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Biodegradation of Unsymmetrical Dimethylhydrazine in Solution and Soil by Bacteria Isolated from Activated Sludge

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Abstract: The biodegradation effect and pathway of unsymmetrical dimethylhydrazine (UDMH), which is a major rocket propellant with highly toxic properties, with two strains isolated from the acclimated activated sludge were investigated in solution and in soil. The results demonstrated that *Stenotrophomonas* sp. M12 (M12) was able to degrade UDMH of 50 mg· L^{-1} as the sole carbon source in aqueous mineral salt medium (MSM), but could not degrade UDMH in soil. *Comamonas* sp. P4 (P4) barely degraded UDMH of 50 mg· L^{-1} as the sole carbon source in aqueous MSM, but the degrading capacity of P4 could be improved by the addition of an extra carbon source. Meanwhile, P4 was able to degrade UDMH of 100–600 mg· kg^{-1} in the soil. The degradation of UDMH in the soil was influenced by organic matter, autochthonous microorganisms, and metal ions. UDMH could inhibit metabolism of M12 and P4, and the inhibition influence was more severe in aqueous MSM than in soil. Oxygen content was important for M12 biodegrading UDMH, and co-metabolism helped P4 to self-detoxify and self-recover. The main intermediates of UDMH were identified by Gas Chromatography-Mass Spectrometer (GC/MS) qualitative analysis, and the concentrations of UDMH and its important transformation products were determined in solution and soil. According to the determination results, the synchronous degradation theory was proposed, and the degradation pathway was discussed.

Keywords: unsymmetrical dimethylhydrazine (UDMH); biodegradation; soil; solution

1. Introduction

Unsymmetrical dimethylhydrazine (1,1-Dimethylhydrazine, UDMH) is an important liquid propellant for space rockets and missiles. At present, countries such as Russia, India, and China still use UDMH, which is a relatively inexpensive fuel also named "heptyl", as a heavy cargo carrier rockets propellant [1]. UDMH is proven to be an highly toxic, carcinogenic, and mutagenic substance [2] that could induce lung and liver tumors [3,4], and digestive system, skin, and mucous membranes could also be damaged at the same time [5]. Several governments have developed comprehensive regulations applied to the hazards of this compound from production, transport, and storage to disposal. In the USA, UDMH was classified as a Group 2B carcinogenic substance [6]. In Russia, the maximum allowable concentration is 0.02 mg· L^{-1} in water and 0.1 mg· kg^{-1} in soil [7,8]. In China, UDMH was listed in the highly toxic chemical index in 2003, and the discharge standard (GB14734) of UDMH in water was set to control its environmental toxicity early in 1993 [9]. Although UDMH has

many flaws, it is still likely to stay in use for the next 25–50 years because it has unique physico-chemical characteristics such as high energy and specific impulse [8].

Because of the water solubility and widespread use of UDMH, the opportunity for spills during storage or transportation creates a potential for environmental contamination [10]. On 1 February 1988, in a railway cargo train near the city of Yaroslavl, three tank cars containing UDMH left the tracks and approximately 740 L was spilled from one overturned tank [7]. In Kazakhstan, the fall of the first stages of rocket-carriers launched from the Baikonur Cosmodrome is accompanied by the spill of 0.6 to 4 tons of unburned propellant, of which 10–30 kg reach the ground and are subsequently spread into the soil and water [11]. It is estimated that the negative influence of space activities influences thousands of square kilometers (more than 7,700,000 km^2) with a fragile and unique ecosystem [12,13]. According to studies of soil samples from fall regions of rockets, UDMH and its transformation products can exist for 30 years after landing [14].

Nowadays, some new treatment technology appear, including oxidation over heterogeneous catalysts [15], catalytic oxidation with dioxygen and hydrogen peroxide over Cu- and Fe-containing catalysts [16], catalytic detoxification in heterogeneous Fenton system [17], reduction with Raney nickel [18], photocatalytic oxidation on TiO_2 [19], oxidation in a microstructured catalytic reactor [20], and so on. They are mainly the target of wastewater treatment, and these methods cannot be used for the environmental remediation of the UDMH spills. Currently, the popular method for the spill of UDMH was using hypochlorite [21]. Information about the bioremediation of UDMH is limited. Although the U.S. Air Force had performed correlative research 30 years ago, little success was achieved in the area of providing a tolerant microbe and revealing the biological mechanisms. Until now, few biotechnologies had been used to dispose of UDMH fuel. Actually, bioremediation, which involves the capabilities of microorganisms in the removal of pollutants, is the most promising, relatively efficient, and cost-effective technology, and is widely used in the soil and water remediation of toxic compounds [22]. Thus it is essential to find out whether biotechnology is able to reverse the environmental contamination of UDMH.

The purpose of this study is to isolate the bacterial strains capable of degrading UDMH and characterize their degradative potential in water and soil. The transformation products of UDMH and the biodegradation pathway were also discussed. This study attempted to provide a fundamental practical way for the bioremediation of soil and water contaminated by UDMH spills at ambient temperature.

2. Materials and Methods

2.1. Chemicals

UDMH (98%, purity) was provided by the Chinese Astronautic Liquid Propellant Research Center, Beijing, China. *N*-nitrosodimethylamine (NDMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were analytical grade and purchased from Beijing Jingwen Chemical Company (Beijing, China).

