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RESEARCH PAPER

Enhanced Sulfate Reduction by *Citrobacter* sp. Coated with Fe_3O_4/SiO_2 Magnetic Nanoparticles

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Abstract A sulfate-reducing *Citrobacter* strain was isolated and coated with Fe_3O_4/SiO_2 magnetic nanoparticles (MNPs) to enhance sulfate reduction. Biolog analysis showed that it utilizes a broad range of electron donors. The findings also showed that this bacteria strain is a facultative anaerobe and can completely reduce 12 mM of sulfate to sulfide in 7 days under anaerobic conditions. Moreover, sulfate reduction was enhanced by 79% under optimized conditions. Different SiO_2 wrap-ratios of the MNPs attached to the cell surface were studied to optimize the sulfate reduction: the surface of cells coated with 300% silica wrap-ratio MNPs showed the highest stability and increased desulfurization batch time, with a 450% increase in sulfate reduction in comparison with uncoated cells cultivated in anaerobic conditions.

Keywords: anaerobic processes, biodesulfurization, cell immobilization, *Citrobacter* sp. HCSR, magnetic particles (MNPs), waste-water treatment

1. Introduction

As a result of the production of leather, paper, and food, as well as fermentation and mining processes, large amounts of industrial wastewater with a high sulfate concentration are produced [1]. Traditional physical-chemical processes, which include neutralization and precipitation, have been widely used to treat sulfate-rich wastewaters [2]. The chemical treatment of sulfate-rich waters involves adding alkali to raise the pH and precipitate sulfate. However, it is generally expensive and produces high sludge volumes. Biological sulfate removal from wastewaters has thus been considered as a promising technology [3,4]. Sulfate-reducing bacteria (SRB) are used for the biological sulfate reduction process.

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that are widespread in anoxic habitats. Under anaerobic conditions, SRB oxidize simple organic compounds such as alcohols and hydrocarbons by utilizing sulfate as an electron acceptor, generating sulfide (S^{2-}) and increasing alkalinity as a result [5]. However, increased toxic effects on SRB cells as well as increased concentrations of S^{2-} are also generated in the sulfate-reducing process [6]. Nanosized magnetic particles (MNPs) are widely applied in the field of biomedicine, for immobilizing cells, among many other fields, due to their chemically liable surface, low cytotoxicity, large surface area, hollow structure and magnetism [7-9]. However, bare MNPs are labile, and can aggregate and biodegrade when exposed to a biological system. Therefore, it is necessary to combine magnetic nanoparticles with other carriers for efficient targeted delivery. SiO_2 is stable under acidic conditions and inert with regards to redox reactions compared with organic coating materials. Consequently, SiO_2 has been considered as an ideal shell composite to protect the inner magnetite core [10].

A traditional Up-flow Anaerobic Sludge Bed (UASB) reactor continuously pumps fresh medium into a sulfate-reducing system to avoid sulfate accumulation, which leads to low water utilization efficiency [11]. In this study, we coated SRB with MNPs in order to enhance their S^{2-} tolerance. More importantly, cells coated with Fe_3O_4/SiO_2

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MNPs can be easily recovered and reused. The sulfate-reducing strain of *Citrobacter* sp., HCSR (CGMCC 4660), reported in this study was isolated from the sediment of papermaking effluent sludge. Although some strains of *Citrobacter* sp. can produce H_2S [12], their sulfate reduction ability has not been studied extensively. We also investigated the biochemical characterization and sulfate reduction ability of the HCSR strain in different air conditions. These traits were thoroughly characterized using Biolog analyses, a Scanning Electron Microscope (SEM), and an ion-chromatogram analyzer. To our best knowledge, this is the first report that discusses a Fe_3O_4/SiO_2 MNP-coated *Citrobacter* sp. strain that enhances sulfate reduction.

2. Materials and Methods

2.1. Growth media and conditions

A modified SRB medium with the following composition was used: 0.60 g of KH_2PO_4/L, 3.0 $MgSO_4$/L, 2.0 g $MgCl_2$/L, 1.0 g of KCl/L, and 1.5 g yeast extract/L. The carbon and energy source was 4.0 g ethanol/L. All incubations were conducted at 30°C. As a facultative anaerobic strain, both anaerobic and aerobic flask cultivation were conducted at 30°C statically. All solutions were made up with sterile deionized water.

