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Sulfolane degrading bacteria from petrochemical plant: activated sludge enrichment, isolation and characterization

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Abstract

Sulfolane is extensively used as an extractive solvent in sour-gas processing plants in the petrochemical industry. After repeated use, deteriorated sulfolane becomes corrosive and may leak into the environment to threaten aquatic and terrestrial organisms or cause shock loading of wastewater treatment systems. It is therefore important to remove sulfolane from the environment and maintain stable wastewater treatment efficiency. To address this issue, bioaugmentation provides a solution by adding specific microorganisms into the biological treatment system to speed up the contaminant degradation rate. Isolation of microbes capable of degrading target contaminant is key point. Understanding the physiological characteristics of isolated microbes is indispensable for subsequent successful applications. In this study, sulfolane degrading bacteria from the activated sludge of a petrochemical plant were enriched using 200–1000 mg L^{-1} sulfolane and then isolated. After confirming their sulfolane degrading ability, sulfolane biodegradation was then investigated under various pHs, sulfolane and sulfate concentrations. Enrichment could enhance the sulfolane degrading rate of the mixed culture from 3.9 to 7.1 times. Three strains (strain Y-a, Y-d and Y-f) capable of degrading sulfolane were isolated. The degrading microbes were identified as *Cupriavidus* sp. using 16S rDNA sequencing. Sulfolane concentration biodegraded by strain *Cupriavidus* sp. Y-d at pH of 7–9 was 1.40–1.45 times higher than that at pH of 6. The optimal pH for strain *Cupriavidus* sp. Y-d to degrade sulfolane was 8. When increasing the sulfolane concentration from 500 to 2000 mg L^{-1}, the specific growth rate and specific substrate utilization rate increased from 0.19 to 0.48 d^{-1} and 0.48 to 0.59 d^{-1}, respectively. Strain *Cupriavidus* sp. Y-d was capable of degrading 2000 mg L^{-1} sulfolane. Sulfate concentrations higher than 2511 mg L^{-1} had a negative effect on sulfolane biodegradation.

Keywords Sulfolane, Biodegradation, Strain *Cupriavidus* sp., Sulfate

1 Introduction

Sulfolane is a sulfur organic compound extensively used in liquid–liquid and liquid–vapor extraction processes because it is a thermally stable solvent and does not react with acids or bases [1]. Due to its selectivity to aromatics, sulfolane is a popular solvent used by the petroleum refining and petrochemical industries to recover benzene, toluene, and other compounds from hydrocarbon mixtures [2]. Sulfolane can be repeatedly recovered and used after removing impurities.

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Improper sulfolane use causes a shock loading on wastewater treatment systems. Without appropriate treatment, its high mobility makes sulfolane migrate offsite and contaminate nearby domestic wells and surface waters [3]. Sulfolane at doses over 200 mg kg^{-1} is acutely toxic to lab rats and guinea pigs [4]. Depending on the dose concentration, sulfolane may cause central nervous system stimulation or depression in mammals [5]. The acute effects of sulfolane on rats include changes in thermoregulation, motor activity and brainwave pattern [4].

In treating sulfolane, the biological strategy is superior to physical and chemical strategies because of economic and environment-friendly concerns. However, sulfolane does not biodegrade quickly under oxygen and nutrients limited conditions [2, 6]. Sulfolane is only biodegraded in the presence of oxygen as the electron acceptor. Nitrate, sulfate, or Fe(III) could not be the electron acceptor to support sulfolane biodegradation [7]. Green and Fedorak indicated that sulfolane contaminated soil microcosms and cultures were stimulated by phosphate addition [8]. In addition, to the environmental and nutrient concerns, poor sulfolane biodegradation may also result from the absence of a specific microorganism or microorganism consortium capable of degrading sulfolane. Bioaugmentation is defined as the process of adding specific bacterial cultures to speed up the degradation rate for a contaminant. After adding strain *Pseudomonas* sp. into the mixed culture from the contaminated aquifer sediment, the sulfolane degrading rate increased from 23 to 96 mg L^{-1} d^{-1} [9]. Chang et al. indicated that bioaugmenting in situ mixed bacteria with sulfolane degrading bacterium in a mixing ratio of 10:3, sulfolane in the contaminated artificial groundwater was effectively biodegraded [10].

Enrichment is often a necessary and critical strategy to obtain target bacterial strains for successful bioaugmentation [11]. The suitable microbial source is from the same ecological niche as the pollutant, so that the bioaugmented microbes are not competed by other indigenous bacterial strains [12]. To stabilize wastewater treatment system and bioremediate groundwater contaminated by sulfolane, it is important to isolate sulfolane degrading bacteria and apply them using the bioaugmentation strategy. However, bacterial isolates capable of degrading sulfolane were seldomly reported in the previous literatures. Matsui e al. isolated strain *Shinella yambaruensis* MS4T capable of degrading 3-methyl-sulfolane and sulfolane from the soil in Japan [13]. Strain *Variovorax* sp. WP1 was isolated from sulfolane contaminated plume aquifer materials [14]. Kasanke et al. confirmed that strain *Rhodoferax* sp. was the dominant sulfolane decomposer in a sulfolane contaminated subarctic aquifer using stable isotope probing combined with metagenomics [15]. After isolating target strains for bioaugmentation, environmental factors such as temperature, pH, dissolved oxygen, nutrient availability, toxicity and osmosis stress must be studied to enhance the possibility of successful bioaugmentation [16]. Previous research indicated that low temperature (8 °C) caused limited sulfolane biodegradation [17]. To isolate sulfolane degrading bacteria for future bioaugmentation, this research enriched and isolated pure sulfolane degrading bacteria. The basic sulfolane degrading characteristics of these bacteria were also investigated.

