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Aerobic biodegradation of odorous dimethyl disulfide in aqueous medium by isolated *Bacillus cereus* GIGAN2 and identification of transformation intermediates

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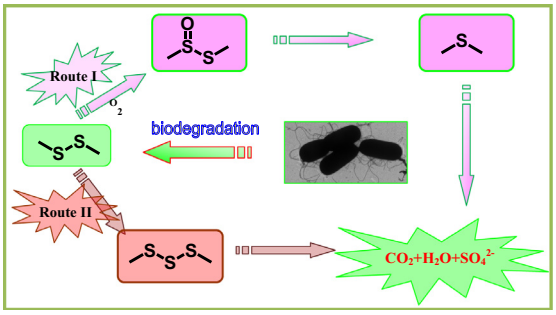
HIGHLIGHTS

- A strain capable of removal DMDS was successfully isolated and identified.
- Optimum conditions with the maximum DMDS biodegradation were obtained.
- Biodegradation kinetics of DMDS was found to follow first-order kinetics model.
- DMDS biodegradation mechanism was proposed based on three identified intermediates.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel, flagellated, rod-shape, Gram-positive facultative aerobe, was isolated and identified as *Bacillus cereus* GIGAN2. It can effectively remove model odorous organics dimethyl disulfide (DMDS) in aqueous solution under aerobic conditions. Initial concentration, pH value and temperature played important role in DMDS biodegradation, and up to 100% of 10 mg L^{-1} of DMDS could be removed within 96 h under the optimum conditions (30 °C, pH 7.0 and 200 rpm) with a maximum biodegradation rate constant of 0.0330 h^{-1} and minimum half-life of 21.0 h, respectively. Three main intermediates were identified using gas chromatography–mass spectrometry during this biodegradation process. Further, a reaction scheme is also proposed to explain the possible DMDS biodegradation mechanism by GIGAN2 based on the above-identified intermediates. Overall, this is the first report to demonstrate a newly isolated strain using high concentrated DMDS as the sole carbon and energy source with high efficiency.

1. Introduction

The odor emission of volatile organic sulfur compounds (VOSCs) like methyl mercaptan, ethanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), methyl phenyl sulfide, carbon disulfide, carbonyl sulfide as well as hydrogen sulfide (H_2S) has kept on growing in the significance and posed a great threat to the public health over recent decades (Gutarowska et al., 2014; Wan et al., 2010). This kind of sulfur-containing odorant frequently occurred during the manufacture, usage and disposal of a variety of materials from the waste and sewage treatment plants, agricultural and food industries, rendering plants, paper mills, oil petroleum refinery industry (Caceres et al., 2012), and so on. Due to extremely low

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nuisance odor threshold, high toxicity and potential corrosive effect, these VOSCs can injure eyes, respiratory tract, skin, liver, and kidney resulting in high psychological stress, insomnia and loss of appetite (Calderon et al., 2012), and even death after long-term exposure. For example, DMDS has the lowest threshold of 0.1 μg/m^3 (Rosenfeld et al., 2001) among all odorous compounds. Research showed that DMDS concentration higher than 25 and 125 ppm would result in a decrease in the body weight gain, food intake, aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen in the male and female rats, respectively (Kim et al., 2006). Therefore, considering the unpleasant odor and toxic nature of sulfur-containing compounds, the abatement of them from water and air has drawn increasing attention and attracted considerable interests (He et al., 2009).

Until now, various effective technologies for eliminating odorous waste gas in aqueous medium have been proposed. In most cases, however, some conventional methods like physical/chemical processes (scrubbing, incineration, adsorption and oxidation) are often found to be unsatisfied with the elimination capability to VOSCs, as well as have often been criticized for their high investment and operating costs, possible generation of hazardous secondary wastes and consumption of high level of reagents and energy (Aroca et al., 2007). Comparatively, biological technology is proved as one of the most effective, sustainable, as well as economical interesting methods for the odorous compounds abatement from the contaminant environment (Li et al., 2013) if proper operational conditions are maintained. So far, biological degradation of a number of sulfur-containing compounds in the water and atmosphere by a newly isolated strain or the microorganisms in active sludge or sediments have been widely reported. For instance, methanethiol in wastewater can be removed by methanogenic bacteria (Sipma et al., 2002). What's more, various microbial species, such as strain Au7 (Wang et al., 2011), *Hyphomicrobium* VS (De Bo et al., 2002) as well as *Bacillus sphaericus* (Giri and Pandey, 2013) were isolated to eliminate DMS from waste air environment. The isolated bacteria with the capability to degrade H_2S were also found to be very popularly investigated, for instance, *Bacillus* sp. TSO3 (Ryu et al., 2009) and *Pseudomonas putida* CH11 (Chung et al., 2005).

