too high, and SRB population exists when the temperature is appropriate. Xu et al.[16] showed that with the increase of temperature, either the amount or activity of SRB increased. However, the rise of temperature also promoted the death of SRB. Li et al.[17] studied that the SRB exhibited strong activity when the environmental temperature was 30 °C, while the SRB activity was weak at 50 °C. Cisse[18] showed that low temperatures and low pH values would reduce the reduction efficiency of SRB to sulfate. Liu et al.[19] found that different initial pH conditions would affect the number and height of crystals of SRB metabolites. Warthmann et al.[20] found that the change of the pH value of solution can affect the formation rate of SRB-induced sulfate mineralization and ultimately affect the mineral species and morphology. S^{2-} had an inhibition effect on SRB growth, and the inhibition went strong with the increase of S^{2-} concentration.[21] Ren et al.[22] reported that when the concentration of H_2S and S^{2-} exceeds 50 and 200 mg/L, the growth of SRB will be severely inhibited. Different COD/SO_4^{2-} will affect the symbiotic environment of SRB and methanogens[23] leading to different dominant growth strains, thus indirectly affecting sulfate reduction efficiency. Liu et al.[24] reported that SRB could be guaranteed to have good desulfurization effect when COD/SO_4^{2-} was 0.67 in theory. However, in actual circumstances, because of the lack of electron donors, SO_4^{2-} cannot be reduced into H_2S effectively by SRB when COD/SO_4^{2-} was among 0.5∼1.5.[24] When COD/SO_4^{2-} was among 1.5∼2.5, SRB had the best removal effect on SO_4^{2-}, and the desulfurization rate was stable at 88.3∼92.1%, with little fluctuation.[24] When COD/SO_4^{2-} was among 2.5∼4, the desulfurization effect of SRB tended to decrease.[24] Zhao[25] explored the effect of temperature (at 20, 30, 35, 40, 50 °C) and environmental pH (3.39, 3.92, 4.30, 5.46, 6.27, 6.66, 7.53, 8.51) on SRB growth based on the complete randomized design, and the results showed that the optimal growth temperature at pH 6.27 was 35 °C. In the present research, the complete randomized design is often used to explore the effects of temperature, pH, and COD/SO_4^{2-} on SRB growth. In the actual growth process of SRB, there is an interaction among the factors such as temperature, pH, and COD/SO_4^{2-} affecting SRB, and the degree of the effect is also different. Therefore, environmental factors have a greater impact on SRB metabolism and sulfate reduction. By preferentially regulating the most obvious factors affecting the growth and metabolism of SRB, combined with the interaction of different factors affecting the growth of SRB, the most suitable growth environment is provided for SRB, therefore regulating the desulfurization effect of SRB. Providing a suitable growth environment can improve the effect of SRB on AMD. However, there are few studies on SRB growth influencing factors and desulfurization performance. Especially, there is a lack of research on the desulfurization performance of SRB under different environmental conditions. Based on the results of the complete randomized design, the RSM can comprehensively analyze the effects of different factors on the SRB growth and select the most suitable environment for SRB desulfurization. At the same time, the RSM can analyze the interaction of different factors affecting SRB and analyze the strength of SRB desulfurization.

Therefore, the effects of temperature, pH, S^{2-} concentration, and COD/SO_4^{2-} on the growth of SRB were analyzed based on the single factor experiment and the response surface methodology (RSM). At the same time, combined with the changes of the OD_{600} value, pH value, ORP value, and SO_4^{2-} removal percentage, the desulfurization performance of SRB in different environments was explored. To summarize the experimental results, the most suitable environment for the growth of SRB was selected and theoretical guidance for SRB repair AMD technology was provided.

2. MATERIALS AND METHODS

SRB: The moist soil around a tailings pond in Huludao City, Liaoning Province, was used as seed mud for SRB enrichment. Five grams of seed mud were added to 120 mL of sterilized Starkey medium.[6] The main components of the Starkey medium were 1 L of distilled water, 0.5 g of K_2HPO_4, 1.0 g of NH_4Cl, 0.5 g of Na_2SO_4, 2.0 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2{\cdot}H_2O$, 1.0 g of yeast extract, 4.0 mL of sodium lactate solution, 0.1 g of ascorbic acid, and 1.2 g of $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, pH = 7.0, sterilized at 121 °C for 30 min.[6] The inoculated liquid was incubated under anaerobic conditions. When the liquid turns black and smells of rotten eggs, SRB has been cultivated. A large amount of SRB can be enriched through continuous culture.

The growth curve determination method of SRB: SRB is inoculated into sterile the Starkey medium according to 5% of the inoculation amount and it is cultured in an incubator at 35 °C and 150 r/min. The experiment was repeated in three groups. After a certain period of culture, the sterile medium without SRB is taken as the blank group and an appropriate amount of bacterial solution is taken to measure the OD_{600} value. The absorbance of bacterial culture at 600 nm was measured by a visible light photometer (V-1600PC). The absorbance value is the OD_{600} value.

The effect of temperature on SRB growth: SRB was inoculated into a series of the Starkey medium with pH = 7 at 5%. The inoculated samples were placed in incubators at 29, 32, 35, 38, and 41 °C and were incubated by shaking at 150 r/min, and each test sample was repeated three times. At a certain interval, an appropriate amount of liquid is taken, the sterilized medium without SRB inoculation is taken as the blank group, the OD_{600} value, pH value, ORP value, electrical conductance (Ec) value, and SO_4^{2-} concentration are measured, the removal percentage of SO_4^{2-} is calculated, and the influence of temperature on the growth of SRB is explored.

The effect of S^{2-} concentration on SRB growth: Na_2S was added to the Starkey medium to form S^{2-} concentrations of 20, 40, 60, 80, and 100 mg/L. SRB is inoculated into the medium (pH = 7) containing S^{2-} according to the above experimental steps. The samples were incubated under the condition of 35 °C and 150 r/min, and samples were taken after a certain interval to determine various indicators.

The Effect of the environmental pH value on SRB growth: The pH value of the medium was adjusted with 1 mol/L HCl and 1 mol/L NaOH solution. A series of medium were formed with pH values of 4, 5, 6, 7, and 8. The medium with a different pH was sterilized at 121 °C for 30 min. The chemical composition of the medium with pH values of 4, 5, 6, and 8

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Table 1. RSM Results of SRB Growth Conditions

	variable			response value			
number	temperature (A)	pH value of initial culture environment (B)	COD/SO_4^{2-} (C)	OD_{600}	pH value	ORP value	removal percentage of SO_4^{2-} /%
1	29	6	2	0.558	6.74	−127	33.65
2	35	6	2	1.159	7.69	−319	70.87
3	29	8	2	1.241	8.14	−346	76.21
4	35	8	2	1.449	9.41	−399	88.54
5	29	7	1	0.634	7.91	−165	36.98
6	35	7	1	1.116	8.71	−308	68.18
7	29	7	3	0.863	7.82	−226	54.58
8	35	7	3	1.311	8.48	−363	80.08
9	32	6	1	0.628	6.91	−165	38.21
10	32	8	1	1.141	8.97	−316	69.73
11	32	6	3	1.032	7.01	−285	63.01
12	32	8	3	1.311	8.77	−363	80.17
13	32	7	2	1.334	8.45	−369	81.54
14	32	7	2	1.329	8.49	−367	81.23
15	32	7	2	1.355	8.39	−374	82.78
16	32	7	2	1.345	8.45	−374	82.16
17	32	7	2	1.343	8.41	−369	82.12

was the same as that of the Starkey medium, respectively. According to the above experimental steps, SRB was inoculated into the same amount of medium with initial environmental pH values of 4, 5, 6, 7, and 8, respectively, at 35 °C and 150 r/min, with a certain interval of time. After a certain interval of time, samples were taken to measure various indicators.

The effect of COD/SO_4^{2-} on SRB growth: The culture medium with COD/SO_4^{2-} of 1.0, 1.5, 2.0, 2.5, and 3.0 was formed by adjusting the content of sodium lactate and SO_4^{2-} in the Starkey medium. The content of other components in the medium with different COD/SO_4^{2-} was consistent with the Starkey medium. SRB was inoculated into different COD/SO_4^{2-} medium at a content of 5% of medium capacity and placed at 35 °C, 150 r/min under the condition of oscillation culture, an interval after a certain time sampling determination of the indicators. The optimum COD/SO_4^{2-} for SRB growth was explored.

Based on the results of the single factor experiment, the SRB culture temperature (A), initial pH (B), and COD/SO_4^{2-} (C) were selected as factors for RSM. The test parameters and results are shown in Table 1.

The detection method of water quality indicators: the electrode method (HJ 1147−2020) is used for pH measurement. The redox potential value was measured with a pen ORP meter. The conductivity value is measured with a pen-type Ec meter. SO_4^{2-} is determined by barium chromate spectrophotometry (HJ/T 342−2007).