2.2. Enrichment Procedure and Isolation of Microorganisms

The active sludge was collected from Beijing Qinghe Wastewater Treatment Plant (Beijing, China). After three days' aeration, the nutrient medium contained glucose of 1.0 g· L^{-1}, NaCl of 1.0 g· L^{-1}, K_2HPO_4 of 1.0 g· L^{-1}, $MgSO_4$ of 0.2 g· L^{-1}, $CaCl_2$ of 0.01 g· L^{-1}, initial UDMH of 30 mg· L^{-1} was used for the enrichment procedure. The final pH value was adjusted to 7.2 with 1 mol· L^{-1} of NaOH or 1 mol· L^{-1} of HCl. After being stirred for 23 h, the whole solution stood for 1 h, 50% supernatant (v/v) was spilled, and the new nutrient solution containing UDMH was added. The experiment was kept running for a period of 7 days and then the concentration of UDMH was increased to 10 mg· L^{-1}. Until the concentration of UDMH was 50 mg· L^{-1} and the degradation percentage of UDMH was stable, the activated sludge was continuously cultivated. Some glass beads with a diameter of 3–5 mm were

added into the sludge sample (20 mL) in a 150 mL Erlenmeyer flask at 32 °C with shaking for 30 min (120 r· min^{-1}). The suspension (1 mL) was separated for 5 min in the speed of 8000 r· min^{-1}, and the supernatant was discarded. Then the final culture was serially diluted and streaked on nutrient agar containing a high UDMH concentration of 50 mg· L^{-1} or a low UDMH concentration of 5 mg· L^{-1}. A single colony was picked and re-streaked for purification three times until each colony exhibited the same morphology on the plates, such as shape, size, color, margin, surface, *etc.* Then the degrading capacity of UDMH was examined.

2.3. Strain Identification

The screened bacteria were characterized by the 16S rDNA sequence analysis [23]. For this purpose, DNA was extracted from the strain collected at the late exponential stage of growth using Genomic DNA Isolation Kit (SBS Genetech Co., Beijing, China). The 16S rDNA genes were amplified using the universal primer pair 27f and 1492r, obtained from Mymbio Co., Beijing, China [24]. PCR amplification was performed in a 50 µL reaction mixture containing 5 µL of 10× PCR buffer, 2.5 mmol· L^{-1} concentration for deoxynucleotide triphosphate, 2 U of rTaq DNA polymerase, and template DNA (2 µL). Amplification was conducted by using a PTC-220 Thermal Cycler PCR (MJ Research Inc, St. Bruno, QC, Canada) under the following conditions: (i) an initial denaturation step of 94 °C for 5 min; (ii) 30 cycles of denaturation, annealing, and extension (94 °C for 30 s followed by 55 °C for 30 s, with an extension step at 72 °C for 45 s); (iii) a final extension at 72 °C for 10 min. The PCR products were purified using the DNA Gel Extraction Kit (OMEGA bio-tek, Norcross, GA, USA) before the amplicons were sequenced. The partial 16S rDNA sequences were compared by the Blast National Center for Biotechnology Information (NCBI) search analysis. Then, the identification to the species level was determined by the 16S rDNA sequence similarity with that of the prototype strain sequence in the GenBank.

2.4. Inoculum Preparation

All the media were autoclaved under the conditions of 121 °C and 0.15 MPa for 20 min and cooled to the range of 40–50 °C. The mineral salt medium (MSM) contained 1.0 g of Na_2HPO_4, 0.8 g of KH_2PO_4, 0.02 g of $CaCl_2$, and 0.1 g of $MgSO_4$ per liter of deionized water. The broth medium contained 10 g of Tryptone, 3.5 g beef extract powder, 0.5 g NaCl per liter of deionized water. The final pH value of the media was adjusted to 7.2.

M12 and P4 were enriched in MSM and the broth medium by shaking in a 100-mL Erlenmeyer flasks at 180 rpm and 28 °C. The optical density of the bacterial biomass was measured at 600 nm with a Varian cary50 spectrophotometer (Varian, Palo Alto, CA, USA). When the OD600 was 2.3 for M12 after about 20 h and 2.0 for P4 after about 16 h, respectively, the strains had been incubated. This was called the incubated bacterium solution.

The incubated bacterium solution of a certain volume was centrifuged to harvest at 8000 r· min^{-1} for 5 min at 4 °C. After discarding the supernatant, the separated cells were washed twice with a potassium phosphate buffer (pH 7.2). Then the solution was diluted to the initial volume with potassium phosphate buffer. This was called the pure bacterium solution.

2.5. Studies on Unsymmetrical Dimethylhydrazine (UDMH) Degradation in Mineral Salt Medium (MSM)

Degradation studies were performed in 250 mL Erlenmeyer flasks containing 100 mL of sterile MSM with 50 mg· L^{-1} of UDMH as the sole carbon source. The pure bacterium solution of M12 and P4 strain was inoculated into MSM in the amount of 1% (v/v). Flasks were shaken on a rotary shaker (140 rpm) in a darkened thermostatic chamber maintained at 30 ± 2 °C for 72 h. In order to study the degradation of UDMH under abiotic conditions, a sample without bacteria was kept as control.

One hundred milliliters of sterile MSM added with 1% (v/v) of the incubated bacterium solution and 50 mg· L^{-1} of UDMH was used to study the effect of an extra carbon source on the degradation of M12 and P4 strain.

2.6. Studies on UDMH Degradation in Soil

The investigated soil was collected in the Beijing Olympic Park, China, 5–10 cm below the ground surface. This natural soil was "clean", and then was contaminated in the laboratory. Before being used in subsequent experiments, the soil samples were passed through a 2-mm sieve, stirred well, and air dried. Detailed physico-chemical properties of the soil are listed in Table 1. The degradation experiment with isolated bacteria was performed in sterile soil (SS) and non-sterile soil (*n*SS). The SS were autoclaved for 1 h at 121 °C and 0.15 MPa to remove the native soil organisms. The non-organic matter (*n*OM) soil was SS with the organic matter removed. It was an SS sample with 30% H_2O_2 added in the ratio of 1:10 (soil:water), and evaporated to dryness in a 85 °C water bath. Then 1 mol·L^{-1} ammonium acetate was added in the ratio of 1:10 (soil:water) and shaken for 2 h. After it was centrifuged, the supernatant was discarded. It was washed twice with deionized water, dried overnight in an 80 °C oven, and refined into powder [25].