However, relatively little information is known about the bacteria which can eliminate DMDS in aqueous solution. Most of available works were mainly focused on the co-treatment of DMDS-containing mixture gas by microorganisms in bioreactors, such as *Lysinibacillus sphaericus* RG-1 (Wang et al., 2011), *Thiobacillus thioparus* (Caceres et al., 2012) and B350 microorganisms (Wang et al., 2011). Few studies concentrated on the isolation of a single strain which can use DMDS as the sole energy, except only one paper about the DMDS degradation by isolated *Pseudomonas fluorescens* strain 76 (Ito et al., 2007). Furthermore, very limited research was carried out about the reaction kinetics and biodegradation mechanisms of sulfur-containing exhaust gas by isolated bacterial strains (Caceres et al., 2012; Gadekar et al., 2006) to evaluate the feasibility and effectiveness of biological degradation technology. Up to now, no similar studies have focused on the biodegradation kinetics and mechanisms of DMDS in aqueous by an individual bacterial strain.

The aim of this study is to isolate and identify a new microorganism, which has the ability to decompose DMDS in aqueous medium, and to investigate its biodegradation behavior and mechanism in aqueous solution. The optimal parameters affecting its removal efficiency were disclosed to obtain the best degradation efficiency by this bacterium. To probe the biodegradation kinetics, the maximum removal rate (V_{max}) and the half saturation concentration (K_m) were also studied using a modified Michealis–Menten type kinetics equation. In addition, a tentative degradation pathway of DMDS by the isolated strain, was also proposed based on the identified intermediates using GC–MS.

2. Methods

2.1. Chemicals and basal medium

Dimethyl disulfide (DMDS, 99.5%, Tianjin, China) was used as the sole carbon source and energy source for the microorganism. Dimethyldisulfide, S-oxide (99%), dimethyl sulfide (99%) were obtained from J&K chemical Ltd. All other chemicals were of analytical grade and purchased from Guangzhou Chemical Reagent Co., Inc., China. In all experiments, the mineral medium (MM) for the isolation of DMDS-degrading microbe was autoclaved at 121 °C for 15 min containing (100 mL): $K_2HPO_4{\cdot}3H_2O$ 0.12 g, KH_2PO_4 0.12 g, NH_4Cl 0.04 g, $MgSO_4{\cdot}7H_2O$ 0.02 g and $FeSO_4{\cdot}7H_2O$ 0.02 g, and 100 μL trace element medium stock solution, which was prepared according to our previous work (Wan et al., 2010).

2.2. Bacterial isolation and identification

The DMDS-degrading microorganism was screened from the sludge of a river collected from Guangzhou city, Guangdong province, China, following the modified isolation procedure (Smith and Kelly, 1988). In general, one gram of wet sludge was added to 100 mL sterilized MM containing 1 mg L^{-1} DMDS in 300 mL flask, and incubated at 37 °C with 200 rpm. To obtain DMDS-acclimating bacteria, 10% (v/v) of the above culture was transferred into the MM with the addition of DMDS ranged from 5 to 20 mg L^{-1} every five day for a month. Then, pure strain was achieved on the solid agar medium with 1 mg L^{-1} DMDS by using spread-plate method with 200 μL of the resultant culture. Finally, a newly isolated strain was characterized by morphological and physiological observation as well as the biochemical identification. In addition, 16s rRNA nucleotide sequence was used to confirm the evolutionary relationship of the isolated bacterium with the help of the BLAST software. The universal primer pair 7 F (5′-CAGAGTTTGATCCTGGCT-3′) and 1540 R (5′-AGGAGGTGATCCAGCCGCA-3′) (Tan et al., 2012) were designed to amplify the 16S rRNA gene with the extracted genomic DNA of the strain as the template. The PCR products were subsequently separated and visualized in 1.0% agarose gel. The detailed reaction system and the conditions of PCR amplification experiments were provided in the Supporting information.