3. RESULTS AND DISCUSSION

3.1. Growth Curve of SRB. It can be seen from Figure 1 that the growth curve of SRB is an "S"-type growth curve. At 0∼14 h, SRB was in a standstill period, its growth and metabolism were relatively slow, and the number of bacteria increased less. At 14∼86 h, SRB was in a logarithmic phase. SRB strains had high activity, vigorous growth, and metabolism, and the number of SRB strains increased significantly. At 86∼146 h, SRB was in a stable period, the number of viable bacteria in SRB was high and relatively stable, the rate of the bacterial division was significantly reduced, and the metabolic activity of the SRB strain was gradually

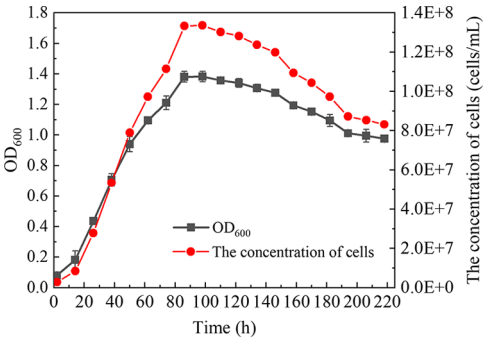

Figure 1. Growth curve of SRB (35 °C, 150 r/min).

weakened. At 146∼218 h, SRB was in the decay period, the metabolic activity of the SRB strain was significantly reduced, and a large number of cells died.

3.2. Effect of Temperature on SRB Growth. It can be seen from Figure 2a that, with the extension of culture time, the OD_{600} values under different culture temperatures increase first and then tend to be stable. The OD_{600} value can indirectly reflect the amount of SRB in the culture medium. At 29 °C, the OD_{600} value was 1.10 after 6 days of culture. At 32 and 35 °C, the OD_{600} value increased rapidly with time, and the maximum values were 1.37 and 1.35, respectively. With the increase of time, the OD_{600} value finally stabilized between 1.25 and 1.35, indicating that the SRB reproduction rate was fast at 32 and 35 °C, and there were a large number of SRB cells in the medium, indicating that 32 and 35 °C were suitable for SRB growth. At 38 and 41 °C, the OD_{600} value rises slowly. At 5 days of culture, the OD_{600} values are 0.58 and 0.41, respectively, indicating that SRB can survive at 38 and 41 °C, but its activity is low. Li et al.[17] showed that SRB activity was strong and that the cell density was high at 30 °C, while SRB activity was low at 50 °C. Xu et al.[16] showed that SRB can survive at 20∼40 °C, and the stable growth period of SRB is the longest at 30 °C. This study shows that the growth activity of SRB at 32 and 35 °C is significantly higher than that at 38 and 41 °C, which is consistent with the above results. Compared with the above studies, this study carefully analyzed

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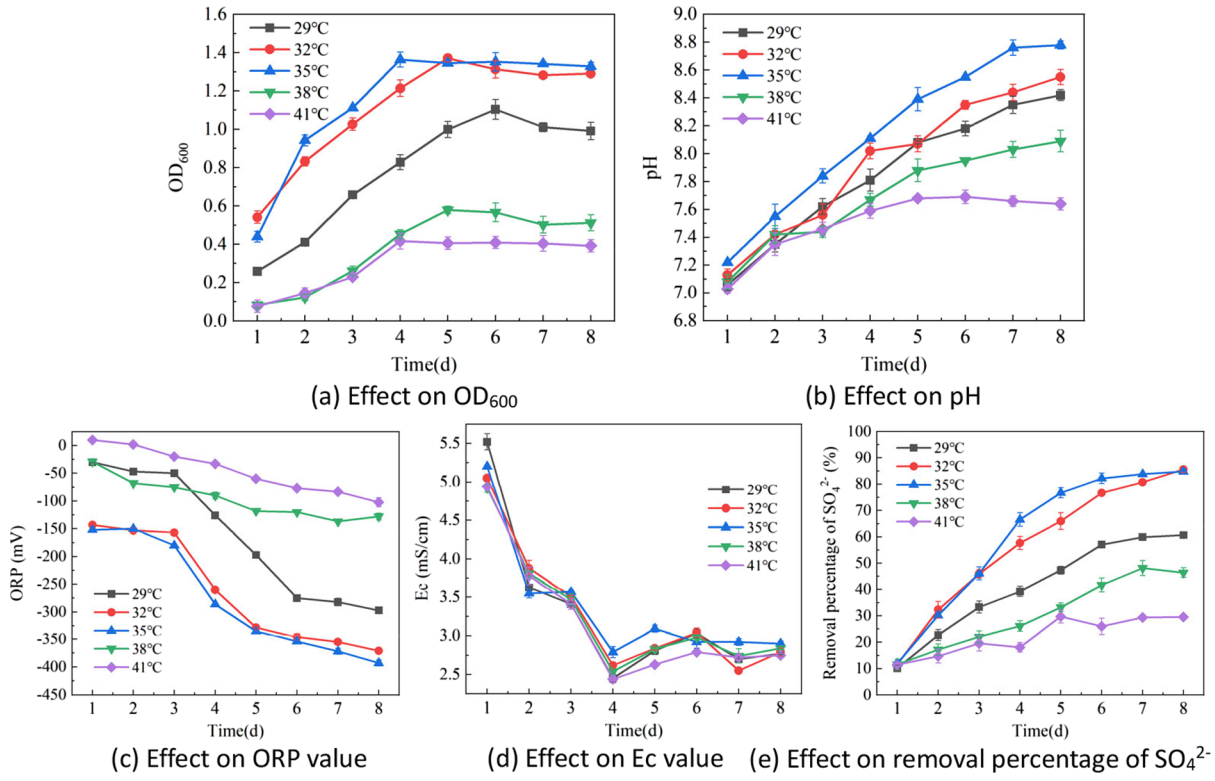

Figure 2. Effect of temperature on SRB growth (pH = 7, 150 r/min). (a) Effect on OD_{600}. (b) Effect on pH. (c) Effect on the ORP value. (d) Effect on the Ec value. (e) Effect on the removal percentage of SO_4^{2-}.

the biological growth quantity of SRB at 30∼40 °C and determined the temperature suitable for SRB growth.

It can be seen from Figure 2b that with the extension of culture time, pH values under different culture temperatures increase significantly. At 29, 32, 35, 38, and 41 °C, the pH values of the solution after 8 days of incubation were 8.42, 8.55, 8.78, 8.09, and 7.64, respectively. At 35 °C, SRB has the highest activity and the most obvious increase in the pH value. Compared with the initial pH = 7, after SRB was cultured at different temperatures, the pH value of the solution increased, mainly because SRB could grow and metabolize normally. During SRB metabolism, SO_4^{2-} is reduced to sulfide, and organic carbon is oxidized to produce HCO_3^-, which improves the overall pH of the solution.[26] At 35 °C, the pH value increases most obviously, mainly because SRB has high growth activity and large reproduction quantity at this temperature, so the alkalinity generated by metabolism is large. At the same time, the alkaline pH value of the solution further promotes the growth and metabolism of SRB.[27]

It can be seen from Figure 2c that the ORP values under different culture temperatures decreased significantly with the extension of culture time. After 8 days of incubation, the ORP values of the solution were −297, −371, −393, −128, and −102 mV, respectively. At 35 °C, the ORP value decreased most obviously. It is reported that when the environmental conditions for microbial growth are ORP < −100 mV and 5 < pH < 9, SRB organisms can dominate.[28] After SRB was cultured at different temperatures, the ORP values of the solution decreased and were all less than −100 mV, indicating that SRB could survive at various temperature gradients. It can be seen from Figure 2d that, with the extension of culture time, the Ec values first decreased and then stabilized at 2.75∼2.90 mS/cm. The decrease of the Ec value is related to SRB growth and metabolism.

It can be seen from Figure 2e that with the extension of culture time, the removal percentage of SO_4^{2-} under different culture temperatures is significantly improved. After 8 days of incubation, the removal percentages of SO_4^{2-} were 60.70, 85.60, 84.77, 46.41, and 29.56%, respectively. When SRB was cultured at 32 and 35 °C, the removal percentage of SO_4^{2-} was relatively large. At 41 °C, the removal percentage of SO_4^{2-} changes slowly. Because temperature will affect the activity of enzymes in bacteria, thereby affecting the efficiency of SRB in treating SO_4^{2-}, the removal percentage of SO_4^{2-} can directly reflect the activity of SRB in metabolizing SO_4^{2-}. It can be seen from the comparison that SRB metabolism is more vigorous at 32 and 35 °C and slower at 41 °C, indicating that temperature has a greater impact on SRB metabolism. With the increase of the culture temperature, the activity of the enzyme system in SRB gradually increased, which enhanced the activity of SRB metabolizing SO_4^{2-} and promoted the removal percentage of SO_4^{2-}. However, when the temperature exceeds 38 °C, it will increase the molecular heat energy that constitutes the protein structure of SRB cells, causing the rupture of some hydrogen bonds, van der Waals force, and other noncovalent bonds, resulting in the reduction of SRB cell activity.[25] Therefore, when the temperature exceeds 38 °C and then continues to rise, the removal percentage of SO_4^{2-} by SRB decreases instead. It is reported that the optimal culture temperature of medium temperature SRB is 28∼38 °C.[29] Zhao[25] isolated an SRB named SST1. The optimum growth temperature of SST1 was 35 °C, and the removal percentage of SO_4^{2-} was 55.03% at 35 °C.[25] The results of this study are consistent with the above studies. Compared with the strain SST1 isolated by Zhao,[25]