Table 1. Physical and chemical properties of soil used in this experiment.

Parameter	Values
Sand (50–2000 μm) (%)	48.83
Silk (2–50 μm) (%)	35.47
Clay (<2 μm) (%)	15.70
Density (g·cm^{-3})	1.55
pH (in water)	7.63
Water-holding capacity WHC (%)	23.04
Organic carbon (%)	1.26
Total nitrogen content (%)	0.11

Because of the low chemical stability of UDMH, a reliable determination of the spiked concentration in the soil could not be obtained [26]. UDMH may react with oxygen in the atmosphere, organic matter, and transition metal ions. In particular, UDMH may be tightly bound with the soil solid, which was a form including the UDMH adsorbed by the mineral phase and chemically adsorbed by the soil's organic matter [27]. In order to get a relatively stable UDMH concentration, the added solution made a water seal by saturating the soil samples. A 10 mL solution containing 1000 μg UDMH was sprayed on the surface of each 5 g soil sample. A micro-syringe was used to spray the surface of the soil so that very small droplets were dispensed to ensure thorough mixing.

Next, different kinds of solution were introduced into SS with added UDMH solution (in quadruplicate). The first SS of the quadruplicate samples with 10 mL of added water was kept as the control. The second had 10 mL of broth medium added to investigate the effect of the nutritive medium on biodegradation. The third and the fourth had 10 mL incubated bacterium solution of M12 and P4 strain added, respectively. The same series was applied to *n*SS, which was contaminated with UDMH to study the degradation potential of autochthonous microorganisms. A 5 g sample of *n*OM contaminated with UDMH had 10 mL of water added, which was used to find out the effect of organic matter on degradation.

All the soil samples were thoroughly mixed and incubated in a darkened thermostatic chamber maintained at 25 ± 2 °C and relative humidity (RH) 90% for five days. Additional water would be supplied if water losses exceeded 2%.

2.7. Sample Pretreatment for Analyses

The pretreatment in MSM for qualitative analysis [28]: the solid phase microextraction method (SPME) was performed mainly to analyze metabolites of UDMH by a manual holder (Supelco, Bellefonte, PA, USA). Four milliliters of aqueous sample were transferred into a 10 mL headspace vial with a pipette. One gram of NaCl was added to the vial, which was stirred to dissolve. The vial was penetrated by the SPME manual holder with 75 μm Carboxen/Polydimethylsiloxane (CAR/PDMS)

SPME fiber. We adjusted the manual holder to expose the fiber to the headspace of the water. The sample was extracted for 10 min with magnetic stirring. The SPME fibers were conditioned in the Gas Chromatography (GC) (Agilent 6890A, Santa Clara, CA, USA) inlet at 250 °C for 30 min before first use. SPME-based sample desorption was carried out at a GC inlet temperature of 250 °C for 15 min.

For the pretreatment in the MSM for determining *N*-nitrosodimethylamine (NDMA) [29], the steam distillation method was used in this study. One hundred milliliters of the aqueous sample were transferred into the distilling flask of the steam distillation apparatus. Thirty grams of NaCl were added to the flask, which was shaken to dissolve. An Erlenmeyer flask containing 40 mL dichloromethane was placed in an ice bath to collect the distillate. When the volume of the distillate was 200 mL, 40 g NaCl and 2 mL sulfuric acid (1 + 3) were added. The Erlenmeyer flask was shaken to make the NaCl dissolve thoroughly, and then the solution was transferred into a 500 mL separatory funnel. After shaking for 5 min, the solution was kept still for 10 min to allow stratification. The dichloromethane layer was transferred into another Erlenmeyer flask. The water layer was extracted by 80 mL dichloromethane twice. All the extractant was merged together and dried by anhydrous sodium sulfate. Finally, the extractant was condensed by the nitrogen blowing instrument to a dry residue. The dry residue was dissolved in 1 mL dichloromethane for subsequent GC/MS determination of NDMA.

The pretreatment in the soil for qualitative analysis [1]: the solid phase microextraction method (SPME) was performed, mainly to analyze the metabolites of UDMH by a manual holder (Supelco). A 5 g soil sample was transferred into a 10 mL headspace vial. One gram of NaCl was added to the vial. The vial was penetrated by the SPME manual holder with 75 μm Carboxen (CAR)/PDMS SPME fiber. We adjusted the manual holder to expose the fiber to the headspace of the soil. The sample was extracted for 18 h at room temperature. SPME-based sample desorption was carried out at a GC inlet temperature of 250 °C for 15 min.

The pretreatment for determining UDMH in the soil: 5 g of soil, 2 g of $Na_2S \cdot 9H_2O$, and 50 mL of a 40% solution of NaOH were placed in a 250 mL distillation flask; the flask was attached to a condenser, and the liquid was distilled to dryness into a receiver containing 10 mL of 0.1 mol·L^{-1} H_2SO_4. The distillate was transferred to a 100 mL flask and diluted to the mark with deionized water [26]. Ten milliliters of liquid from the 100 mL flask and 0.2 mL salicylaldehyde were added into a 25 mL colorimetric tube with a stopper. The derivatization reaction was catalyzed by ultrasonic for 1 h under the following conditions: 50 kHz of ultrasonic frequency, 80 W·cm^{-2} of ultrasonic power, and 50 °C. Then the tube was taken out and cooled to room temperature. Afterwards 2.5 g NaCl was added to dissolve and 5 mL dichloromethane was used to extract the derivative product, named salicylaldehyde dimethylhydrazone. After vigorous shaking, all the liquid was transferred into a 15 mL separating funnel. After shaking for 5 min, the liquid was still for 10 min to allow stratification. One milliliter of extractant of dichloromethane layer was separated to be analyzed by GC/MS [30].