2 Materials and methods

2.1 Bacterial source and media

The activated sludge was from an activated sludge aeration tank of one petrochemical production plant located in Yunlin Country, Taiwan. Mineral salt medium (MSM) was used for the enrichment and batch experiments in this research. MSM was composed of 0.334 g L^{-1} NH_4Cl, 0.015 g L^{-1} $CaCl_2$, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 4 g L^{-1} K_2HPO_4, 4 g L^{-1} KH_2PO_4 and 10 mL L^{-1} Trace element. Trace element contained 300 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 40 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 58 mg L^{-1} $CoCl_2$, 50 mg L^{-1} $MnSO_4{\cdot}H_2O$, 34 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$ and 50 mg L^{-1} $CuSO_4{\cdot}5H_2O$. Differential medium agar [18] used for sulfolane degrading bacteria isolation contained 2.3 g L^{-1} bacto plate count agar, 17 g L^{-1} Baco agar, 0.1 g L^{-1} Bromothymol Blue and 1 g L^{-1} sulfolane (99% purity).

2.2 Enrichment of sulfolane degrading consortium

To obtain pure culture capable of degrading high sulfolane concentration, the activated sludge was enriched using 200–1000 mg L^{-1} sulfolane. Ten mL washed activated sludge was added into a 500-mL flask containing 240 mL MSM with 200–1000 mg L^{-1} sulfolane supplied as the sole carbon and energy sources. The mixed culture was incubated at 28 °C and 120 rpm in the dark. Sulfolane and pH were measured periodically to identify sulfolane degradation. During enrichment, sulfolane was repeatedly added and pH was kept at 7.2 using sterilized 3 N NaOH. The enrichment period was separated into three phases according to the sulfolane degrading rate. The enrichment periods for the three phases were Day 1–48, Day 49–62 and Day 63–70.

2.3 Isolation and identification of sulfolane degrading bacteria

After sulfolane biodegradation became significant and stable during enrichment, a series of tenfold dilutions (10^{-1}–10^{-5}) of the enriched mixed culture were made for target bacterial isolation. For bacterial isolation, 300 µL of each dilution was spread onto the differential medium agar. The agar plates were incubated at 28°C. The loopfuls of single colony producing yellow

rings were repeatedly streaked onto the fresh differential medium agar until a pure culture was obtained. Each isolate was then checked for its ability to degrade sulfolane by inoculating the isolate into a 250-mL flask containing 100 mL MSM and 1000 mg L^{-1} sulfolane. The flasks were periodically sampled to measure sulfolane, pH and sulfate. The theoretical aerobic mineralization of sulfolane could be according to the following equation. $C_4H_8O_2S + 6.5O_2 \rightarrow 4CO_2 + 3H_2O + 2H^+ + SO_4^{2-}$ [14]. Sulfolane biodegradation accompanies sulfate release, so the sulfate concentration was additionally analyzed to obtain sulfolane mineralization efficiency. The potential bacterial isolates were chosen for further strain identification. Bacteria identification was carried out by the Mission Biotech Company (Taipei, Taiwan). The DNeasy plant kit (Qiagen, Germany) was used to extract total genomic DNA of pure culture. The 16S rDNA gene of the bacterial isolates was amplified with primer pair 16 s-F1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 16 s-R1 (5'-GGTTACCTTGTTACGACTT-3') [19]. The DNA amplicon was then sequenced with a DNA sequencer (ABI 3730XL DNA Analyzer, Perkin-Elmer, California, USA). The sequence data were compared by the BLAST algorithm of the National Center for Biotechnology Information (NCBI).

2.4 Batch reactor experiments

To apply sulfolane degrading bacteria for further bioaugmentation, the important factors influencing bacterial performance and function in the real wastewater should be studied. The real wastewater contained high sulfate concentration. Moreover, sulfolane was suddenly discharged into the wastewater treatment system, causing the sulfolane shock loading problem. In this study, pH, sulfolane and sulfate concentrations were chosen as the parameters for batch bioreactor experiments. The culture experiment was conducted using a series of 500-mL flasks. The initial biomass amount was started at a cell concentration yielding 0.1–0.2 optical density ($O.D._{600}$). The pure culture was incubated at 28˚C under different initial pHs (6, 7, 8, 9), sulfolane concentrations (500, 1000, 1500, 2000 mg L^{-1}) and sulfate concentrations (2511, 3020, 3534, 4020 mg L^{-1}). The $O.D._{600}$, pH, sulfolane and sulfate concentrations were periodically monitored during the whole experimental period.