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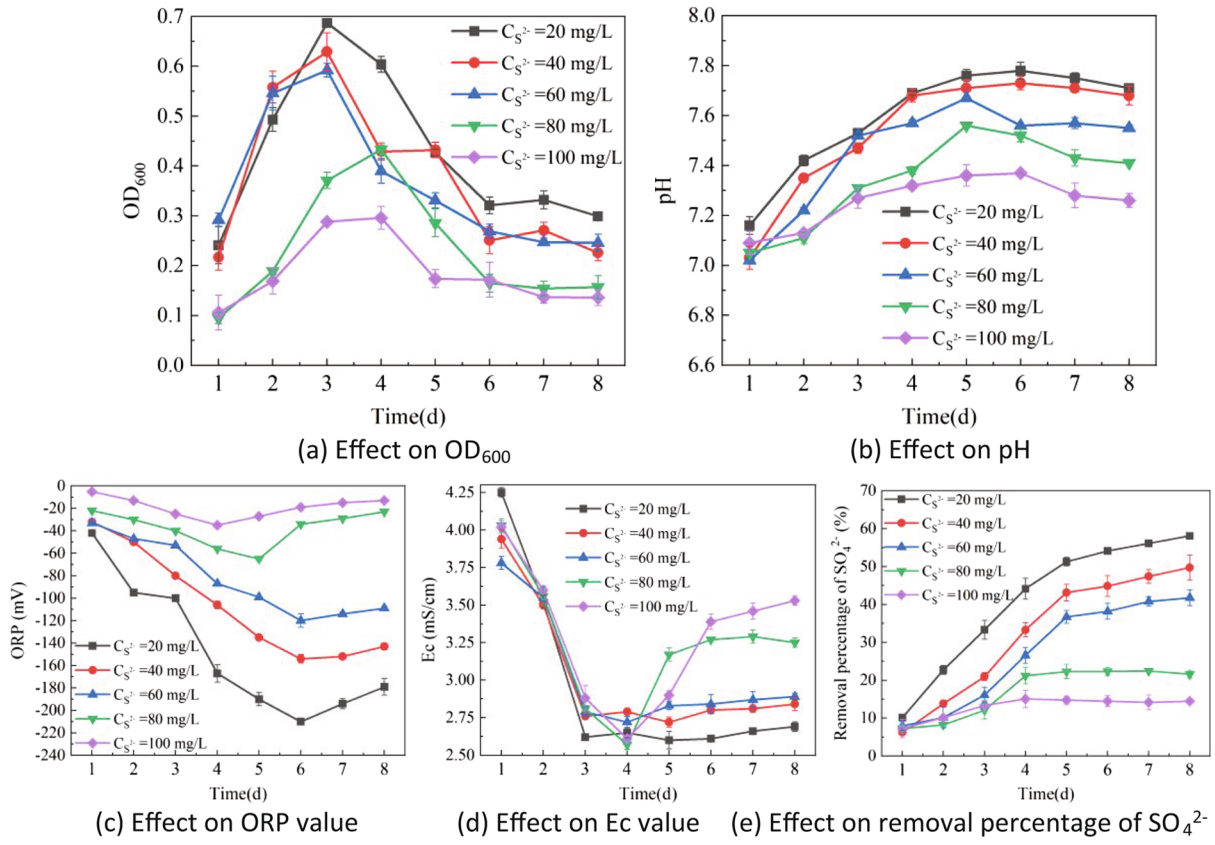

Figure 3. Effect of the S^{2-} concentration on SRB growth (pH = 7, 35 °C, 150 r/min). (a) Effect on OD_{600}. (b) Effect on pH. (c) Effect on the ORP value. (d) Effect on the Ec value. (e) Effect on the removal percentage of SO_4^{2-}.

the SRB in this study can remove 84.77% of SO_4^{2-} at 35 °C, and the removal percentage of SO_4^{2-} is significantly increased.

To sum up, SRB has the highest activity when the temperature is 32∼35 °C. At 35 °C, after 8 days of SRB inoculation, the OD_{600} value, pH value, ORP value, Ec value, and SO_4^{2-} removal percentage of the medium were 1.33, 8.78, −393 mV, 2.90 mS/cm, and 84.77%, respectively.

3.3. Effect of S^{2-} Concentration on SRB Growth. The effect of the S^{2-} concentration on SRB growth when pH = 7, 35 °C, 150 r/min is shown in Figure 3.

It can be seen from Figure 3a that when the initial concentration of S^{2-} in the culture medium is 20, 40, 60, 80, and 100 mg/L, respectively, the OD_{600} is 0.69, 0.63, 0.59, 0.37, and 0.29, respectively, after inoculation with SRB for 3 days. OD_{600} was 0.30, 0.23, 0.25, 0.16, and 0.14, respectively, after 8 days of culture. It is reported that when the sulfide dissolved in the solution accumulates to a certain concentration, it will inhibit the growth and metabolism of SRB.[21] Xu[30] reported that the growth of SRB would be inhibited when the concentration of S^{2-} in the system was >97 mg/L. Ren et al.[22] reported that when the concentration of free H_2S and sulfide in the system exceeds 50 and 200 mg/L, respectively, the growth activity of SRB will be severely inhibited. When cultured for 3 days, the OD_{600} value decreased with the increase of S^{2-} concentration, indicating that the high concentration of S^{2-} could significantly inhibit the growth of SRB at this time. At 3−6 days, the OD_{600} value decreased, mainly because SRB metabolized sulfate, which also led to an increase in the concentration of S^{2-} in the system. The accumulation of a high concentration of S^{2-} in the culture solution would inhibit the growth of SRB and even lead to its death. It is reported that H_2S at a lower concentration is toxic, and with the increase of H_2S concentration, it will not only inhibit SRB sulfate reduction but also significantly reduce biodiversity.[31−33] When cultured for 8 days, the cumulative concentration of S^{2-} was high and the OD_{600} values in the five systems were small and the numerical difference was small, indicating that most SRB strains in the system had lost their activity.

As shown in Figure 3b, when SRB was inoculated for 8 days, the pH values were 7.71, 7.68, 7.55, 7.41, and 7.26, respectively. Compared with the initial pH value, the pH value of the solution after 8 days of SRB culture increased, but the increase amplitude was small, especially in the medium with the initial S^{2-} concentration of 100 mg/L. The increase of pH in the system with initial S^{2-} concentrations of 20, 40, and 60 mg/L is mainly related to SRB metabolism. When the initial S^{2-} concentration is low, SRB will metabolize to produce alkalinity, but with the progress of the metabolic reaction, metabolite S^{2-} will be produced, and the accumulated S^{2-} will inhibit SRB metabolism and even lead to SRB death. After SRB died, the substances in the cells were released into the solution, which made the pH value of the system decrease slightly.

It can be seen from Figure 3c that SRB metabolism at the early stage of culture promotes ORP values to drop to −210, −154, −120, −65, and −35 mV, respectively. However, with the increase of the H_2S concentration, the activity of SRB was inhibited until SRB died, and the ORP value of the system increased. After 8 days of SRB culture, the ORP values of the solution were −179, −143, −109, −23, and −13 mV, respectively. At this time, in the culture system with the initial S^{2-} concentration of 80 and 100 mg/L, the ORP value of the