The pretreatment for determining NDMA in the soil: the 10 g soil sample was placed in a Soxhlet apparatus (Beijing Glass Group Company, Beijing, China) containing 50 mL dichloromethane. The extraction was performed for 8 h from the moment of boiling the extractant. The obtained extractant was dried by anhydrous sodium sulfate, filtered through a 0.40 μm membrane, and condensed to a dry residue using a nitrogen blowing instrument. The dry residue was dissolved with 1 mL of dichloromethane for subsequent analysis.

2.8. Chemical Analyses

UDMH in the aqueous MSM was determined by Chinese standard GB/T14376 method with a UV spectrophotometer (CARY 50, Varian, Palo Alto, CA, USA) [31]. Other analyses mainly used the GC/MS method. Agilent GC/MS (6890A-5973N, Santa Clara, CA, USA), equipped with a capillary column (0.25 μm × 0.25 mm × 30.0 m, HP-5-MS), was used to identify potential degradation intermediates and to determine UDMH and NDMA.

Conditions of GC/MS: The GC carrier gas was helium, whose flow velocity was 1.0 mL min^{-1} in constant flow mode. The ionization mode of the MS was electron ionization with an ionization energy

of +70 eV. The ion source temperature was 230 °C, the quadrupole temperature was 150 °C, and the tuning mode was autotune. The Interface temperature was 280 °C. For the compounds considered, the following characteristic ions were used (m/z) in the selected ion mode (SIM): 42 and 74 for NDMA, and 164, 120, 74, and 50 for salicylaldehyde dimethylhydrazone.

Qualitative analysis in aqueous MSM: the initial temperature of the column was 40 °C (for 9 min), and the temperature program was 20 K· min^{-1} to 280 °C (for 5 min). The injection mode was splitless. The MS detector worked in the SCAN mode.

Determination of NDMA in aqueous MSM: the temperature of the injector was 180 °C, the initial temperature of the column was 40 °C (for 8 min), and the temperature program was 20 K· min^{-1} to 280 °C (for 5 min). The injection mode was splitless. The injection volume was 1 μL. Retention time of NDMA was 4.75 min. The MS detector worked in the SIM mode. The cathode was turned on at 4.0 min after the sample injection.

Qualitative analysis in the soil: the temperature of the injector was 250 °C, the initial temperature of the column was 40 °C (for 9 min), and the temperature program was 20 K· min^{-1} to 250 °C (for 5 min). The injection mode was splitless. When working in the SCAN mode, the qualitative analysis was carried out.

Determination of UDMH in the soil: the temperature of the injector was 180 °C, the initial temperature of the column was 40 °C, and the temperature program was 20 K· min^{-1} to 100 °C. Then the temperature was raised to 250 °C (for 2 min) at a rate of 6 K· min^{-1}. The injection mode was splitless. The injection volume was 1 μL. The SIM mode was carried out. The cathode was turned on at 8 min after the sample injection. Retention time of salicylaldehyde dimethylhydrazone was 12.42 min.

Determination of NDMA in the soil: the temperature of the injector was 180 °C, the initial temperature of the column was 40 °C (for 9 min), and the temperature program was 20 K min^{-1} to 250 °C (for 5 min). The injection mode was splitless. The injection volume was 1 μL. The SIM mode was carried out. The cathode was turned on at 4 min after the sample injection. Retention time of NDMA was 4.75 min.

3. Results

3.1. The Biodegradation of UDMH by Activated Sludge

Figure 1 shows the change of UDMH concentration per day before and after treatment, and the change in the daily degradation rate. In Figure 1, the degrading percentage of UDMH (24 h) increased gradually. At the beginning of the seven days, the degrading percentage was only just over 30%, which suggests the activated sludge did not form the dominant bacteria consortium to biodegrade UDMH. As the acclimation continued, the bacteria that had the capacity of biodegrading UDMH formed the domain bacterial consortium, and the degrading percentage rose to 70% after 15 days. On the 28th day, the degrading percentage steadily increased to about 80% and the acclimation could be accomplished. At first, the addition of UDMH prevented the growth of bacteria and made a delay in the start of the log growth phase [32]. As soon as the bacteria adapted to UDMH, they smoothly entered the log growth phase in the course of acclimation. The concentration of chemical oxygen demand (COD) was also detected with the potassium dichromate ($K_2Cr_2O_7$) method [33]. The final degrading percentage of COD (24 h) was 84.5%, which proved that the treatment by activated sludge system was effective.

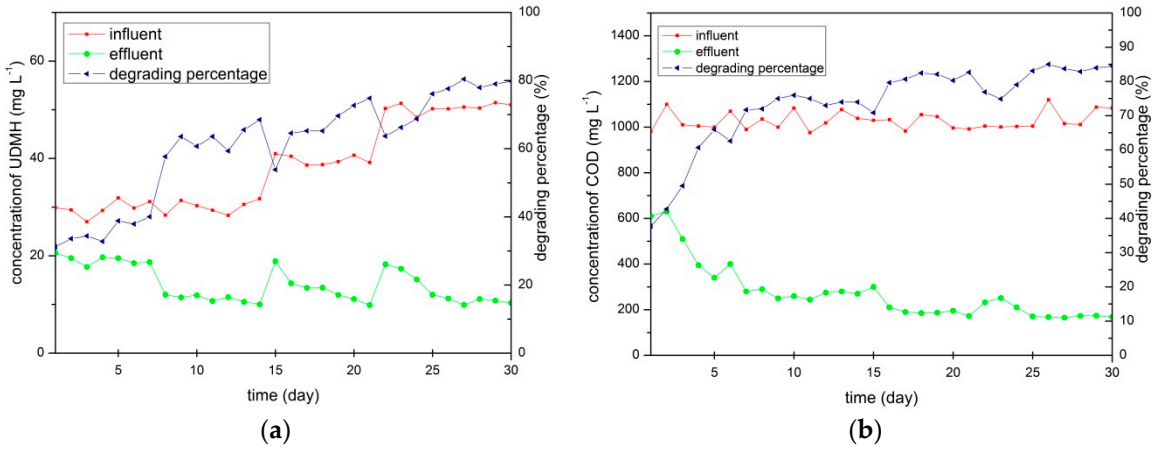

Figure 1. The degradation of unsymmetrical dimethylhydrazine (UDMH) by activated sludge: (**a**) the removal of UDMH; (**b**) the removal of chemical oxygen demand (COD).