2.2. Enrichment and isolation of the HCSR strain

The isolation of SRB was performed using enrichment culture. Sediment for the enrichment cultures was collected from the papermaking effluent sludge of a paper mill located in Tianjin, China. For isolation, the enrichment culture was incubated in a 150 mL anaerobic flask. The anaerobic flask contained 50 mL of a previously prepared basal medium and a gas phase of 1.5 bar N_2. After autoclaving, 0.05% sterile $FeSO_4 \cdot 6H_2O$ was added to the flask to indicate hydrogen sulfide formation and to buffer the pH. After inoculating with 5% (wt/vol) papermaking effluent sludge sediment, the flask was cultivated in the dark in an anaerobic chamber at 30°C. The inoculated liquid medium turned black after three days, and 0.1 mL of enrichment culture was then cultivated in an anaerobic flask at 30°C. Plates were prepared with the previous medium. Black colonies were purified three times.

2.3. Sequencing of the 16S rDNA and biolog analyses

The genomic DNA of the HCSR strain was isolated, and the 16S rRNA gene was amplified from the genomic DNA using eubacterial primers 27F and 1492R [13]. The PCR products were purified with the QIA quick PCR purification kit (Qiagen) according to the manufacturer's instructions. An online similarity analysis of the 16S rRNA gene sequences was performed with the BLAST program based on the GenBank and EMBL databases.

The cellular Biolog profile of the HCSR strain was determined at the Identification Service of the CAAS (The Chinese Academy of Agricultural Sciences). Isolated colonies of the HCSR strain were prepared according to the manufacturer's instructions. Colonies were picked up with a sterile wooden Biolog Streakerz stick and rubbed around the walls of a sterile and glass tube. The suspension materials were subsequently used to inoculate culture wells of Gellan Negative Microplates.

2.4. Synthesis of Fe_3O_4/SiO_2 MNPs and coating of cells with MNPs

To prepare silica-coated Fe_3O_4 nanoparticles, 2.5 g/L of Fe_3O_4 nanoparticles were suspended in 500 mL deionized water, which was then heated to 85°C under nitrogen flow (30 mL/min). 40 mL of 1.0 M sodium silicate solution was then added to the Fe_3O_4 suspension under vigorous stirring, and the mixture pH was adjusted to 5.8 with 2.0 M HCl solution within 3 h. The reaction mixture was further stirred at 85°C for 2 h. The resulting silica-coated Fe_3O_4 MNPs were thoroughly washed with deionized water and then collected by magnetic separation, which was followed by drying at 50°C under vacuum for 12 h. The materials obtained are referred to as Fe_3O_4/SiO_2 nanoparticles. MNPs with different ratios of Fe_3O_4/SiO_2 were synthesized by modifying the amount of Fe_3O_4 and SiO_2.

To ensure anaerobic conditions, all samplings were carried out using sterilized flask flushed with N_2. The prepared MNPs had been washed three times with sterilized cultivation solution, then 5% (wt/vol) of culture (mixed with MNPs) were injected into the experimental flask, in which cells had already been cultivated for 2 days under aerobic conditions. In order to make the MNPs and cells contact sufficiently, the flasks were placed in the shaker (HYG-A from Taicang Equipment Factory, China) for 15 min (rotary speed was conducted at 80 rpm). All samplings were cultivated in anaerobic chamber at 30°C in the dark at last. Upon completion of the sulfate reduction process, the cells were recovered by magnetic separation and fresh medium was added to the anaerobic flask to commence the next sulfate reduction process.

2.5. Scanning electron microscopy

The type of instrument used was a Hitachi S-3400N Scanning Electron Microscope (Hitachi, Japan), operated at 15 kV. Samples were prepared by using double-sided tape to mount specimens coated with a thin layer of gold. Images were obtained at magnifications ranging from 20,000 × to

60,000 ×, depending on the feature to be studied.