2.3. DMDS biodegradation

In the biodegradation experiments, the newly isolated strain was pre-cultured to late logarithmic growth phase in Luria–Bertani at 37 °C and 200 rpm of shaking for 18 h and collected by centrifuged at 8000*g* for 15 min if not specified. Also, there is no replication in cultures. After washing with MM twice, 20 mL of harvested cultures were added into a 300 mL of serum bottle with 100 mL of MM containing 5 mg L^{-1} DMDS to perform the DMDS biodegradation experiments under the conditions of 37 °C, pH 7.4 and 200 rpm. The serum bottles were sealed with a rubber stopper to avoid the volatilization of DMDS. To further investigate the DMDS biodegradation capability of this strain, the effect of three operating parameters, such as initial DMDS concentration, pH value and the temperature on the removal of DMDS was analyzed. For each batch experiment, one of the parameters was changed while the others keeping constant. At biodegradation intervals of 24, 36, 48, 72 and 96 h, 300 μL of the upper gas samples were collected from the bottles using a 500 μL gas-tight locking syringe (Agilent, Australia) for parameter analysis. All samples were measured in triplicate. Moreover, the removal efficiency (RE) of DMDS was calculated according to the following kinetic equation: $RE(\%) = (C_0 - C_t)/C_0 \times 100\%$, where C_0 and C_t are the initial and residual concentration of DMDS at the time indicated, respectively. In addition,

biodegradation rate constant (K) and half-life degradation time ($t_{1/2} = 0.693/K$) were measured using the algorithm $\ln(C_0/C_t) = Kt$ in according to the fittings of pseudo first-order kinetic model, where t is the decomposing time.

The gaseous DMDS was collected with the method of headspace gas chromatograph (Higgins et al., 2006), while the aquatic DMDS was extracted by dichloromethane, then injected into the gas chromatography (GC, HP 5890, series II), equipped with a HP-5 MS capillary column (0.25 mm × 0.25 μm × 30 m) and a flame ionization detector in splitless mode. The injector and detector temperature were defined at 200 °C and 250 °C, respectively. Besides, the temperature of the column was maintained at 80 °C for 2 min, then programmed to 150 °C with the rate of 10 °C/min.

2.4. Identification of metabolites

Metabolites produced during biodegradation process were detected both in the solution and gas phase analyzed using a GC (Agilent 7890A) – mass spectrometer detector (Agilent 5975C with Triple-Axis Detector) (GC-MSD) with a DB-5 capillary column (0.32 mm × 0.25 μm × 30 m). The samples in aqueous medium were extracted with dichloromethane three times, then concentrated to 1 mL by a rotary evaporator, and dried completely with gentle flow of ultra-high purity nitrogen after transferring into a 1.5 mL vial. Finally, metabolites were re-dissolved in 0.1 mL hexane and detected using GC–MS. However, unlike metabolites in aqueous medium, the intermediates in gas could be directly detected using GC–MS with 500 μL of gas sample. The analysis method and programmed procedure were as follows: the column was held at 40 °C for 2 min, then increased to 150 °C with a rate of 5 °C min^{-1} and to 250 °C with 10 °C min^{-1}. The temperature of ion source was set at 230 °C, and the MSD was functioned in full scan mode with m/z 30 to 500 amu. With the help of Wiley database, the metabolites were identified via mass spectrum. The concentration of the final metabolite, sulfate, in the aqueous medium was determined every 24 h using standard baryta yellow spectrophotometric method.