2.5 Analytical methods and calculation

The pH and $O.D._{600}$ were measured using a pH meter (Mettler Toledo, Schwerzenbach, Switzerland) and spectrophotometer (ChromTech, Singapore) at 600 nm, respectively. To translate the $O.D._{600}$ value for microbial kinetic calculation, cell biomass was determined from a standard curve relating $O.D._{600}$ to dry weight. $O.D._{600}$ values of 0.5 represented 0.27 mg (dry weight) per mL for bacterial cells. This relationship was linear up to at least an $O.D._{600}$ value of 1.0. For quantification of the produced sulfate from sulfolane biodegradation, the ion chromatography (IC) (Metrohm AG, Herisau, Switzerland) equipped with the conductivity detector was applied. Sample was filtered with 0.22 μm PTFE filter, and then the filtrate was injected into the IC with the column of Metrosep A Supp 5–150/4.0. The mobile solvent was the 3.2 mM Na_2CO_3 and the flow rate was 0.7 mL min^{-1}. Sulfolane was analyzed using gas chromatography (GC) (Shimadzu, Tokyo, Japan) after extraction. For sulfolane extraction, 0.1 g NaCl was added into 5 mL sample and then 0.5 mL dichlormethane was added for sulfolane extraction followed by the previous studies [20, 21]. One μL organic liquid was then analyzed with GC equipped with the flame ionization detector. The GC column was Agilent DB-1. Nitrogen gas was used as the carrier gas and flow rate was 2 mL min^{-1}. The injection, oven and detector temperatures were 200, 150 and 250˚C, respectively. Split injection was conducted with a split ratio of 1:20. Sulfolane removal efficiency was calculated by the removed sulfolane concentration divided by the reaction time. Sulfolane degrading rate was obtained using sulfolane concentration interval with the steepest slope and linear range ($R^2 > 0.97$) divided by the time of the interval.

2.5.1 Nucleotide sequence accession numbers

The 16S rDNA sequences of strain *Cupriavidus* sp. Y-a, Y-d and Y-f have been deposited in NCBI GenBank under accession no. MG725955.

3 Results and discussion

3.1 Sulfolane degrading bacteria enrichment

Enrichment is a commonly used strategy to effectively increase the population of target microorganisms. After enrichment, the target microorganisms are isolated easier for further study and application. Enrichment was performed in this study under various sulfolane concentrations (200, 400, 600, 800 and 1000 mg L^{-1}) before bacterial isolation. Based on the sulfolane degrading rate, the entire sulfolane enrichment period was separated into three phases. Figure 1 presents the sulfolane removal efficiency and degrading rate on the 7^{th} day of each phase. In the 1^{st} enrichment phase, the removal efficiency for each sulfolane concentration was less than 45% within 7 days. As the sulfolane concentration increased, the sulfolane removal efficiency showed a downward trend, except for 1000 mg L^{-1} sulfolane. The sulfolane degrading rates from low to high sulfolane concentration in the 1^{st} enrichment phase were 14.1, 11.2, 8.4, 9.2 and 24.7 mg L^{-1} d^{-1}. In the 2^{nd} enrichment phase, the sulfolane removal efficiency significantly increased. Sulfolane

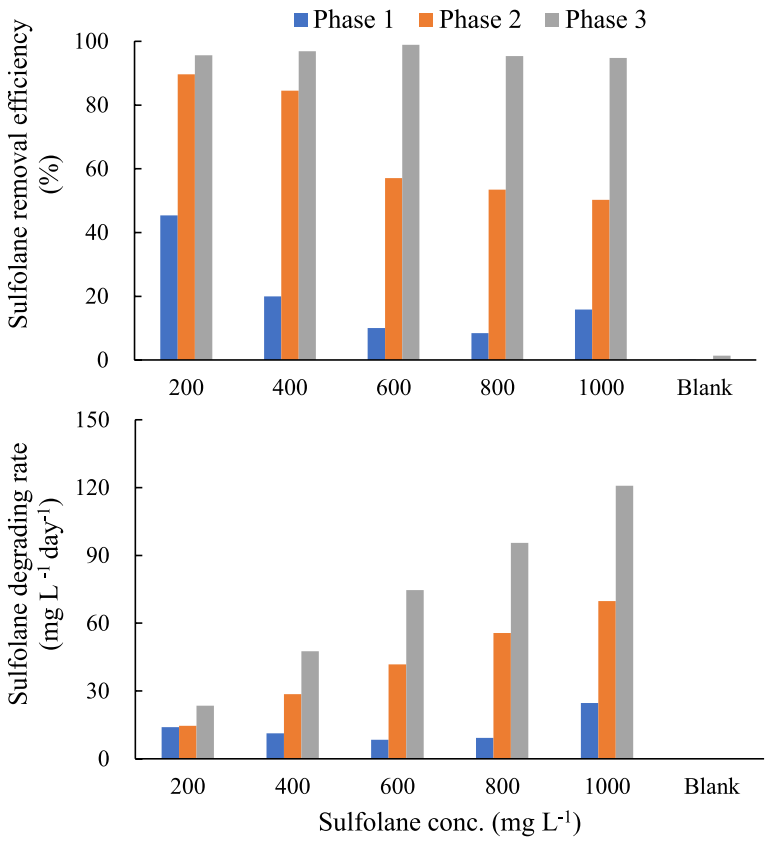

Fig. 1 Sulfolane removal efficiency and degrading rate of three enrichment phases (Phase I: Day 1–48; Phase II: Day 49–62; Phase II: Day 63–70) on the 7th day