2.6. Analytical methods

Bacterial growth was determined by measuring the optical density at 600 nm using a U-2910 spectrophotometer (Hitachi High-Technology Company, Japan). In order to determine the sulfate reduction by the HCSR strain, a series of solutions with different $MgSO_4$ concentrations (15, 20, and 25 mM) was added. After two days of cultivation in a constant temperature shaker (30ºC, 180 rpm), the anaerobic flask was vacuum sealed and then injected with 1.5 bar N_2, to ensure anaerobic conditions within the flask. Samples were centrifuged at 2,510 × *g*, filtered through 0.22 μm pore size nylon filters (Membrane, USA), and then prepared for sulfate analysis. Levels of sulfate and sulfide were determined by an ICS-900 ion-chromatogram analyzer (Dionex Corporation, USA). All experiments were performed in triplicate. Bars represent standard deviations in graph.

3. Results and Discussion

3.1. 16S rRNA gene sequence analysis of the HCSR strain

Phylogenetic analysis of the 16S rRNA gene sequence of the HCSR strain consistently grouped the strain with the family *Citrobacter*. The phylogenetically closest relative of the HCSR strain, the *C. freundii* strain, shares 100% 16S rRNA gene sequence similarity (Fig. 1), while the *C. braakii* strain shares 99% similarity. Hence, the isolate is referred to as *Citrobacter* sp. strain HCSR. Based on comparative analysis of 16S rRNA sequences, the known SRB can be grouped into seven phylogenetic lineages, five under Bacteria and two under Archaea [1]. However, the *Citrobacter* sp. strain HCSR was not among the known SRB, which indicates that the diversity of SRB still has the possibility of expanding.

3.2. Biolog analysis and phenotypic characteristics of the HCSR strain

Table 1 shows phenotypic characteristics, utilized electron donors, and utilized electron acceptors of the HCSR strain compared with other SRB. The strain is mesophilic, and grows at an optimum temperature of 30ºC. The HCSR strain also utilizes a broad range of electron donors. For instance, this strain can grow on propionate, succinate and citrate, which, respectively, are non-utilized by the SRB *Desulfococcus Multivorans*, *Desulfococcus Biacutus* [14], and *Desulfobacterium Indolicum* [15]. In addition, compared with the other three SRB, the HCSR strain is unable to use sulfate as a terminal electron acceptor.

3.3. Growth and sulfate reduction of the HCSR strain in anaerobic and aerobic conditions

The growth and sulfate reduction of the HCSR strain in anaerobic and aerobic conditions were studied. It was apparent that the HCSR strain grew more quickly in aerobic conditions. Changes in ORP (oxidation-reduction potential) were measured in both conditions. In anaerobic conditions, the ORP decreased from 50 to -52 mV in 7 days (Fig. 2A). In aerobic conditions, the ORP increased from 50 to 197 mV in 7 days (Fig. 2B).

In anaerobic conditions, the rate of sulfate removal reached a maximum after 0.6 days (Fig. 2A). By 6 days, the sulfate concentration had dropped from an initial value of 11.8 ~ 0.2 mM, indicating that the HCSR strain had completely reduced the sulfate. On the other hand, after 2 days of growth in aerobic conditions, sulfate reduction by the HCSR strain stopped (Fig. 2B).

3.4. Optimized sulfate reduction of the HCSR strain

Fig. 2 indicates that the growth and maximum cell dry weight of the HCSR strain is about 4 times higher in

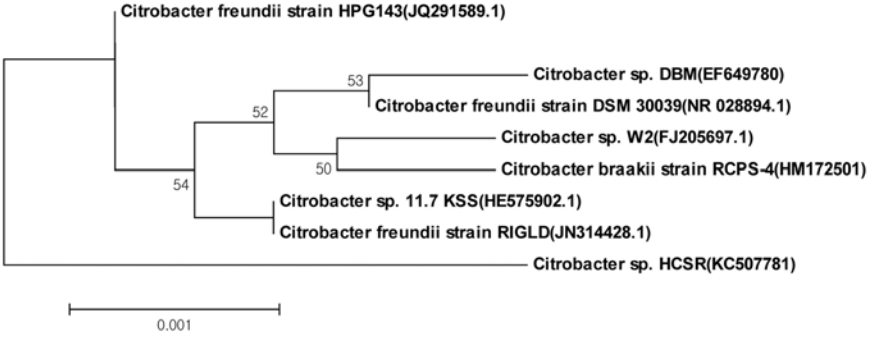

Fig. 1. Unrooted phylogenetic tree showing the phylogenetic relationship of the HCSR strain (*Citrobacter sp.*) with some members of the genus *Citrobacter*. The tree was constructed using the neighbor-joining method based on a comparison of approximately 1350 nucleotides. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar: 0.1% sequence divergence.