3. Results and discussion

3.1. Isolation and identification of DMDS-degrading bacterium

After one month acclimatization, a pure colony with the capability of degrading DMDS was isolated using DMDS as the sole carbon and energy source. The scanning electron microscope was used for the morphological observation, and a rod-shaped strain with flagellum can be found (Fig. S1). Furthermore, according to the physiological and biochemical characterization (Table 1), this strain is a Gram-positive facultative aerobes and the surface colonies on solid agar plate are creamy, circle in shape, smooth on the surface, trim on the edge with a diameter of 2–3 mm on agar plate. In addition, sugar utilization tests exhibited positive except for arabinose, xylose and mannitol.

To further confirm the phylogeny relationships of the newly isolated strain, the 16sRNA was amplified by polymerase chain reaction (PCR) using genomic DNA, which was extracted by bacterial genomic DNA isolation kit (Sangon Biotech Co., Ltd., Shanghai) based on the manufacturer's instructions. The results of gel electrophoresis of PCR product from strain GIGAN2 in Fig. 1 indicated that a 1396 bp nucleotide gene (GenBank accession number: KM284695) was obtained and used to construct a phylogenetic tree by neighbor-joining method after alignment with other relevant gene sequences derived from the GenBank database of National Center for Biotechnology Information (NCBI) using ClustalW. As Fig. 2 shows, strain GIGAN2 belongs to *Bacillus cereus* of genus *Bacillus*,

Table 1
The biochemical characteristics of *Bacillus cereus* GIGAN2.

Biochemical and culture conditions	Results
Gram-staining	+
Gelatin liquefaction	+
Catalase	+
Oxidase	–
Aerobic growth	+
Anaerobic growth	+
Hydrolysis of starch	+
V-P test	+
Growth at 40 °C	+
Growth at 50 °C	–
Nitrate reduction	+
Citrate utilization	+
Glucose utilization	+
Arabinose utilization	–
Mannitol utilization	–
Xylose utilization	–

+ positive reaction, – negative reaction.

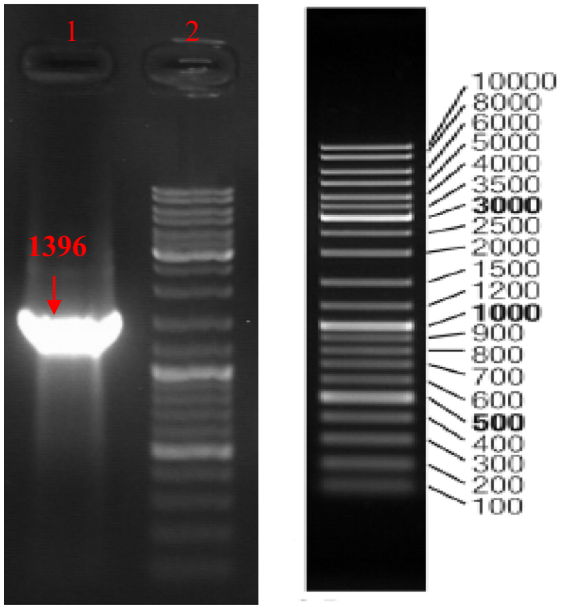


Fig. 1. The 16 sRNA agarose gel electrophoresis image of *Bacillus cereus* GIGAN2. Line 1: 16 sRNA of *Bacillus cereus* GIGAN2, line 2: molecular weight Marker.

which has the highest similarity (98%) with *Bacillus cereus* strain ML267 (KC692161). Besides, it is also much closer to *Bacillus* sp. A57 (JX010962) with a homology of 96%. Thus, based on the phenotypic characteristics and genotypic investigation above, the newly isolated microorganism could be grouped as a member of genus *Bacillus* and was named as *Bacillus cereus* GIGAN2.