removal efficiencies were 90 and 85% when the sulfolane concentrations were 200 and 400 mg L^{-1}, respectively. The sulfolane removal efficiencies in the remaining three sulfolane concentration groups were between 50–57%. In the 3rd enrichment phase, the sulfolane removal efficiency for all concentration groups on the 7th day could reach more than 95%. The sulfolane degrading rates in the 3rd phase increased to 24, 48, 77, 96, and 121 mg L^{-1} d^{-1}, respectively. Comparing the 1st and 3rd enrichment phases, the sulfolane degrading rate increased 1.7, 4.3, 8.9, 10.3 and 4.9 times from low to high sulfolane concentration. The pH values decreased as sulfolane biodegradation increased, especially in the 3rd phase (data not shown). This phenomenon might imply that sulfate was released by sulfolane biodegradation and sulfolane could be utilized as the carbon source to increase the population of sulfolane degrading bacteria in the enriched mixed cultures.

3.2 Sulfolane degrading bacteria screening and identification

After enrichment, sulfolane degrading bacteria isolation was performed. Eighteen pure strains grew on the differential medium agar. Among all strains, 5 colonies that formed yellow rings were considered as possible sulfolane degrading bacteria. Thses colonies were designated as strains Y-a, Y-c, Y-d, Y-f and Y-h. To further ensure sulfolane utilization, each isolated strain was incubated in MSM containing 1000 mg L^{-1} sulfolane as the sole carbon and energy source. Table 1 presents the sulfolane biodegradation and sulfate production for the 5 bacterial isolates. Strain Y-c could not utilize sulfolane and the other four strains were capable of degrading sulfolane. The sulfolane removal efficiencies were 93, 90, 90 and 74% for strain Y-a, Y-d, Y-f and Y-h, respectively. Strain Y-a had the best sulfolane degrading rate of 34 mg L^{-1} d^{-1} and produced the highest sulfate concentration of 758 mg L^{-1}. Strains Y-a, Y-d and Y-f showed higher sulfolane removal efficiencies and were then identified based on 16S rDNA sequencing. All of them were identified as *Cupriavidus* sp. with similarities of 99%. Few research was conducted on the isolation of pure sulfolane degrading bacteria and only strain *Shinella yambaruensis* MS4T and *Variovorax* sp. WP1 were published [13, 14]. The sulfolane removal ability of strain *Cupriavidus* sp. was first demonstrated and published in this research.

Table 1 Sulfolane biodegradation and sulfate production of 5 sulfolane degrading bacteria as 1000 mg L^{-1} sulfolane as the sole carbon source at pH of 7

Sulfolane degrading bacteria	Sulfolane			Sulfate
	Removed conc. (mg L^{-1})	Degrading rate (mg L^{-1} d^{-1})	Removal efficiency (%)	Produced conc (mg L^{-1})
Y-a	945	33.75	93	758
Y-c	3	0.11	0.3	6
Y-d	913	32.60	90	713
Y-f	922	32.92	90	741
Y-h	758	27.06	74	551

This research provides an alternative candidate for bioaugmentation. Strain *Cupriavidus* sp. Y-a obtained the highest sulfolane removal efficiency after the screening test. However, compared to strain *Cupriavidus* sp. Y-a, strain *Cupriavidus* sp. Y-d had the greatest sCOD and sulfolane removal efficiencies when using real wastewater as the substrate (data not shown). Thus, strain *Cupriavidus* sp. Y-d was chosen as the target strain for the following batch experiments.

3.3 Effect of pHs on sulfolane biodegradation

Figure 2 shows the sulfolane, sulfate, pH and $O.D._{600}$ variations during sulfolane biodegradation using strain *Cupriavidus* sp. Y-d at various pHs. When strain *Cupriavidus* sp. Y-d was incubated at the initial pH of 6, 7, 8 and 9. The removed sulfolane concentrations were 655, 951, 952 and 914 mg L^{-1}, respectively, referring to 66, 95, 95 and 91% sulfolane removal efficiencies. The sulfolane removal efficiency at the pH of 7 to 9 was significantly greater that at the pH of 6. This means that acidic environments are not appropriate for strain *Cupriavidus* sp. Y-d to degrade sulfolane. Sulfate concentration in MSM was considered as background value and subtracted at the beginning. The produced sulfate concentrations were 520, 760, 760 and 732 mg L^{-1} when the initial pHs were 6, 7, 8 and 9, respectively. According to the removed