3.2. Isolation and Identification of M12 and P4

Two stains, numbered M12 and P4, were found to have a strong capability of degrading UDMH. The M12 strain was screened from the medium containing a high concentration (50 mg· L^{-1}) of UDMH, and the P4 strain was screened from the medium containing a low concentration (5 mg· L^{-1}) of UDMH.

DNA was extracted from the pure culture of the two strains, and 16S rDNA sequence was analyzed. Partial sequencing with both primers revealed the closest matches. Determined by a BLAST search, the strain M12 was named as *Stenotrophomonas* sp. M12 with a similarity of 99%, and the strain P4 was named as *Comamonas* sp. P4 with a similarity of 99%. A 16S rDNA sequence has already been submitted to GenBank with accession numbers of KP663381 and KT779283.

3.3. Biodegradation of UDMH in Aqueous MSM

The degrading percentage in MSM with UDMH as the sole carbon source for M12 and P4 is shown in Figure 2a. M12 could effectively degrade UMDH with a high percentage of 86.0% (72 h); however, P4 barely biodegraded UDMH, approaching the control sample with the degrading percentage of 53.0%. The control sample degradation rate was 50.8%. It was shown that P4 was intensively inhibited by UDMH without an extra carbon source.

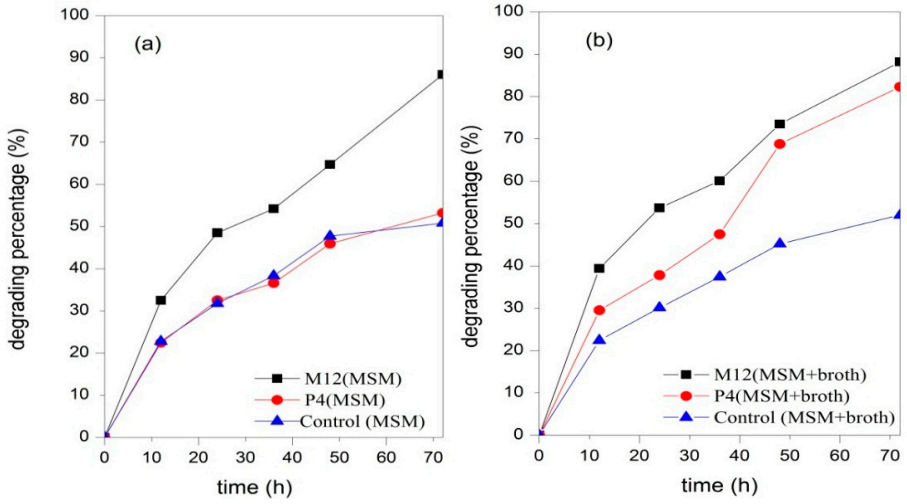

Figure 2. The degradation of UDMH in aqueous mineral salt medium (MSM): (**a**) UDMH as sole carbon source; (**b**) with the addition of broth medium.

When M12 and P4 were added with extra carbon sources of broth medium, as shown in Figure 2b, the degrading capacity of M12 was not obviously influenced, but the degrading capacity of P4 was significantly increased to 82.3%. This showed that broth medium could be used as co-metabolism substrate of P4 degrading UDMH. The conventional carbon sources at suitable concentration could stimulate microbial growth, which led to higher levels of enzymes being available for the degradation of toxic compounds [34].

Compared with P4, M12 had a stronger ability to tolerate and degrade UMDH in solution. This kind of bacterium is not unique. The research group of Street [35] isolated a bacterium named *Achromobacter* sp. that could oxidize hydrazine which had chemical properties similar to UDMH, in a solution of 100 mg· L^{-1}, early in 1987.

3.4. Biodegradation of UDMH in Soil

The preliminary experiments had shown that the degradation percentage of UDMH with M12 and P4 without the addition of broth medium in SS was no higher than the sample with no added broth medium, so M12 and P4 without broth medium were not discussed in this study. The biodegrading percentage of UDMH in the soil is shown in Figure 3.