Table 1. Characteristics of the HCSR strain and traditional SRB representatives of the genera Desulfococcus and Desulfobacterium

Characteristic	Strain HCSR	*Desulfococcus Multivorans*	*Desulfococcus Biacutus*	*Desulfobacterium Indolicum*
Morphology	Oval to rod	Spherical	Lemon	Oval to rod
Optimum temperature (°C)	30 ~ 37	35	20 ~ 30	28
Optimum pH	6.5 ~ 6.8	NR	6.8 ~ 7.8	7.0
Optimum salinity (NaCl g/L)	4	5	2	20
Electron donors				
Ethanol	+	+	+	+
Formate	+	+	NR	+
Acetate	+	+	+	+
Pyruvate	–	NR	+	+
Lactate	–	+	–	–
Acetone	–	+	+	NR
Propionate	+	NR	–	–
Fumarate	+	–	–	+
Succinate	+	–	–	NR
Malate	–	–	–	+
Butanol	+	NR	+	+
Butyrate	+	+	+	–
Benzoate	+	+	–	–
Palmitate	+	NR	NR	NR
Citrate	+	NR	NR	NR
Electron acceptors				
Sulfate	+	+	+	+
Thiosulfate	+	+	NR	+
Sulfite	–	+	+	+
Nitrate	–	–	–	NR

NR: Not Reported.

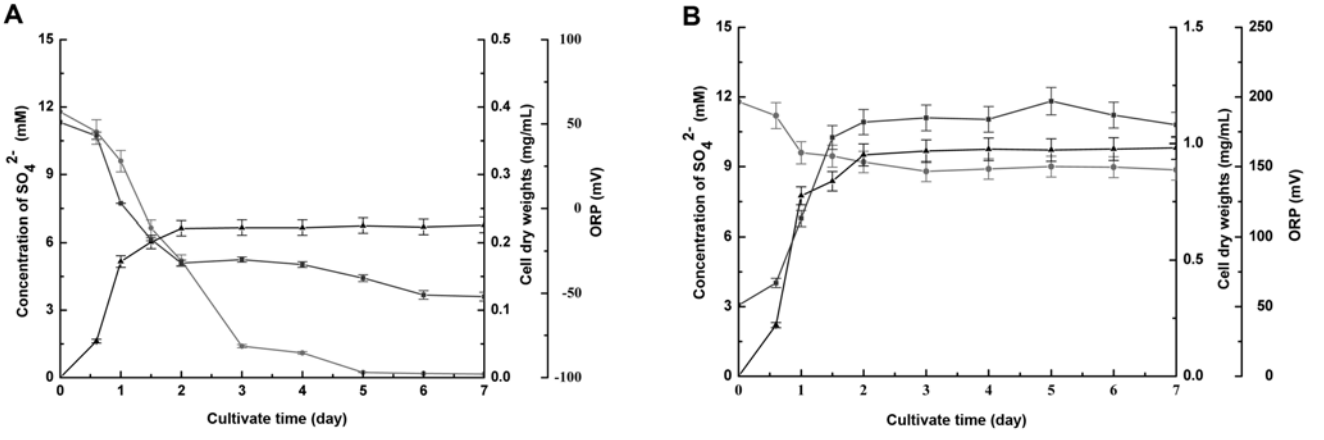

Fig. 2. (A) Growth and sulfate reduction of the HCSR strain in anaerobic conditions. (B) Growth and sulfate reduction of the HCSR strain in aerobic conditions. (SO_4^{2-} ●, Cell dry weight ▲, Oxidation Reduction Potential, ORP ■).

aerobic conditions than in anaerobic conditions. On the contrary, sulfate reduction reached only 24% of the maximum capability in aerobic conditions. Therefore, we changed the conditions from aerobic to anaerobic when the cell dry weight reached its maximum after 2 days, in order to enhance the amount of sulfate reduction by the HCSR strain.