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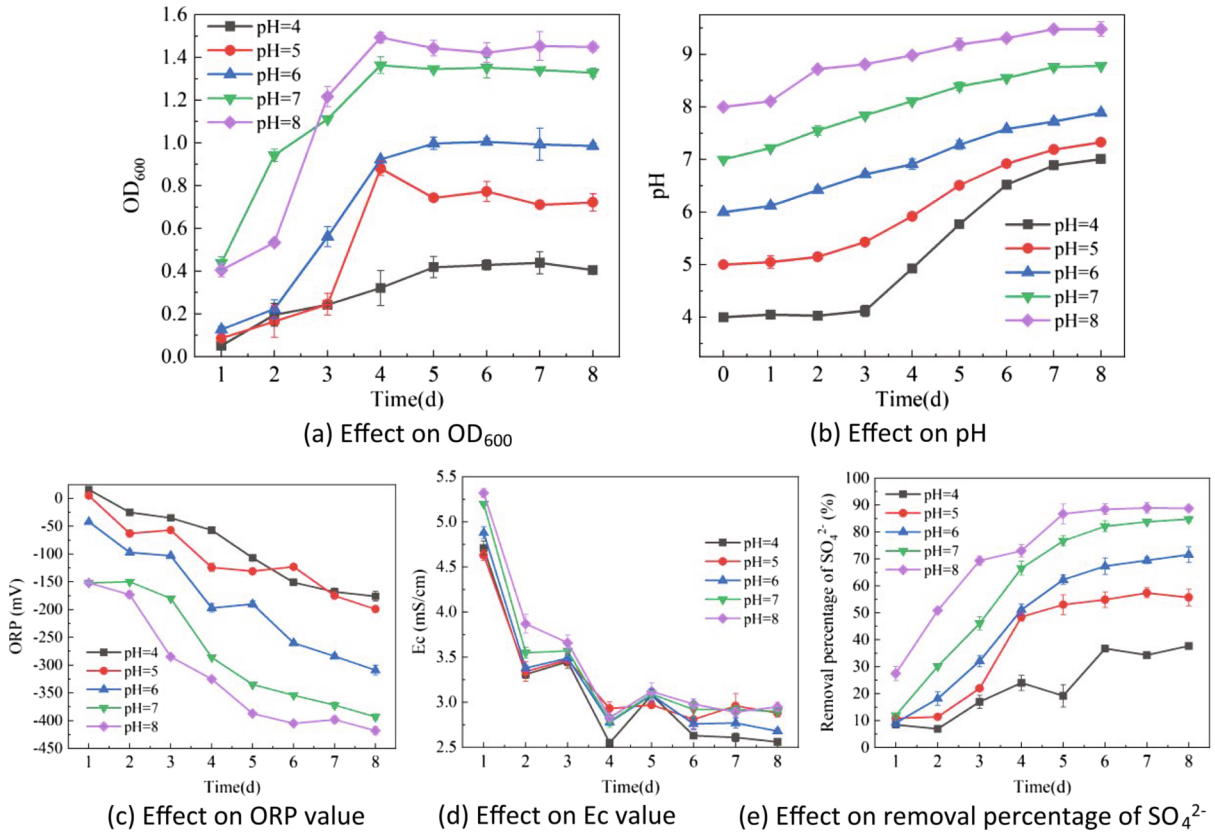

Figure 4. Effect of pH on SRB growth (35 °C, 150 r/min). (a) Effect on OD_{600}. (b) Effect on pH. (c) Effect on ORP value. (d) Effect on the Ec value. (e) Effect on the removal percentage of SO_4^{2-}.

solution has been > −100 mV, and SRB in the system can hardly survive, indicating that the high concentration of S^{2-} accelerates the death of SRB cells.

It can be seen from Figure 3d that after 8 days of culture, the Ec values of the solution are 2.69, 2.84, 2.89, 3.25, and 3.53 mS/cm, respectively. At the initial stage of culture, the decrease of the Ec value in the culture medium was related to the growth and metabolism of SRB. At the later stage of culture, the increase of the Ec value in the culture medium was related to the acceleration of SRB cell death by the high concentration of S^{2-}. After SRB cell death, substances in the cells leaked into the solution, leading to the increase of the Ec value in the culture system.

It can be seen from Figure 3e that, with the extension of culture time, when the initial concentration of S^{2-} in the culture medium is 20, 40, and 60 mg/L, respectively, the SO_4^{2-} removal in the culture medium shows a decreasing trend, and the SO_4^{2-} removal rate is higher at 1∼5 days, while significantly reduced at 5∼8 days. It indicates that SRB activity is higher in the early stage and metabolizes more SO_4^{2-}. At the later stage, SRB metabolism was restricted by S^{2-}, and the rate of metabolizing SO_4^{2-} decreased significantly. With the extension of culture time, when the initial concentration of S^{2-} in the medium was 80 and 100 mg/L, respectively, the removal percentage of SO_4^{2-} in the medium was first increased and then stabilized. It shows that the high concentration of S^{2-} in the early stage inhibited SRB, and only a small amount of SRB could metabolize SO_4^{2-}. In the later period, a large number of SRBs died, leading to no reduction of SO_4^{2-} in the system, and the removal percentage of SO_4^{2-} tended to be stable. When the initial concentrations of S^{2-} in the medium were 20, 40, 60, 80, and 100 mg/L, respectively, the removal percentages of SO_4^{2-} after 8 days of culture were 58.11, 49.74, 41.77, 21.59, and 14.48%, respectively.

In conclusion, with the gradual increase of the S^{2-} concentration in the system, the SRB activity will be inhibited, even leading to SRB cell death.

3.4. Effect of the Environmental pH Value on SRB Growth. The influence of the environmental pH value on SRB growth under the condition of 35 °C and 150 r/min is shown in Figure 4.

It can be seen from Figure 4a that, with the extension of incubation time, the OD_{600} values under different initial environmental pH values show a trend of first increasing and then tending to be stable. It is reported that SRB is greatly affected by environmental pH.[34] When the initial environmental pH values were 4, 5, 6, 7, and 8, respectively, the OD_{600} values of the solution were 0.41, 0.72, 0.99, 1.33, and 1.45, respectively, after 8 days of incubation. When the initial environment pH is 4∼8, the OD_{600} value gradually increases with the increase of the initial pH value. When the initial environment pH is 4, the OD_{600} value changes slowly, indicating that the acidic pH value will inhibit the growth of SRB, and even lead to the death of SRB. It is reported that SRB is sensitive to weak acidic conditions. When pH is lower than 5, SRB is not easy to survive.[35] The results of this study are consistent with the above studies. The concentration of H^+ in the culture medium with different initial pH is different. The interaction between H^+ and the enzyme in SRB indirectly affects the activity of SRB cells,[36] resulting in that the culture medium with different initial pH has a greater impact on the growth of SRB. When the environmental pH is 7 and 8, the

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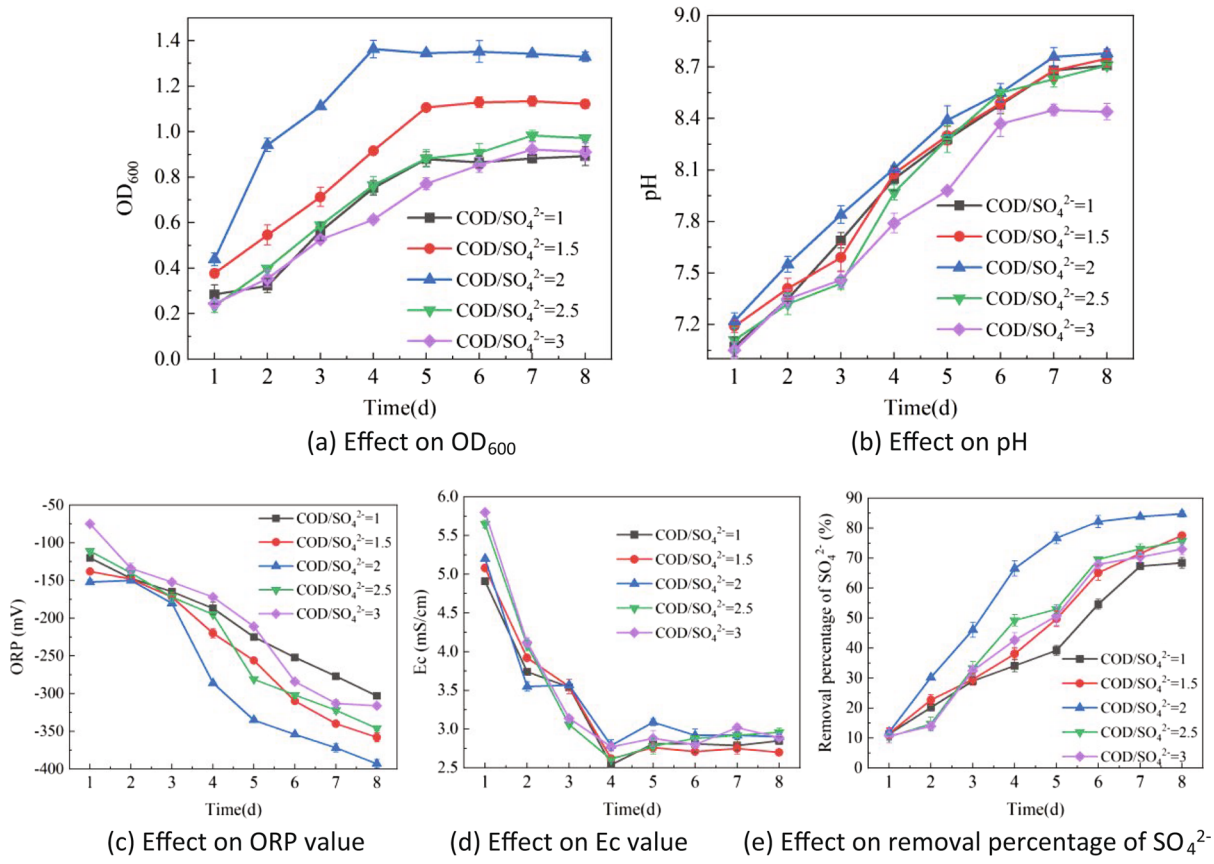

Figure 5. Effect of COD/SO_4^{2-} on SRB growth (pH = 7, 35 °C, 150 r/min). (a) Effect on OD_{600}. (b) Effect on pH. (c) Effect on the ORP value. (d) Effect on the Ec value. (e) Effect on the removal percentage of SO_4^{2-}.

OD_{600} value increases significantly, indicating that the neutral and weakly alkaline pH values will promote the growth of SRB.