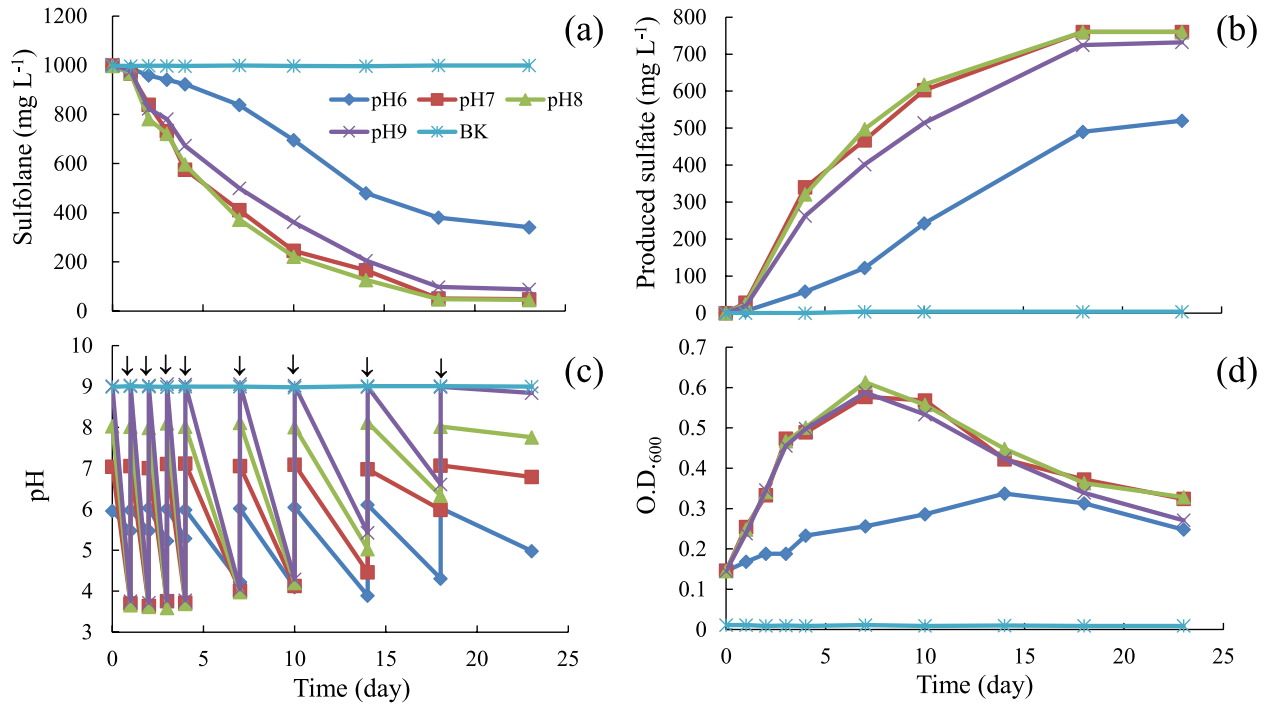

Fig. 2 Sulfolane (**a**), sulfate (**b**), pH (**c**) and $O.D._{600}$ (**d**) variations during 1000 mg L^{-1} sulfolane biodegradation by strain *Cupriavidus* sp. Y-d at various initial pHs. Arrow indicates pH adjustment. BK refers to no added bacteria

sulfolane concentrations at various pHs, the theoretical sulfate concentrations should be 523, 761, 762 and 731 mg L^{-1} at the initial low to high pH. The difference between the produced and theoretical sulfate concentrations was less than 1%. This implies the sulfolane was mineralized and no intermediate was accumulated during sulfolane biodegradation. Sulfate release accompanied pH decline from the initial pH to 3.6–3.9. To avoid the negative effect of low pH on sulfolane biodegradation, pH was adjusted as sampling. The maximum $O.D._{600}$ values were 0.58, 0.61 and 0.59 at the 7th day when the pHs were 7, 8 and 9, respectively. The maximum $O.D._{600}$ value at the pH of 6 was 0.34, about half of the highest $O.D._{600}$ of the other groups, at the 14th day. Strain *Cupriavidus* sp. Y-d could utilize sulfolane as the carbon and energy sources and mineralize sulfolane. The optimum pH range for sulfolane biodegradation was from neutral to slightly alkaline. Feng et al. [22] pointed out that when the strain *Cupriavidus lacunae* sp. was incubated in R2A broth with pH range between 5 and 9 for 3 d, the optimal pH for bacterial growth was between 7–8, which was consistent with the results in this study.

3.4 Effect of sulfolane concentrations on sulfolane biodegradation

The sulfolane, sulfate, pH and $O.D._{600}$ variations during the biodegradation of various sulfolane concentrations using strain *Cupriavidus* sp. Y-d are presented in Fig. 3. When strain *Cupriavidus* sp. Y-d was incubated in the presence of 500 and 1000 mg L^{-1} sulfolane, the sulfolane removal efficiencies were 98 and 99% and the released sulfate were 402 and 813 mg L^{-1}, respectively. Based on stoichiometry, the released sulfate concentration was 0.8-fold the sulfolane biodegradation concentration [14]. Thus, the theoretical concentrations of released sulfate were 399 and 798 mg L^{-1} from the removed sulfolane concentrations of 499 and 998 mg L^{-1}, respectively. The difference between the theoretical and produced sulfate concentrations was less than 2%. When further increasing the sulfolane concentration to 1500 and 2000 mg L^{-1}, the removed sulfolane concentrations were 1297 and 1535 mg L^{-1} and the sulfolane removal efficiencies decreased to 84 and 75%, respectively. The released sulfate concentrations were 1173 and 1385 mg L^{-1} when the suloflane concentrations were 1500 and 2000 mg L^{-1}, respectively. The substrate utilization rates in the order of low to high sulfolane concentrations were 90, 93, 114 and 129 mg L^{-1} d^{-1}. Fedorak and Coy investigated sulfolane biodegradation using aerobic shake-flask slurry cultures and the most rapid degradation rate obtained at 26 °C was 8 mg L^{-1} d^{-1} [23]. Strain *Cupriavidus* sp. Y-d had much better sulfolane degrading ability than that in the literature. The sulfolane in the high concentration sulfolane groups (1500 and 2000 mg L^{-1}) kept decreasing