Fig. 3 shows that the cell dry weight decreased slightly after shifting to an anaerobic condition. In contrast, the ORP dropped dramatically from 188 to -32 mV. The sulfate concentration decreased from 24 to 19 mM in 2 days. However, sulfate reduction occurred quickly following the

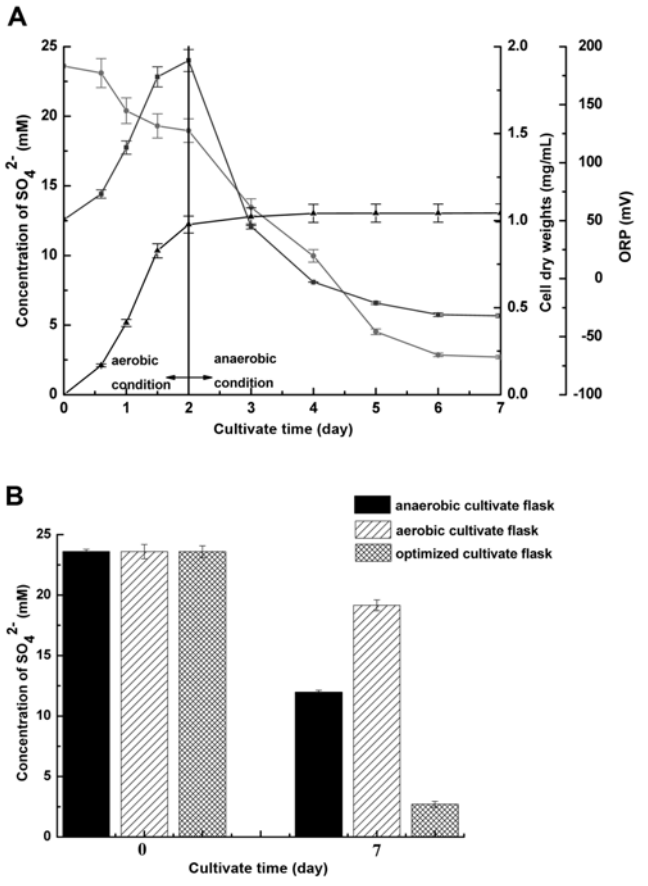

Fig. 3. (A) Dual-phase controlled growth and sulfate reduction of the HCSR strain. (SO_4^{2-} ●, Cell dry weight ▲, Oxidation Reduction Potential, ORP ■) (B) Comparison of sulfate reduction in anaerobic, aerobic and optimized condition of the HCSR strain, over a period of 7 days.