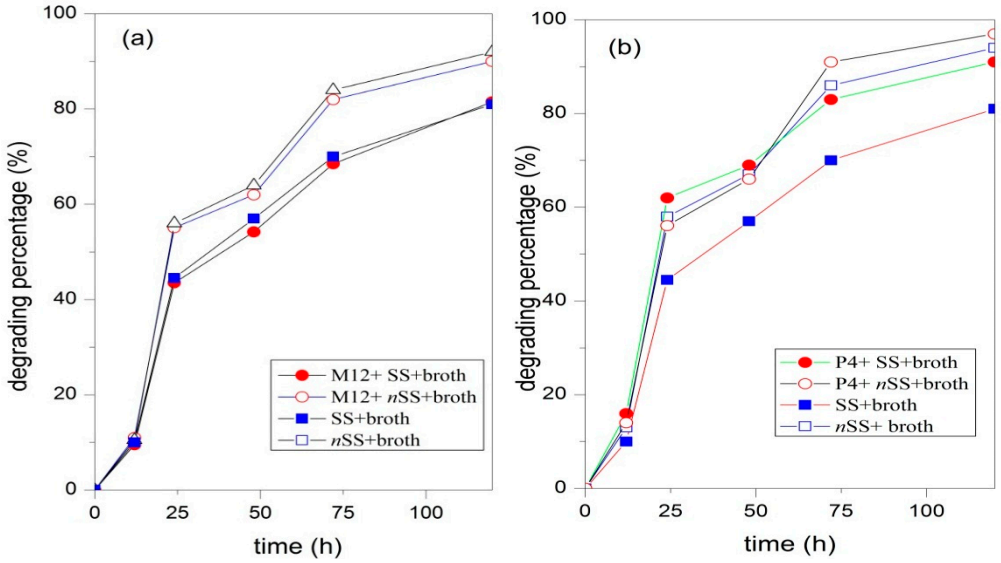

Figure 3. The degradation of UDMH in soil: (**a**) the degradation in sterile soil (SS) and non-sterile soil (*n*SS) with the M12 strain; (**b**) the degradation in SS and *n*SS with the P4 strain.

It is worth noting that the M12 strain, which had the best degradation ability of UDMH in water, could not degrade UDMH in soil. In Figure 3a, no matter whether there was an added external carbon source or not, M12 degradation capability could not exceed that of the control samples, and M12 + *n*SS degradation rate was even lower than *n*SS. Because M12 did not reveal a good degrading capacity in water, it could be inferred that M12 might lack some biodegradation conditions in soil.

In Figure 3b, the P4 strain had a good degradation ability with the broth medium and could co-metabolize with the UDMH in soil. During the first 48 h of degradation, P4 + SS showed the strongest degradation ability, and the degrading percentage was obviously higher than SS + broth and M12 + SS + broth, and even higher than P4 + *n*SS + broth. This showed that UDMH in soil could be degraded by the P4 strain, which had high adaptability to UDMH in soil environment and was inhibited by indigenous microorganisms in the early growth period. After 48 h, P4 adapted to UDMH, and the degradation rate of the P4 + *n*SS + broth samples significantly improved. The final degrading percentage of P4 in the *n*SS sample was 97.0%, which was the highest in all the samples. The final UDMH concentration of the P4 + *n*SS + broth sample was 5.8 mg· kg^{-1}, and in the *n*SS + broth

sample it was 12.3 mg· kg^{-1}. Given that UDMH could remain stable for a long time in the soil [27], the reduction in UDMH concentration can be attributed to the participation of the P4 strain. More experiments showed that the concentration of UDMH in soil underwent little change after five days, and the improvement of UDMH degrading percentage in the contaminated soil was a difficult process.

3.5. Degradation Intermediates

If UDMH was spilled into the environment, the oxidative transformation would form a wide range of oxidation intermediates simply because of its strong reducing properties [36]. Recent studies showed that UDMH would transform a number of compounds such as dimethylamine (DMA), 1,1,4,4-tetramethyltetrazene (TMT), formaldehyde dimethylhydrazone (FADMH), *N*-nitrosodimethylamine (NDMA), 1-methyl-1H-1,2,4-triazole (MTA), and others [1]. Those compounds both constitute transformation products, which are formed directly from UDMH, as well as compounds that are formed in various consecutive processes [37]. Among all the transformation products, NDMA is a particularly harmful nitrosamine and potential carcinogen classified by the U.S. Environmental Protection Agency (EPA). Current concerns with NDMA in ground water began more than a decade ago when the compound was detected at various military and aerospace facilities that previously handled liquid propellants containing UDMH [38]. Studies conducted on the containment systems indicate that NDMA is not being effectively removed from the extracted groundwater. Concentrations of NDMA in the Rocky Mountain Arsenal groundwater, which was used to produce rocket fuel for the U.S. military, generally range from 0.87 to 2.7 μg· L^{-1} [39]. The degradation intermediates of UDMH biodegraded in aerobic activated sludge had been studied by the SPME-GC/MS analytic method in 2005. DMA, FADMH, TMT, DMF (*N*,*N*-dimethylfomamide), acetaldehyde dimethylhydrazone, and MTA were found [28]. However, the SPME-GC/MS method could not detect low concentration NDMA in an aqueous solution. This problem was resolved by adopting the GC/MS method [29].

UDMH and its degradation intermediates could bind tightly to the soil absorbing complex. When soil substances were introduced into an aqueous solution, absorption of a significant portion of the dissolved substances proceeds quickly, which is indicative of the high absorption capacity of the substance in question [40]. The standard UV spectrophotometer method could not detect UDMH in the soil by dissolving and filtering if the concentration of UDMH was not high enough. So the pretreatment method of alkali distillation was used to extract UDMH from the soil [41].

Chemical analysis of the UDMH degradation intermediates revealed some differences between aqueous MSM and the soil samples (Figure 4). In the aqueous MSM, M12 could effectively biodegrade both UDMH and NDMA. The same also happened in the soil for P4. In particular, the NDMA concentration of the biodegradation sample was far lower than that of the control, which showed that the bacteria M12 and P4 were involved in the degradation of NDMA at the same time.