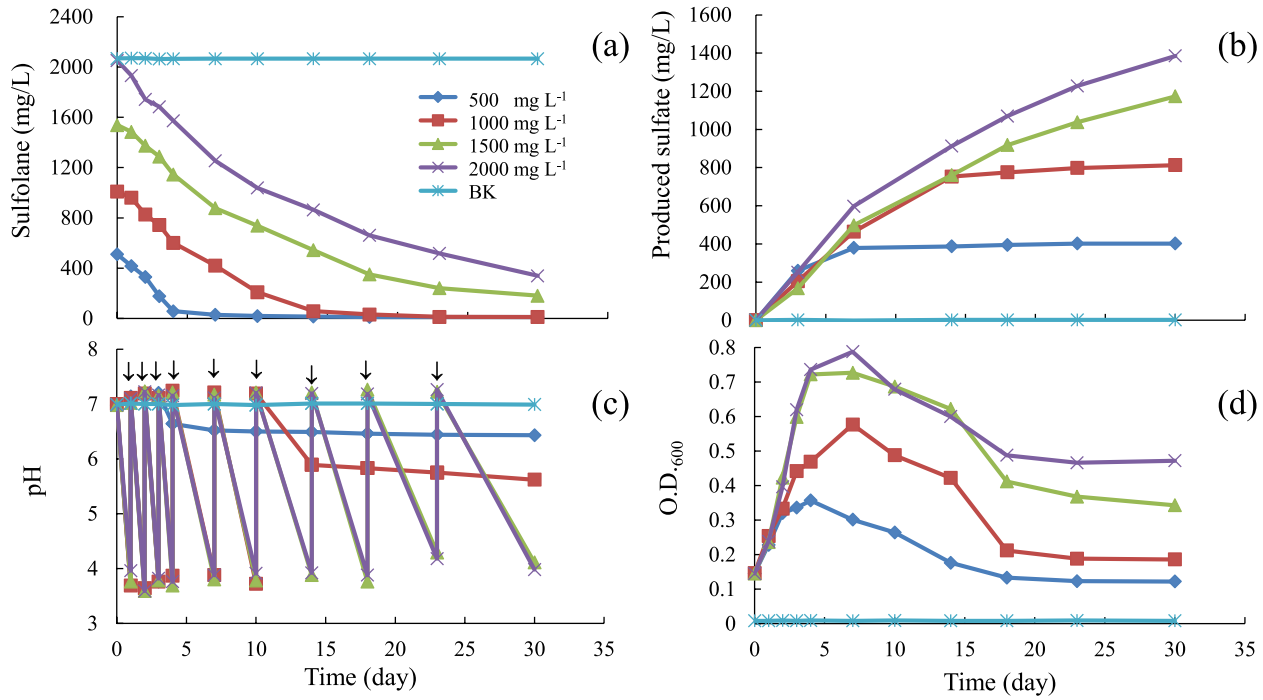

Fig. 3 Sulfolane (**a**), sulfate (**b**), pH (**c**) and $O.D._{600}$ (**d**) variations during different sulfolane concentrations biodegradation by strain *Cupriavidus* sp. Y-d at the pH of 7. Arrow indicates pH adjustment. BK refers to no added bacteria

and sulfate kept producing in the later stage of the experiment. If the experiment time was extended, sulfolane might be completely biodegraded. Sulfate release caused pH decline. Strain *Cupriavidus* sp. Y-d reached the maximum O.D.$_{600}$ value under different sulfolane concentrations in the 4th to 7th days of incubation, which was 0.36 for 500 mg L^{-1} sulfolane, 0.58 for 1000 mg L^{-1} sulfolane, 0.73 for 1500 mg L^{-1} sulfolane and 0.79 for 2000 mg L^{-1} sulfolane.