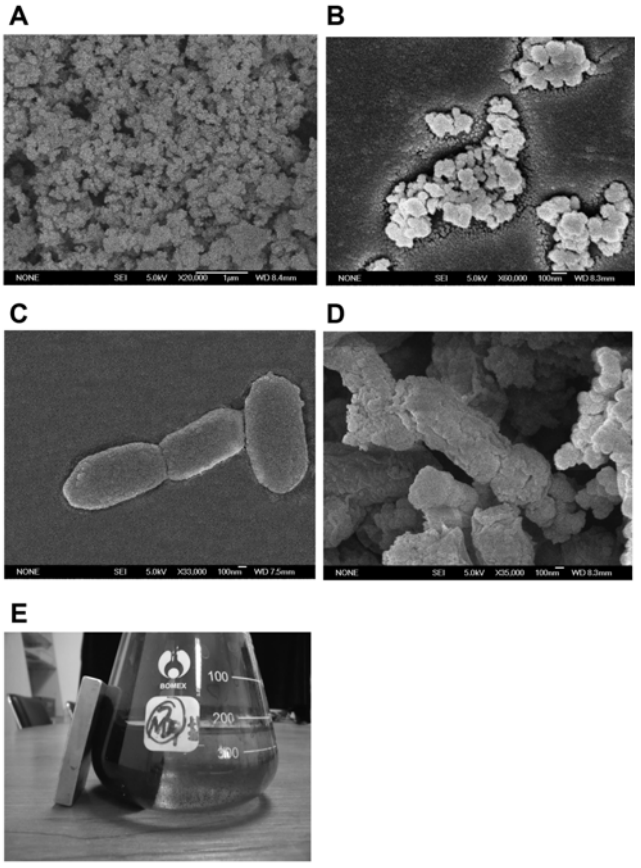

Fig. 4. (A) SEM photos of Fe_3O_4 without a coating of SiO_2 magnetic nanoparticles. (B) SEM photos of Fe_3O_4/SiO_2 magnetic nanoparticles. (C) Cells of the HCSR strain. (D) Cells of the HCSR strain coated with Fe_3O_4/SiO_2 magnetic nanoparticles. (E) cell separation using magnets during sulfate reduction process.

change in conditions from aerobic to anaerobic, indicating that over 90% of the sulfate had been removed.

3.5. Morphological analysis of MNPs and cells coated with MNPs

Electron microscopy (Fig. 4A) shows that Fe_3O_4 MNPs have a great degree of aggregation in the nano-structure. Following the coating of the Fe_3O_4 core by the silica layer (Fig. 4B), the MNPs showed considerable dispersion compared with Fig. 4A. The morphology of the HCSR strain cells can be observed clearly (Fig. 4C). After 2 days of cultivation in an aerobic condition, the surface of the cells had been fully coated by the Fe_3O_4/SiO_2 magnetic nanoparticles (Fig. 4D). This indicates that Fe_3O_4/SiO_2 MNPs attach firmly to the cell surface.

3.6. Enhanced sulfate reduction after coating of cells with Fe_3O_4/SiO_2 MNPs

To improve cell endurance to high concentrations of S^{2-}, the cell surface of the HCSR strain with attached MNPs was studied. Fig. 5A shows sulfate removal capability of the HCSR strain with/without nanomaterials. In 0 ~ 3 days, the desulfurization by HCSR strain with & without MNPs showed a similar velocity. However, sulfate removal rate of the strain without MNPs decreased drastically since 4th day and the sulfate concentration had dropped from an initial value of 40 ~ 20 mM by 14 days. On the contrary, strain with MNPs kept high sulfate reduction rate from 1 to 9 days and the sulfate concentration decreased from 40 to 5 mM in 14 days.

Fig. 5B shows that sulfate removal capability increased with an increasing SiO_2 wrap-ratio of the MNPs. Under a low SiO_2 wrap-ratio condition, no enhancement of sulfate reduction was observed. More importantly, MNPs with a 100% silica wrap-ratio were degraded within 6 days under optimized cultivation. When the silica wrap-ratio rose to 300%, the MNPs showed a great resistance to high concentrations of S^{2-}, and the degradation occurred at 28 days since inoculation. The experimental results showed a similar enhancement in sulfate reduction with a constant