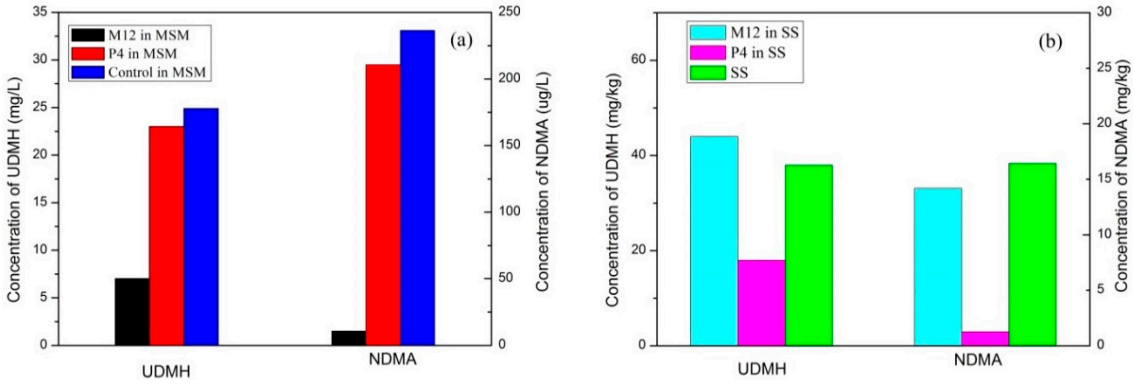

Figure 4. The final concentrations of UDMH and *N*-nitrosodimethylamine (NDMA) (**a**) in aqueous MSM and (**b**) in SS.

The qualitative analysis results of the degradation intermediates in the aqueous MSM and soil are shown in the total ion chromatograms (TIC) (Figure 5). Table 2 lists seven kinds of final compound identified by the NIST MS library match; any compounds that were not clearly identified (probability match <85%) are not listed.

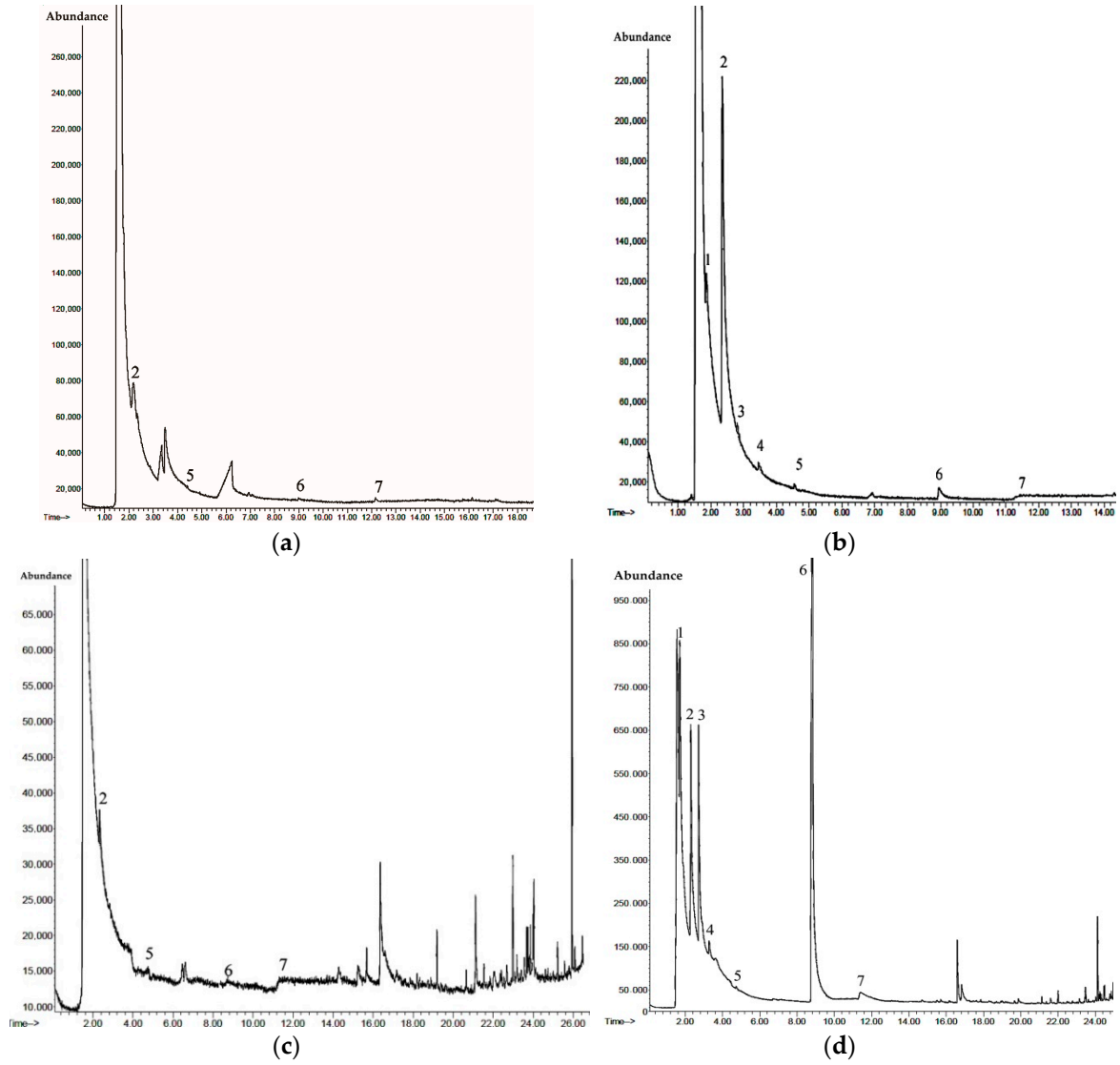

Figure 5. GC-MS data of degradation intermediates: (**a**) final degrading total ion chromatograms (TIC) of M12 strain in aqueous MSM; (**b**) final degrading TIC of control sample in aqueous MSM; (**c**) final degrading TIC of P4 strain in SS; (**d**) final degrading TIC of control sample in SS.

Whether in the aqueous solution or in the SS, the kinds of main final intermediates of the control did not have a visible difference. FADMH, NDMA, TMT, and MTA were detected in all the TICs. The concentration of these compounds biodegraded was also far lower than those of the controls. Although other compounds such as DMA, tetramethylhydrazine, and acetaldehyde dimethylhydrazone could not be detected in the TICs of biodegradation in the SS and aqueous solution, their characteristic ion peaks were clearly found in the extract ion chromatograms. For the control sample, it was mainly oxidized by the air, and the exogenous bacteria did not change the course of natural oxidation of UDMH, but the exogenous bacteria could degrade UDMH and other intermediate products at the same time.