It can be seen from Figure 4b that when the initial pH values of the culture medium are 4, 5, 6, 7, and 8, respectively, the pH values of the solution are 7.01, 7.33, 7.89, 8.78, and 9.48, respectively, after 8 days of culture. The pH value of the solution increased, mainly related to the metabolism of SRB. Hao et al.[37] found that the reduction of SO_4^{2-} by SRB was accompanied by an increase of pH. The increase of pH value is mainly due to the oxidation of organic carbon to produce HCO_3^{-}[38] when SRB metabolizes sulfate.

It can be seen from Figure 4c that when the environmental pH values are 4, 5, 6, 7, and 8, respectively, the ORP values of the solution after 8 days of incubation are −176, −199, −309, −393, and −418 mV, respectively. When the environmental pH value is 7 and 8, the ORP value decreases most obviously, indicating that the neutral and weakly alkaline environment will promote the growth of SRB. It can be seen from Figure 4d that with the extension of incubation time, the Ec value of SRB solution cultured under different environmental pH values shows a trend of first decreasing and then stabilizing. It can be seen from Figure 4e that the removal percentage of SO_4^{2-} under different initial environmental pH values increases significantly with the extension of incubation time. When the environmental pH values were 4, 5, 6, 7, and 8, respectively, the removal percentages of SO_4^{2-} after 8 days of incubation were 37.71, 55.74, 71.59, 84.77, and 88.78%, respectively. It is reported that the optimal pH for SRB growth and sulfate reduction is 7∼8. Both acidic pH and alkaline pH will inhibit the metabolic activity of SRB.[14] It can be seen from the removal percentage of SO_4^{2-} that the initial environmental pH value has a great impact on SRB's metabolism of SO_4^{2-}. When the environmental pH value is acidic, the efficiency of SRB's metabolism of SO_4^{2-} is relatively slow. When the environmental pH values are 7 and 8, the efficiency of SRB's metabolism of SO_4^{2-} exceeds 80%. The main mechanism that the change of environmental pH value affects the metabolism of SO_4^{2-} by SRB is that H^+ in solution will cause the change of surface charge of the SRB cell membrane, thus affecting the absorption capacity of SRB to the substrate. At the same time, the environmental pH value will also affect the activity and stability of the internal enzyme of SRB, thus affecting the progress of the metabolism of the SO_4^{2-} reaction of SRB.

Therefore, the environmental pH value that SRB can tolerate is 5∼8. When the environmental pH value is 7∼8, SRB has the strongest activity.

3.5. Effect of COD/SO_4^{2-} on SRB Growth. The effect of initial COD/SO_4^{2-} on SRB growth when pH = 7, 35 °C, 150 r/min is shown in Figure 5.

It can be seen from Figure 5a that when the initial COD/SO_4^{2-} in the culture medium is 1.0, 1.5, 2.0, 2.5, and 3.0 respectively, and SRB is inoculated for 8 days, the OD_{600} values are 0.89, 1.12, 1.33, 0.97, and 0.91, respectively. When the initial COD/SO_4^{2-} in the medium is 1∼2, the OD_{600} value in the solution system gradually increases with the increase of COD/SO_4^{2-}. When the initial COD/SO_4^{2-} in the medium is 2∼3, with the increase of COD/SO_4^{2-}, the OD_{600} value in the solution system gradually decreases. It shows that when the initial COD/SO_4^{2-} is 1∼2, properly increasing COD/SO_4^{2-} is beneficial to the growth and metabolism of SRB. However,

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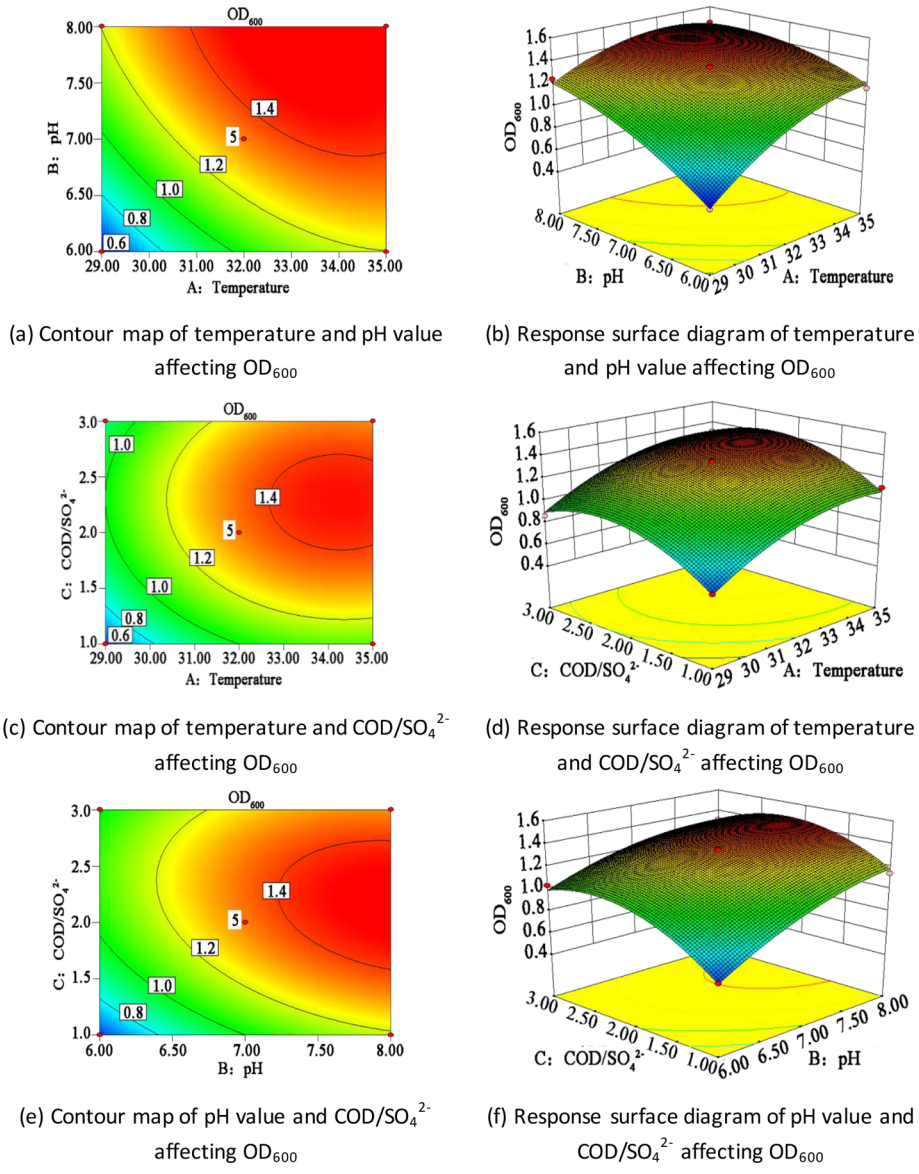

Figure 6. Response surface and contour map of OD_{600} for SRB under different culture conditions. (a) Contour map of temperature and pH value affecting OD_{600}. (b) Response surface diagram of temperature and pH value affecting OD_{600}. (c) Contour map of temperature and COD/SO_4^{2-} affecting OD_{600}. (d) Response surface diagram of temperature and COD/SO_4^{2-} affecting OD_{600}. (e) Contour map of the pH value and COD/SO_4^{2-} affecting OD_{600}. (f) Response surface diagram of the pH value and COD/SO_4^{2-} affecting OD_{600}.

when the initial COD/SO_4^{2-} is 2∼3, proper reduction of COD/SO_4^{2-} is conducive to SRB growth and metabolism. It is reported that theoretically, 0.67 g of COD[24] is required to reduce 1 g of SO_4^{2-}. However, considering the competition between SRB and methanogenic bacteria on the substrate in the actual bacterial culture process, the required COD/SO_4^{2-} is far greater than the theoretical value of COD/SO_4^{2-}.[39] This experiment shows that when the initial COD/SO_4^{2-} is 2, the OD_{600} value is the largest, that is, the number of SRB cells is the largest.

It can be seen from Figure 5b that with the extension of incubation time, pH values under different initial COD/SO_4^{2-} showed an increasing trend. When the initial COD/SO_4^{2-} in the medium was 1.0, 1.5, 2.0, 2.5, and 3.0, respectively, and the SRB was inoculated for 8 days, the pH values were 8.71, 8.75, 8.78, 8.71, and 8.44, respectively. When the initial COD/SO_4^{2-} = 2 in the medium, the increase of the pH value is the largest. When the initial COD/SO_4^{2-} = 3 in the medium, the increase of the pH value is the smallest. It shows that the initial COD/SO_4^{2-} = 2 in the medium is conducive to SRB growth and metabolism and the production of alkalinity.