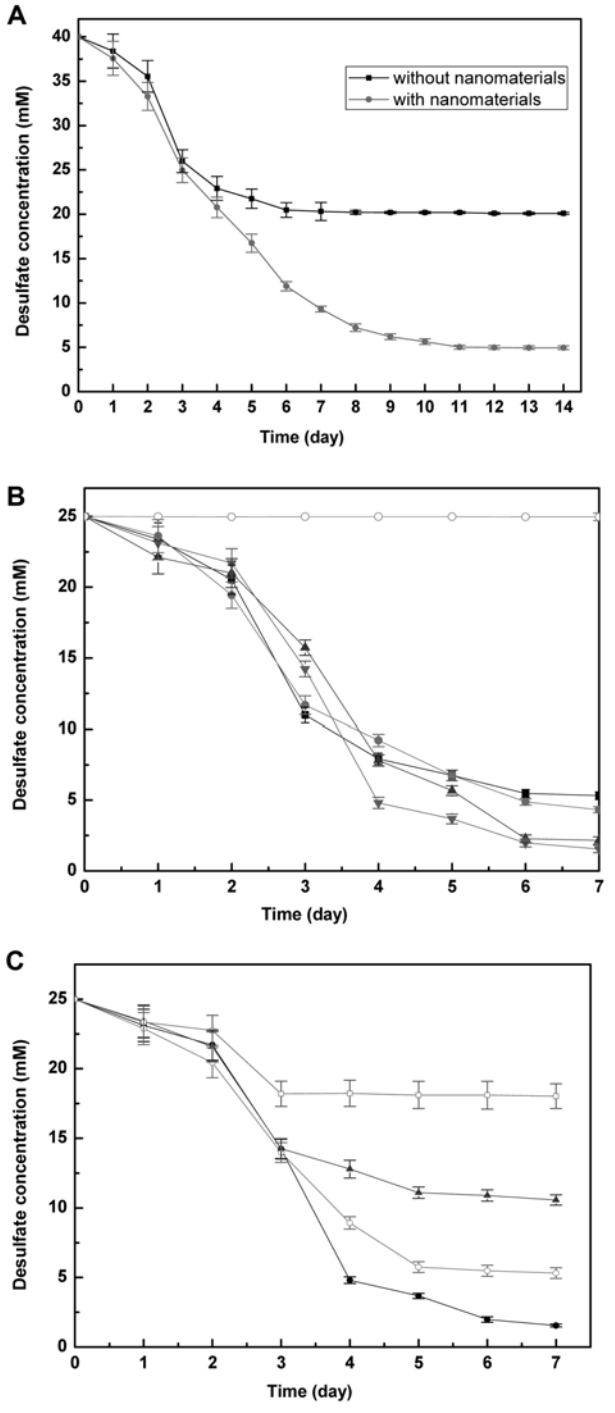

Fig. 5. (A) sulfate reduction by the HCSR strain with/without nanomaterials. (B) Sulfate reduction by the HCSR strain coated with different Fe_3O_4/SiO_2 ratio MNPs, and a negative control (SiO_2/Fe_3O_4 ratio of MNPs: 100% ■, 200% ●, 300% ▲, 400% ▼, negative control ○). (C) Repeat desulfurization effect by the HCSR strain coated with 300% Fe_3O_4/SiO_2 ratio MNPs (desulfurization batch 1st ●, 2nd ○, 3rd ▲, 4th □).

increase of the cover ratio of the silica layer to 400%, when compared with 300%. In a negative control experiment, heated cells of the HCSR strain coated with 300% silica wrap-ratio MNPs showed no sulfate reduction ability, which indicates that living cells are the basis of sulfate reduction activity, and an MNP/SiO_2 coating alone cannot catalyze the reaction.

Fig. 5C shows multiple batch desulfurization by the HCSR strain coated with 300% silica wrap-ratio MNPs. A sulfate removal rate of 93.8% after 7 days of optimized cultivation in the first batch can be observed. The cells were recovered by magnetic separation (Fig. 4E) and then re-injected with fresh medium. Compared with the first batch, the sulfate removal rate decreased slightly in the second desulfurization batch. The 7-day sulfate removal rate of the third desulfurization batch decreased drastically to 57.8%. Furthermore, for the fourth desulfurization batch, the 7-day sulfate removal rate only reached 28.0%, and sulfate reduction activity stopped at 24 days. In addition, the degradation of the MNPs occurred at 25 days.The total sulfate removal capability was 65.54 mM, and sulfate reduction was enhanced by 450% in comparison with a non-optimized anaerobic condition.

4. Conclusion

SRB play a critical role in the bio-treatment of sulfate-rich wastewater. However, these bacteria are almost strictly anaerobic. In contrast, the *Citrobacter* sp. strain HCSR is a facultative sulfate-reducing strain and can utilize a broad range of electron donors, allowing it to occupy a new niche in sulfate-rich wastewater treatment. In this study, the cell surface of the HCSR strain coated with Fe_3O_4/SiO_2 nano-sized magnetic particles (MNPs) showed a great enhancement in sulfate reduction in comparison with uncoated cells. Application of a modified facultative sulfate-reducing strain may thus be a novel option in the bio-treatment of sulfate-rich wastewater.

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