3.5 Effect of sulfate concentrations on sulfolane biodegradation

Since the petrochemical plant wastewater contains high sulfate concentration, it is necessary to realize the effect of sulfate concentration on sulfolane biodegradation. The experiment explored the sulfolane biodegradation in the presence of various sulfate concentrations. Figure 4 shows the variations in sulfolane, sulfate, pH and O.D.$_{600}$ when strain *Cupriavidus* sp. Y-d was incubated under different sulfate concentrations. Increasing the sulfate concentration was not conducive to sulfolane removal by strain *Cupriavidus* sp. Y-d, especially when the sulfate concentration was higher than 3500 mg L^{-1}. The removed sulfolane concentrations were 941, 918, 828 and 739 mg L^{-1} and the sulfolane removal efficiencies were 94, 92, 83 and 74%, when the sulfate concentrations were 2511, 3020, 3534, 4020 mg L^{-1}, respectively. The sulfolane biodegradation accompanied sulfate release. The produced sulfate concentration was proportional to the degraded sulfolane concentration. The produced sulfate concentrations were 779, 745, 689 and 601 mg L^{-1} in the presence of sulfate from low to high concentrations. Calculating the mass balance according to stoichiometry, the sulfolane mineralization efficiencies were 100, 100, 100 and 95% in the presence of sulfate from low to high concentrations. This implied that high sulfate concentration suppressed sulfolane mineralization. The pH value decreased with the sulfate release, and the degree of pH fluctuation was proportional to the produced sulfate concentration. The bacterial growth rate significantly declined with the increase in sulfate concentration. The O.D.$_{600}$ value reached the maximum value at 0.69 for 2511 mg L^{-1} sulfate, 0.58 for 3020 mg L^{-1} sulfate, 0.49 for 3534 mg L^{-1} sulfate after 7 to 10 days incubation. The maximum O.D.$_{600}$ was 0.34 in the presence of 4020 mg L^{-1} sulfate. This indicated that the growth of strain *Cupriavidus* sp. Y-d was inhibited by high sulfate concentration. Xue et al. explored the role of sulfate in aerobic granular sludge process to treat emerging sulfate-laden wastewater. When the sulfate concentration was higher than 200 mg L^{-1}, phosphate removal was inhibited and phosphate removal deterioration was observed in the presence of 1000 mg L^{-1} sulfate [24]. This indicated that high sulfate concentration negatively impacted biological wastewater treatment efficiency, and this negative influence was also observed in this study.

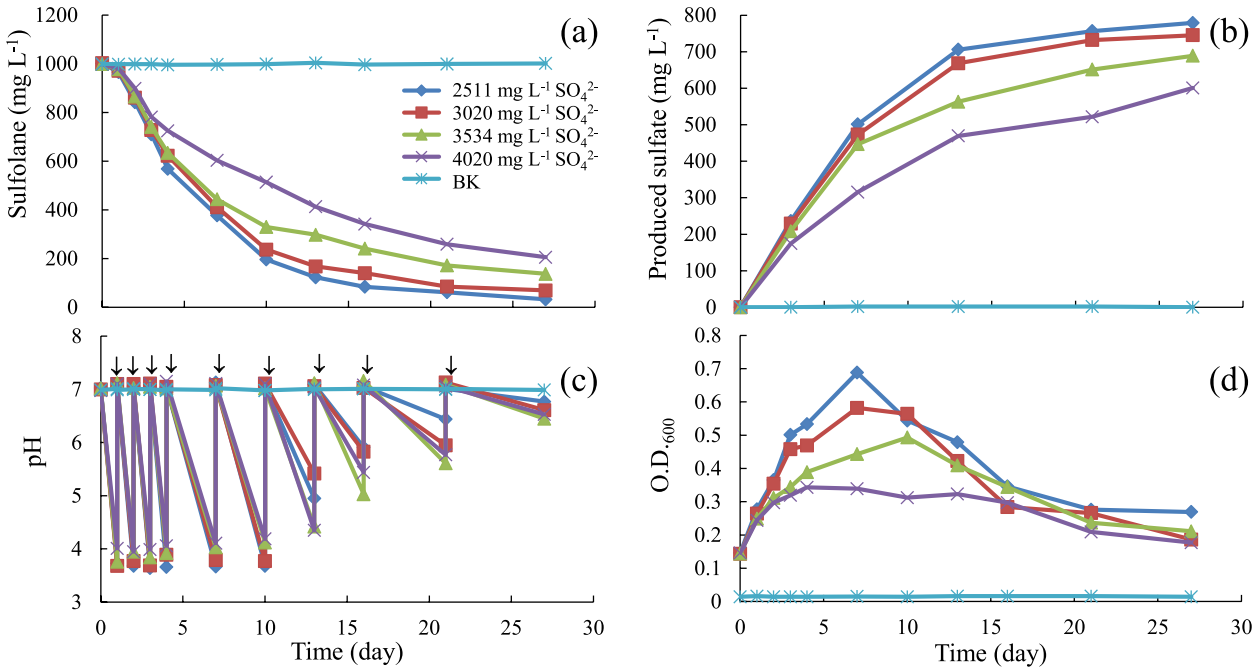

Fig. 4 Sulfolane (**a**), sulfate (**b**), pH (**c**) and O.D.$_{600}$ (**d**) variations during 1000 mg L^{-1} sulfolane biodegradation by strain *Cupriavidus* sp. Y-d in the presence of different sulfate concentrations. Arrow indicates pH adjustment. BK refers to no added bacteria