It can be seen from Figure 5c that the ORP values under different initial COD/SO_4^{2-} decreased with the extension of incubation time. It is reported that as SRB continuously metabolizes and consumes the substrate SO_4^{2-} and continuously generates metabolites such as H_2S, HS^-, and S^{2-}, the ORP value in the solution shows a decreasing trend.[40,41] When the initial COD/SO_4^{2-} in the medium was 1.0, 1.5, 2.0, 2.5, and 3.0, respectively, the ORP values decreased to −303, −358, −393, −346, and −316 mV, respectively, after 8 days of SRB inoculation. Xu et al.[42] reported that when ORP < −350 mV, compared with the SO_4^{2-} concentration and pH value, the ORP value will no longer be the main factor affecting SRB to reduce SO_4^{2-}. Therefore, when the initial COD/SO_4^{2-} = 2 in the medium, the ORP value decreases the most, indicating that SRB metabolism is most vigorous at this time, and the ORP

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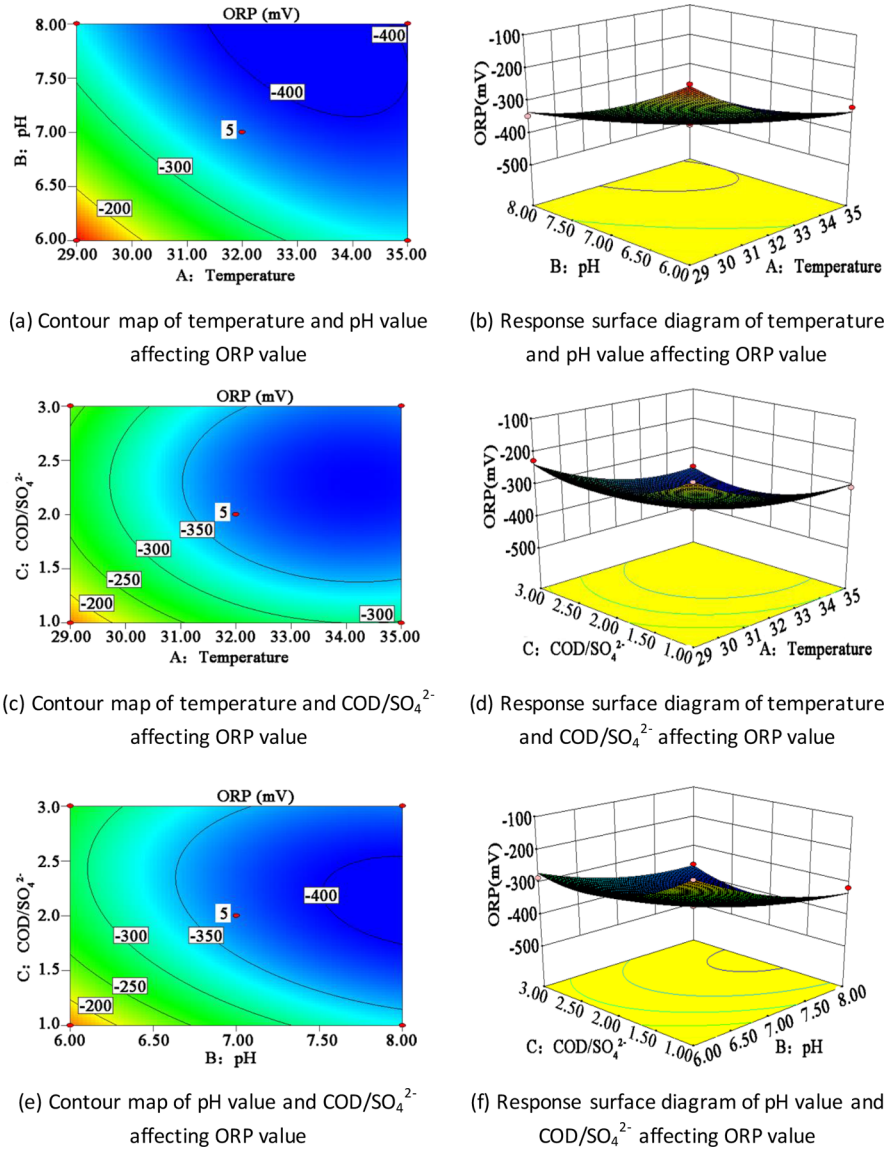

(a) Contour map of temperature and pH value affecting ORP value

(b) Response surface diagram of temperature and pH value affecting ORP value

(c) Contour map of temperature and COD/SO_4^{2-} affecting ORP value

(d) Response surface diagram of temperature and COD/SO_4^{2-} affecting ORP value

(e) Contour map of pH value and COD/SO_4^{2-} affecting ORP value

(f) Response surface diagram of pH value and COD/SO_4^{2-} affecting ORP value

Figure 7. Response surface and contour map of the ORP value of SRB under different culture conditions. (a) Contour map of temperature and pH value affecting the ORP value. (b) Response surface diagram of temperature and pH value affecting the ORP value. (c) Contour map of temperature and COD/SO_4^{2-} affecting the ORP value. (d) Response surface diagram of temperature and COD/SO_4^{2-} affecting the ORP value. (e) Contour map of the pH value and COD/SO_4^{2-} affecting the ORP value. (f) Response surface diagram of the pH value and COD/SO_4^{2-} affecting the ORP value.

value is not the main factor affecting the rate of SRB reducing SO_4^{2-}. It can be seen from Figure 5d that when the initial COD/SO_4^{2-} in the medium is 1.0, 1.5, 2.0, 2.5, and 3.0 after 8 days of SRB inoculation, the Ec values of the medium are 2.85, 2.70, 2.90, 2.96 and 2.89 mS/cm, respectively. It can be seen from Figure 5e that when the initial COD/SO_4^{2-} in the culture medium is 1, 1.5, 2, 2.5, and 3, the removal percentages of SO_4^{2-} in the solution are 68.41, 77.56, 84.77, 75.70, and 73.03%, respectively, after 8 days of inoculation with SRB. When the initial COD/SO_4^{2-} = 2 in the medium, the removal percentage of SO_4^{2-} is the largest, indicating that SRB metabolism is most vigorous at this time. SRB can metabolize the substrate SO_4^{2-} and organics to generate metabolites such as HCO_3^-, H_2S, HS^-, and S^{2-}, achieving the effect of increasing the pH value, reducing the ORP value, and removing SO_4^{2-}.

To sum up, the COD/SO_4^{2-} most suitable for SRB growth is 2. After SRB was incubated in the medium with initial COD/SO_4^{2-} for 8 days, the OD_{600} value, pH value, ORP value, Ec value, and SO_4^{2-} removal percentage of the medium were 1.33, 8.78, −393 mV, 2.90 mS/cm, and 84.77%, respectively.

3.6. RSM of SRB under Optimal Growth Conditions.

The experimental results of RSM are shown in Table 1 and Figures 6789.

The design expert 8.0 software was used to evaluate the experimental parameters of the RSM. Through regression analysis of the Box Behnken test results, the quadratic multiple regression models of the OD_{600} value, ORP value, pH value, and removal percentage of SO_4^{2-} under different SRB culture conditions were obtained as follows:

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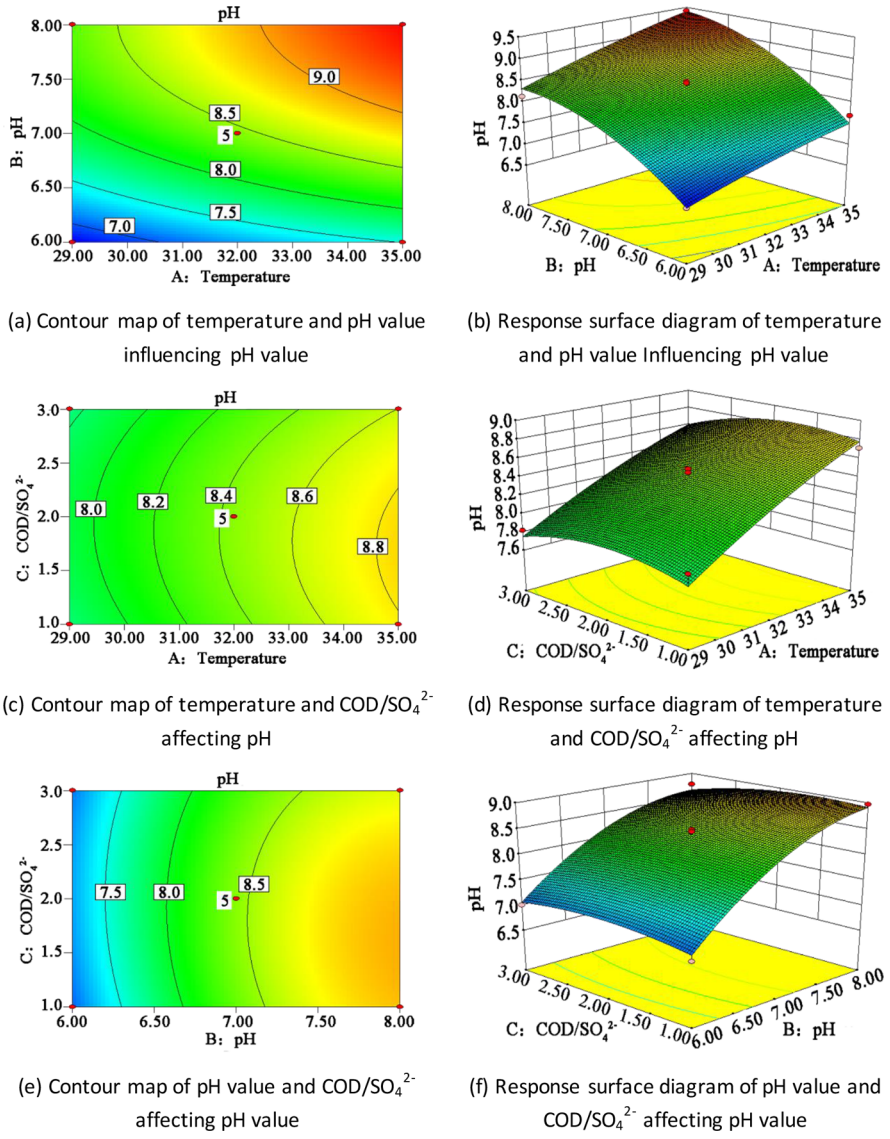