3.2. Biodegradation kinetics of DMDS

In the present work, DMDS was provided as the sole carbon and energy source for the *Bacillus cereus* GIGAN2. Therefore, the effect of three parameters on the biodegradation performance including initial DMDS concentration, temperature and pH value, which were related to DMDS treatment performance, were evaluated in detail at a fixed rotational speed of 200 rpm. Fig. 3a shows that, with the initial DMDS concentrations increased from 5 to 25 mg L^{-1}, the removal efficiencies of DMDS declined steadily from 100% to 62.3% after 4 days treatment at 30 °C, pH 7.0. It also can be found that the biodegradation efficiencies initially increase rapidly

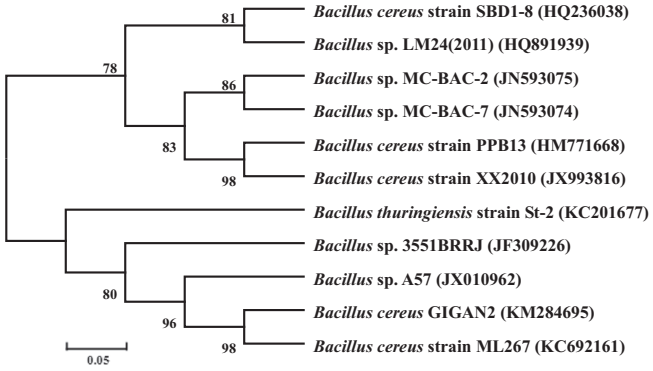


Fig. 2. Phylogenetic tree for the strain of *Bacillus cereus* GIGAN2 based on 16S rRNA nucleotide sequence with other reference gene sequences by the neighbor-joining method on the program MEGA5.2. The numbers shown next to the nodes indicated the bootstrap values of 1000 for the confidence level.

during first 2 days and then level off gradually. Furthermore, the relationship between the DMDS removal efficiencies by *Bacillus cereus* GIGAN2 and the reaction temperature was also investigated (Fig. 3b). At the temperatures of 20, 25, 30, 35 and 40 °C, the degradation efficiencies of 10 mg L^{-1} DMDS by the newly isolated strain were obtained as 73.2, 86.6, 100, 92.8 and 79.2%, respectively, after 96 h treatment at pH 7.0. This demonstrated that either too high or too low temperature would inhibit the microorganism growth and then the biodegradation activity. From the above information, it can be found that much higher abatement activity could be maintained with the temperatures ranging from 25 to 35 °C, and optimum temperature was 30 °C under the reaction conditions of pH 7.0, 200 rpm and 10 mg L^{-1} DMDS. This result was in good agreement with other previous work that the unsatisfied removal capacity was attained at lower and higher temperature because of the temperature sensitivity to microorganism (Moghanloo et al., 2010). In addition, the effect of pH value on the biodegradation ability of the strain was also conducted. Seen from Fig. 3c, it can be found that, after 96 h treatment at 30 °C, the total removal efficiencies of 10 mg L^{-1} DMDS by the strain increased dramatically from 79.9% to 100% with the pH value increase from 5.0 to 8.0, then decreased to 83.3% at pH = 9.0. This is due to that appropriate pH value may enable higher activity of enzyme associated with DMDS degradation and subsequently result in higher biodegradation efficiency. This result agreed with the viewpoints that the strain with the ability of biodegradation sulfur-containing contaminants did not function very well in overly basic or acidic conditions, and optimum pH value was achieved at 7.0 as previous report (Hwang et al., 2007; Ramírez et al., 2009).

To obtain full insight into the effect of different variables on the removal efficiency of DMDS by *Bacillus cereus* GIGAN2, the kinetic equations were used to model the biodegradation rate constant and half-life of DMDS in solution. As Table S2 shows, the biodegradation of DMDS follows pseudo-first order kinetic reaction and it is in accordance with the Langmuir – Hinshelwood (L-H) mode. What's more, it also revealed that higher rate constant and lower half-life are beneficial to the removal of DMDS. For example, with the DMDS concentrations rose steadily from 5 to 25 mg L^{-1}, the biodegradation rate constants (K) dropped quickly from 0.0648 to 0.0101 h^{-1}. However, the half-life exhibited the opposite changing tendency with an apparent increase from 10.6 to 68.6 h because the high concentration of contaminant would suppress the enzyme of the bacteria (Cox et al., 2013; Pol et al., 2007).