3.6 Implication

Enrichment is a technique to enhance the density of target microorganisms and an essential step for microbial isolation [25]. After enrichment, strain *Cupriavidus* sp. Y-d capable of degrading sulfolane was isolated from the enriched activated sludge obtained from the petrochemical plant. This strain is a novel sulfolane degrading bacterium and different from previous bacterial isolates *Shinella yambaruensis* MS4T [13], *Variovorax* sp. WP1 [14], *Pseudomonas maltophilia* [26] and *Pseudomonas* sp. [27].

pH is a crucial factor influencing bacterial activity and sulfolane biodegradation. The petroleum wastewater pH value was between 6.5–9.5 [28]. Strain *Cupriavidus* sp. Y-d was capable of degrading sulfolane at the pH between 7–9 and achieved the 91–95% sulfolane removal efficiency. This means the novel strain *Cupriavidus* sp. Y-d has potential to treat petroleum wastewater using bioaugmentation. The sulfolane degrading rates of strain *Cupriavidus* sp. Y-d at the pH of 6, 7, 8 and 9 were 18, 93, 94 and 66 mg L^{-1} d^{-1}, respectively. The released sulfate concentration met the theoretical stoichiometric concentration, which meant that the strain *Cupriavidus* sp. Y-d could mineralize sulfolane.

The petrochemical wastewater is characterized by high organic pollutants and sulfate concentration [29]. The petrochemical wastewater analysis result indicated that the sodium sulfate concentration was not more than 2% (w/v) [30]. In our research, high sulfate concentration resulted in a negative effect on sulfolane biodegradation. However, sulfolane removal efficiency maintained higher than 90% when the sulfate concentration was lower than 3000 mg L^{-1}. To link bacterial growth and substrate utilization, specific growth, and specific substrate utilization rates of each batch experiment were calculated for further discussion. Table 2 presents the specific growth, and specific substrate utilization rates of strain *Cupriavidus* sp. Y-d in the presence of various sulfolane and sulfate concentrations at different pHs. The specific growth rate (0.07 d^{-1}) and specific substrate utilization rate (0.16 d^{-1}) at the pH of 6 were much lower than those at pH 7, 8, and 9. The specific growth rates were 0.23, 0.32 and 0.25 d^{-1} at the pH of 7, 8, and 9, respectively. The specific substrate utilization rates were 0.43, 0.51 and 0.33 d^{-1} in the order of the pH of 7 to 9. The specific substrate utilization rates were 0.48, 0.49, 0.54 and 0.59 d^{-1} in order of low to high sulfolane concentration. The specific growth rate increased with the increase in sulfolane concentration. However, when the sulfolane concentration was higher than 1500 mg L^{-1}, the increase in specific growth rate was not significant. This implied that sulfolane concentrations higher than 1500 mg L^{-1} might affect cell growth. The specific growth rates were 0.23, 0.20, 0.14 and 0.11 d^{-1} and the specific substrate utilization rates were 0.45, 0.40, 0.34 and 0.33 d^{-1} in the presence of 2511, 3020, 3534, 4020 mg L^{-1} sulfate, respectively. Results showed that the increase in sulfate would lead to the decrease in specific growth rate and specific substrate utilization rate. This indicated that high sulfate concentration would inhibit the bacterial growth since the bacteria could not adapt to the permeated pressure from high sulfate. For strain *Cupriavidus* sp. Y-d bioaugmentation, dilution is needed when the petrochemical wastewater contains sulfate concentrations higher than 3020 mg L^{-1}.

Table 2 The specific growth and specific substrate utilization rates of strain *Cupriavidus* sp. Y-d in the presence of various sulfolane and sulfate concentrations at the different pHs

Sulfolane (mg L^{-1})	pH	Sulfate[a] (mg L^{-1})	Specific growth rate (d^{-1})	Specific substrate utilization rate (d^{-1})
1000	6	0	0.07	0.16
1000	7	0	0.23	0.43
1000	8	0	0.32	0.51
1000	9	0	0.25	0.33
500	7	0	0.19	0.48
1000	7	0	0.28	0.49
1500	7	0	0.47	0.54
2000	7	0	0.48	0.59
1000	7	2511	0.23	0.45
1000	7	3020	0.20	0.40
1000	7	3534	0.14	0.34
1000	7	4020	0.11	0.33

[a] Sulfate in the MSM was subtracted at the beginning

4 Conclusions

Novel strain *Cupriavidus* sp. Y-d capable of degrading sulfolane was isolated from the activated sludge from a petrochemical plant after enrichment. Batch experiments were performed to investigate the effects of pHs, sulfolane and sulfate concentrations on sulfolane biodegradation. Sulfolane was biodegraded at the pH of 6–9, and the optimal pH for sulfolane biodegradation was 8. Strain *Cupriavidus* sp. Y-d completely biodegraded sulfolane concentration lower than 2000 mg L^{-1} and the sulfate was released. The difference between the theoretical and produced sulfate concentrations was less than 2%. Sulfolane removal efficiency maintained higher than 90% when the sulfate concentration was lower than 3000 mg L^{-1}.

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Authors' contributions
C. C. Wang served as a consultant on research concepts and methodology. C. M. Liang wrote the manuscript. Y. H. Liu performed the experiments and analyzed the data. C. F. Yang conceived and designed the experimental approach and wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare they have no competing interests.

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