(a) Contour map of temperature and pH value influencing pH value

(b) Response surface diagram of temperature and pH value Influencing pH value

(c) Contour map of temperature and COD/SO_4^{2-} affecting pH

(d) Response surface diagram of temperature and COD/SO_4^{2-} affecting pH

(e) Contour map of pH value and COD/SO_4^{2-} affecting pH value

(f) Response surface diagram of pH value and COD/SO_4^{2-} affecting pH value

Figure 8. Surface and contour chart of SRB pH response under different culture conditions. (a) Contour map of temperature and pH value influencing the pH value. (b) Response surface diagram of temperature and pH value influencing the pH value. (c) Contour map of temperature and COD/SO_4^{2-} affecting pH. (d) Response surface diagram of temperature and COD/SO_4^{2-} affecting pH. (e) Contour map of the pH value and COD/SO_4^{2-} affecting the pH value. (f) Response surface diagram of the pH value and COD/SO_4^{2-} affecting the pH value.

$$\begin{aligned} OD_{600} = {} & 1.34 + 0.22 \times A + 0.22 \times B + 0.12 \times C \\ & - 0.098 \times A \times B - 0.0085 \times A \times C \\ & - 0.058 \times B \times C - 0.14 \times A^2 - 0.096 \times B^2 \\ & - 0.22 \times C^2, R^2 \\ = {} & 0.9930 \end{aligned}$$

$$\begin{aligned} \text{pH value} = {} & 8.44 + 0.46 \times A + 0.87 \times B - 0.053 \times C \\ & + 0.080 \times A \times B - 0.035 \times A \times C \\ & - 0.075 \times B \times C - 0.064 \times A^2 - 0.38 \times B^2 \\ & - 0.14 \times C^2, R^2 \\ = {} & 0.9832 \end{aligned}$$

$$\begin{aligned} \text{ORP value} = {} & -370.60 - 65.63 \times A - 66.00 \times B \\ & - 35.38 \times C + 34.75 \times A \times B \\ & + 1.50 \times A \times C + 18.25 \times B \times C \\ & + 44.80 \times A^2 + 28.05 \times B^2 \\ & + 60.30 \times C^2, R^2 \\ = {} & 0.9903 \end{aligned}$$

$$\begin{aligned} & \text{Removal percentage of } SO_4^{2-} (\%) \\ = {} & 81.97 + 13.28 \times A + 13.61 \times B + 8.09 \times C \\ & - 6.22 \times A \times B - 1.43 \times A \times C - 3.59 \times B \times C \\ & - 8.74 \times A^2 - 5.91 \times B^2 - 13.27 \times C^2, R^2 \\ = {} & 0.9943 \end{aligned}$$

The F values of the second-order models of OD_{600}, ORP, pH, and removal percentage of SO_4^{2-} under different SRB culture conditions were 110.62, 79.28, 45.64, and 134.89, respectively, $P < 0.0001$. It shows that the regression of the

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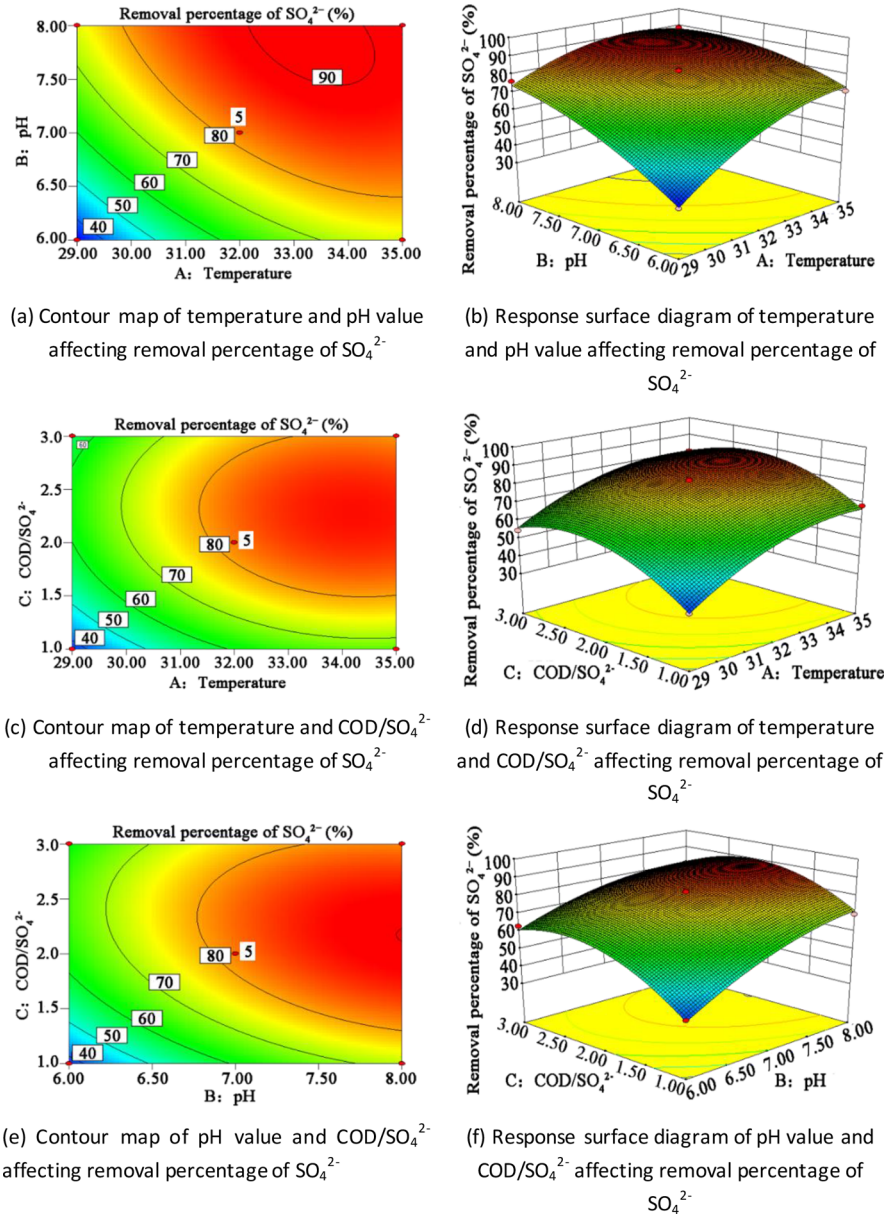

(a) Contour map of temperature and pH value affecting removal percentage of SO_4^{2-}

(b) Response surface diagram of temperature and pH value affecting removal percentage of SO_4^{2-}

(c) Contour map of temperature and COD/SO_4^{2-} affecting removal percentage of SO_4^{2-}

(d) Response surface diagram of temperature and COD/SO_4^{2-} affecting removal percentage of SO_4^{2-}

(e) Contour map of pH value and COD/SO_4^{2-} affecting removal percentage of SO_4^{2-}

(f) Response surface diagram of pH value and COD/SO_4^{2-} affecting removal percentage of SO_4^{2-}

Figure 9. Response surface and contour map of the SO_4^{2-} removal percentage of SRB under different culture conditions. (a) Contour map of temperature and the pH value affecting the removal percentage of SO_4^{2-}. (b) Response surface diagram of temperature and the pH value affecting the removal percentage of SO_4^{2-}. (c) Contour map of temperature and COD/SO_4^{2-} affecting the removal percentage of SO_4^{2-}. (d) Response surface diagram of temperature and COD/SO_4^{2-} affecting the removal percentage of SO_4^{2-}. (e) Contour map of the pH value and COD/SO_4^{2-} affecting the removal percentage of SO_4^{2-}. (f) Response surface diagram of the pH value and COD/SO_4^{2-} affecting the removal percentage of SO_4^{2-}.

above four second-order models is good, and the models are extremely significant. The R^2 values of the four models are 0.9930, 0.9903, 0.9832, and 0.9943 and R^2_{Adj} values are 0.9840, 0.9778, 0.9617, and 0.9869, which indicates that the model has a high fitting degree and a small error. The accuracies of the four models are 31.60, 26.99, 24.00, and 34.68, all of which are >4, and the fitting models are reasonable. The coefficients of variation of the four models were 3.21, 4.13, 1.77, and 2.94%, all of which were less than 10%, indicating that the models had credibility and precision. Therefore, the above four models are suitable for analysis and prediction of the OD_{600} value, ORP value, pH value, and removal percentage of SO_4^{2-} under different SRB culture conditions.