Similarly, with the pH values increased from 5.0 to 7.0 then to 9.0, the rate constants increased from 0.0165 to 0.033 h^{-1} and finally dropped to 0.0185 h^{-1}. Besides, such a changing trend can also be found for the effect of temperature on the rate constant. That is, the rate constants rose steadily from 0.0133 to 0.033 h^{-1} when the temperatures increased from 20 to 30 °C, and then fall sharply to 0.0154 h^{-1} at 40 °C. Nevertheless, there is an opposite trend for the half-life, which drop steeply from 42.0 to 21.0 h as the pH value jumped from 5.0 to 7.0, and then went up to 37.4 h with further rose pH to 9.0. Moreover, the table also reveals that, the half-lives obtained as 52.1, 35.9, 21.0, 26.6 and 45.0 h when the temperatures were set at 20, 25, 30, 35 and 40 °C. Thus, it can be concluded that pH 7.0 and 30 °C were the optimum conditions for the biodegradation of DMDS by the isolated strain, which agreed well with our experimental results described above. With the evidence that the supreme biodegradation rate constant of 0.033 h^{-1} and the minimum half-life of 21.0 h were achieved at pH 7.0 and 30 °C, it can be found that too lower and higher temperature, or too acidic and alkaline environment would lead to lower biodegradation rate constant and longer half-life.

3.3. Identification of the biodegradation intermediates

To fully understand the DMDS environmental fate in aqueous medium by newly-isolated bacterium under aerobic condition, the possible biodegradation pathways and metabolic mechanism were elucidated based on the GC–MS analysis results. The chemical

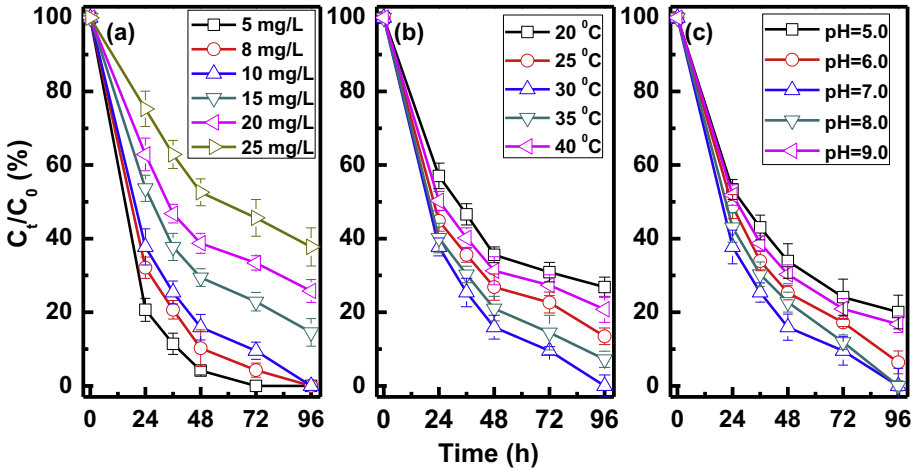
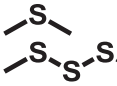


Fig. 3. The effect of (a) initial concentration of DMDS, (b) temperature, and (c) pH value on the DMDS biodegradation efficiencies in batch culture. The error bar value stands for the standard deviation of triplicates.

Table 2
Retention time, mass spectra and structure of DMDS as well as identified metabolites by GC-MSD analysis.

Intermediates		Retention time (min)	Chemical structure	*m/z* of observed fragment ions
A	Dimethyl disulfide	4.015		94* [M^+], 79 [M^+-CH_3],
B	Dimethyldisulfide, S-oxide	6.043		110* [M^+], 95 [M^+-CH_3],
C	Dimethyl sulfide	1.487		62* [M^+],
D	Dimethyl trisulfide	9.542		126* [M^+], 79 [M^+-CH_3S],