Figure 6 shows the interaction of temperature (A), pH value of the initial culture environment (B) and COD/SO_4^{2-} (C) of culture medium on the OD_{600} value of SRB in the SRB culture system under different culture conditions. It can be seen from Figure 6a,b that the OD_{600} value increases significantly with the increase of temperature and environmental pH value. It can be seen from Figure 6c,d that the OD_{600} value increases significantly with the increase of temperature. With the increase of COD/SO_4^{2-}, the OD_{600} value first increases and then slowly decreases. It can be seen from Figure 6e,f that the OD_{600} value increases significantly with the increase of environmental pH. With the increase of COD/SO_4^{2-}, the OD_{600} value increases gradually. When the temperature is 34.74 °C, the environmental pH value is 8.00, the initial COD/

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ACS Omega 2023, 8, 4046−4059

SO_4^{2-} is 1.98, and the OD_{600} value in the SRB culture system is 1.45. Analysis of variance showed that the effects of single factor temperature, pH value of the initial culture environment, and COD/SO_4^{2-} on the OD_{600} value of SRB in different culture conditions were extremely significant ($P < 0.001$). From the value of F, it can be seen that the order of influence of single factor temperature (A), pH (B), and COD/SO_4^{2-} (C) is environmental pH > temperature > COD/SO_4^{2-}. Among the effects on the OD_{600} value of SRB in different culture conditions, the interaction between the environmental pH value and temperature, COD/SO_4^{2-} exists, especially the impact of environmental pH value and temperature on the OD_{600} value reaches a highly significant level, and the significance level is 0.001. However, there is no interaction between the temperature and COD/SO_4^{2-} response to the OD_{600} value.

Figure 7 shows the interaction of temperature, environmental pH value, and COD/SO_4^{2-} on the ORP value in the SRB culture system. When the temperature is 34.74 °C, the environmental pH value is 8.00, the initial COD/SO_4^{2-} is 1.98, and the pH value is 9.37. The influence of single factor temperature, environmental pH value, and COD/SO_4^{2-} on the ORP value reached an extremely significant level ($P < 0.001$). The order of influence on the ORP value is environmental pH > temperature > COD/SO_4^{2-}. Among the impacts on the ORP value, the interaction between the environmental pH value and temperature, COD/SO_4^{2-} exists, especially the impact of pH value and temperature on ORP value reaches a very significant level, and the significance level is 0.0009. However, there is no interaction between the temperature and COD/SO_4^{2-} response to ORP.

Figure 8 shows the interaction of temperature, environmental pH value, and COD/SO_4^{2-} on the pH value in the SRB culture system. It can be seen from Figure 8a,b that the pH value increases slowly with the increase of temperature. With the increase of the environmental pH value, the pH value increases significantly. It can be seen from Figure 8c,d that the pH value increases significantly with the increase of temperature. With the increase of COD/SO_4^{2-}, the pH value does not change significantly. It can be seen from Figure 8e,f that with the increase of the environmental pH value, the pH value increases significantly. With the increase of COD/SO_4^{2-}, the pH value does not change significantly. When the temperature is 34.74 °C, the environmental pH value is 8.00, the environmental COD/SO_4^{2-} is 1.98, and the ORP value is −399 mV. The analysis of variance showed that the influence of temperature and environmental pH value of a single factor on the pH value of SRB culture system reached a very significant level ($P < 0.001$), while the influence of COD/SO_4^{2-} on the pH value of the single factor was not significant. The order of influence is environmental pH > temperature > COD/SO_4^{2-}. There was no interaction between temperature and environmental pH, between environmental pH and COD/SO_4^{2-}, and between temperature and COD/SO_4^{2-} in response to pH in the SRB culture system, with P values of 0.30, 0.33, and 0.64, respectively.

Figure 9 shows the interaction of temperature, pH value, and COD/SO_4^{2-} on the removal percentage of SO_4^{2-} in the SRB culture system. It can be seen from Figure 9a,b that the removal percentage of SO_4^{2-} increases significantly with the increase of temperature. With the increase of the environmental pH value, the removal percentage of SO_4^{2-} increased significantly. It can be seen from Figure 9c,d that the removal percentage of SO_4^{2-} increases significantly with the increase of temperature. With the increase of COD/SO_4^{2-}, the removal percentage of SO_4^{2-} increased. It can be seen from Figure 9 that the removal percentage of SO_4^{2-} increases significantly with the increase of environmental pH. With the increase of COD/SO_4^{2-}, the removal percentage of SO_4^{2-} increased. When the temperature is 34.74 °C, the environmental pH value is 8.00, the initial COD/SO_4^{2-} is 1.98, and the removal percentage of SO_4^{2-} is 88.74%. The analysis of variance showed that the effects of single factor temperature, environmental pH value, and COD/SO_4^{2-} on the removal percentage of SO_4^{2-} were extremely significant ($P < 0.001$). The order of influence is environmental pH > temperature > COD/SO_4^{2-}. Among the influences on the removal percentage of SO_4^{2-}, the interaction between the environmental pH value and temperature, COD/SO_4^{2-} exists, especially the influence of pH value and temperature on removal percentage of SO_4^{2-} reaches a very significant level, and the significance level is 0.0005. However, there is no interaction between the temperature and the response of COD/SO_4^{2-} to the removal percentage of SO_4^{2-}.

To sum up, based on the single factor experiment and response surface experiment, the optimal growth conditions of the SRB theory were obtained as follows: the culture temperature was 34.74 °C, the initial pH value was 8.00, and the initial COD/SO_4^{2-} was 1.98. Under this condition, OD_{600} is 1.45, the pH value is 9.37, the ORP value is −399 mV, and the removal percentage of SO_4^{2-} is 88.74%. Considering the temperature adjustment range of a constant temperature oscillation incubator in the actual culture, the temperature is adjusted to 35 °C in experimental verification. That is to say, under the conditions of 35 °C culture temperature, initial pH value of 8.00, and initial COD/SO_4^{2-} of 1.98, three repeated tests were conducted, and the results showed that OD_{600} was 1.51, the pH value was 9.43, the ORP value was −414 mV, and the removal percentage of SO_4^{2-} was 89.21%. The difference between the experimental value and the theoretical value is small, which shows that the model is effective.

4. CONCLUSIONS

(1) SRB is enriched in the soil around the lead-zinc tailings pond, and the growth curve of SRB is "S" type. SRB was in the logarithmic phase when cultured for 14−86 h, with high activity and vigorous growth metabolism.

(2) When the temperature is 32∼35 °C, the activity of SRB is the highest. At 35 °C, the OD_{600} value, pH value, ORP value, Ec value, and removal percentage of SO_4^{2-} after SRB inoculation were 1.33, 8.78, −393 mV, 2.90 mS/cm, and 84.77%, respectively. With the gradual increase of the S^{2-} concentration in the culture system, the SRB activity will be inhibited and even lead to SRB cell death. The environmental pH value that SRB can tolerate is 5∼8, and when the environmental pH value is 7∼8, the SRB activity is the strongest. The most suitable COD/SO_4^{2-} for SRB growth is 2. Under this condition, the OD_{600} value, pH value, ORP value, Ec value, and removal percentage of SO_4^{2-} after SRB growth are 1.33, 8.78, −393 mV, 2.90 mS/cm, and 84.77%, respectively.

(3) The results of RSM showed that culture temperature (A), environmental pH (B), and COD/SO_4^{2-} (C) had an effect on the desulfurization performance of SRB, which was extremely significant. The performance of

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ACS Omega 2023, 8, 4046−4059

affecting SRB desulfurization performance among temperature, environmental pH, and COD/SO_4^{2-} was SO_4^{2-} removal percentage (%) = 81.97 + 13.28 × A + 13.61 × B + 8.09 × C − 6.22 × A × B − 1.43 × A × C − 3.59 × B × C − 8.74 × A^2 − 5.91 × B^2 − 13.27 × C^2, R^2 = 0.9943. The order affecting SRB to remove SO_4^{2-} was as follows: environmental pH > temperature > COD/SO_4^{2-}. However, in the process of removing SO_4^{2-} by SRB, there was an interaction among environmental pH and temperature, pH value, and COD/SO_4^{2-}. Especially, the interaction between the pH value and temperature which has an effect on SRB desulfurization reached an extremely significant level, and there was no interaction between temperature and COD/SO_4^{2-} on the SRB desulfurization process. The optimal growth conditions of SRB obtained from RSM were as follows: culture temperature 34.74 °C, initial pH 8.00, initial COD/SO_4^{2-} = 1.98. Under this condition, the OD_{600} value is 1.45, the pH value is 9.37, the ORP value is −399 mV, and the removal percentage of SO_4^{2-} is 88.74%.

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