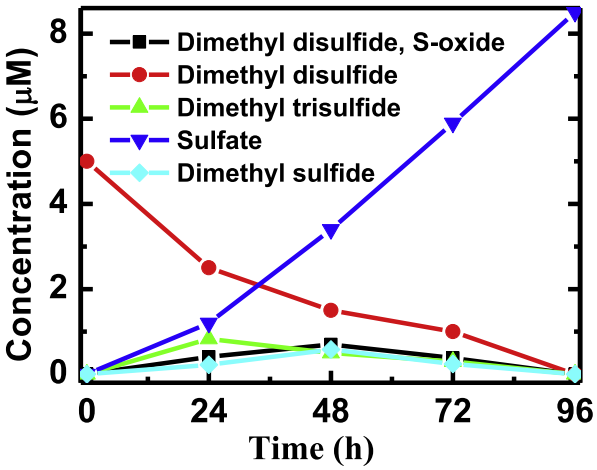


Fig. 4. Evolution curves of DMDS biodegradation intermediates identified by GC-MSD analysis and sulfate concentration versus time.

structure and retention time of the three intermediates as well as DMDS are listed in Table 2. Results showed that a peak with retention time of 4.015 min was observed for substrate without biodegradation, and with the decrease of initial concentration of DMDS, the peaks of other three intermediates [M^+] at *m/z* 110, *m/z* 62 and *m/z* 126 with the retention time of 6.043, 1.487 and 9.542 min could be detected (Fig. S2). After validating with the authentic standards, these three intermediates were identified as dimethyldisulfide, S-oxide, dimethyl sulfide and dimethyl trisulfide at the biodegradation time of 24 h, respectively (Figs. S2 and S3). As seen from the evolution curves of Fig. 4, during the first 36 h of DMDS biodegradation, the intermediate dimethyl trisulfide accounted for a large proportion, then the concentration of dimethyldisulfide, S-oxide and dimethyl sulfide rose slightly with the further decline of the concentration of DMDS. Finally, all the intermediates would be completely mineralized by strain GIGAN2. Further, the mass balance from the accumulation of sulfate during the biodegradation process were conducted quantitatively. As operation time increasing from 24 to 48 h, the accumulated amount of sulfate slightly increased from 1.2 to 3.4 μM, then swiftly rose to 5.9 μM at the reaction time of 72 h, and finally up to 8.5 μM. This indicated that sulfate was the main final metabolite, and desulfurization may be the primary biodegradation route.

According to the identified intermediates, a feasible biodegradation mechanism was worth tentatively discussing. As seen from the Scheme S1, two routes could be proposed. In route I, in the presence of oxygen, the S–S bond of DMDS was first attacked by oxygen and cleaved to form CH_3SSOCH_3 (dimethyldisulfide, S-oxide) with the action of enzyme of *Bacillus cereus* GIGAN2, which was agree well with other previous studies (Jee and Tao, 2006; Jensen et al., 1992; Yin et al., 1990). Then, followed by the CH_3SO radical substitution by methyl group of DMDS, the above intermediate could be converted to DMS. It should be pointed out that dimethyl sulphoxide served as an electron donor (DMSO) might then be produced by the oxidation of dimethyl sulfide, although DMSO had not been detected in the GC–MS due to its low concentration and high polar and solubility in water. However, this step was also achieved and validated in other previous reports (McDevitt et al., 2002; Zhang et al., 1991). Otherwise, in route II, dimethyl trisulfide was detected, which might be derived from intermolecular interaction of DMDS. Similar route could also be found in some previous studies (Chin and Lindsay, 1994; van den Bosch et al., 2009). Meanwhile, according to the kinetics results, both routes can lead to the mineralization of DMDS into CO_2, H_2O and sulfate after a series of degradation steps (Wei et al., 2013).

4. Conclusions

An acclimated single species, *Bacillus cereus* GIGAN2, with capability to eliminate DMDS, was isolated and identified for the first time. DMDS could be successfully degraded by this newly isolated strain within 96 h under the optimum conditions. The biodegradation kinetics of DMDS followed the pseudo first-order kinetics model with the highest biodegradation rate constant of 0.330 h^{-1} and the half-life of 21.0 h, respectively. Main by-products were identified as dimethyl trisulfide and dimethyldisulfide, S-oxide after validating by authentic standards. All results demonstrated that this newly-isolated strain was of great importance in the DMDS elimination in aqueous solution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.11.002.

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