Table 2. The list of transformation compounds of unsymmetrical dimethylhydrazine (UDMH) detected in aqueous mineral salt medium (MSM) and soil.

No.	Compound Name	CAS No.	Formula	Characteristic Ions, *m*/*z* (Relative Abundance, %)
1	Dimethylamine	124-40-3	C_2NH_7	44 (100); 45 (56); 28 (26); 46 (3)
2	Formaldehyde dimethylhydrazone	2035-89-4	$C_3N_2H_8$	72 (100); 71 (71); 42 (61); 57 (15)
3	Tetramethylhydrazine	6415-12-9	$C_4N_2H_{12}$	88 (100); 73 (89); 44 (73); 42 (63)
4	Acetaldehyde dimethylhydrazone	7422-90-4	$C_4N_2H_{10}$	86 (100); 44 (51); 42 (48); 85 (34)
5	*N*-Nitrosodimethylamine	62-75-9	$C_2N_2H_6O$	74 (100); 42 (37); 43 (15); 41 (4)
6	1,1,4,4-Tetramethyltetrazene	6130-87-6	$C_4N_4H_{12}$	116 (100); 43 (53); 72 (42); 42 (32)
7	1-methyl-1H-1,2,4-triazole	6086-21-1	$C_3N_3H_5$	83 (100); 56 (29); 84 (5); 40 (4)

Compounds with the NIST MS library match >85% both in soil and solution.

4. Discussion

4.1. Capability of Biodegradation UDMH

In 1979, London [32] selected the *Paracoccus denitrificans* sp. named D-31 from the soil, and discussed its tolerance of UDMH. From then on, *Achromobacter* sp., *Bacillus* sp., *Psuedomonas* sp. [35], and *Enterobacter cloacae* sp. [42] were used to research the toxicity and tolerance of UDMH. According to the study, Kane [43] thought that UDMH was not suitable to be biodegraded because of the toxicity and inhibition effect of UDMH on the environmental bacteria, so UDMH in the environment was rarely adopted as a biodegradation method for a long time [44]. It is worth noting that these strains were screened from nature directly, rather than from the acclimated environment.

Entering the 21st century, as bioremediation technology became more mature, the treatment of microorganisms on the UDMH gained attention again. Actually, UDMH is a highly reactive compound that undergoes oxidation reduction reactions and acts as a potent reducing agent [8]. If bacteria that are UDMH tolerant could be found, the biodegradation of UDMH might be realized. The acclimated activated sludge method attempted to treat wastewater containing UDMH of 1000 mg$\cdot$ L^{-1}, and the degrading percentage of UDMH (72 h) was high at 90% [45]. The EOW/MBR (Electrolyzed Oxidizing Water/Membrane Biological Reactor) technology succeeded in treating wastewater containing UDMH of 500 mg$\cdot$ L^{-1}, which also proves that UDMH can be treated by biodegradation [46]. A biodegradation technology of bioaugmentation in the fixed-film bioreactor has been adopted by NASA to treat wastewater containing methylhydrazine and hydrazine fuel, which has similar properties to UDMH [47].

Almost all studies have been focused on wastewater treatment; only Ou and Street [35] studied the special strain biodegradation of hydrazine in soil, but the results showed that the UDMH in soil could not be degraded by adding bacteria. In this study, under the process of co-metabolism, P4 had good adaptability in soil, which could withstand 600 mg$\cdot$ kg^{-1} UDMH and exhibited high biodegradation activity. More importantly, the concentration of the intermediate products obviously decreased in this biodegrading process.

4.2. The Complexity of UDMH Biodegradation in Soil

From Figure 6, one can see that the extra bacteria obviously improved the biodegrading ability of autochthonous microorganisms. Usually, a high concentration of UDMH, bonded with the soil solids in the form including the UDMH adsorbed by the mineral phase and chemically adsorbed by the soil organic matter, could exist for approximately 30 years [27,48]. Therefore, biodegradation of UDMH in the soil was a complicated process still influenced by organic matter, autochthonous microorganisms, and metal ions [49]. The prerequisite release of a contaminant from the adsorbed phase to the aqueous phase for its degradation by microorganisms determined the susceptibility to microbial degradation,

thereby influencing the effectiveness of the biodegradation process [50]. The fate of UDMH was largely influenced by the competing processes of degradation and sorption [22]. The extra bacterial culture should be added as soon as possible to biodegrade UDMH against the chemical absorption.

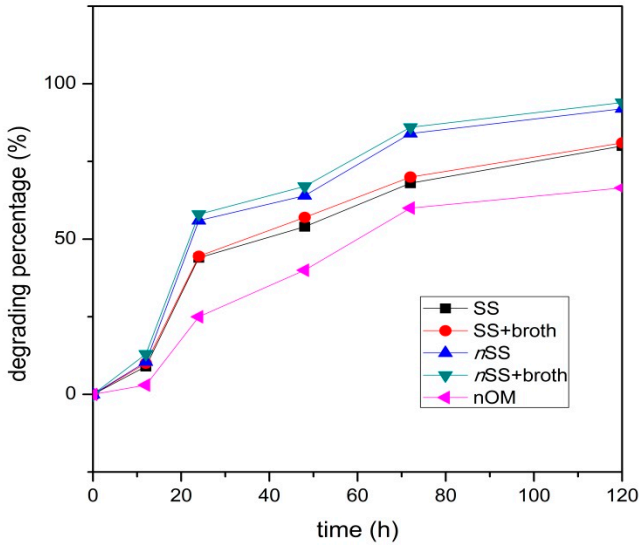

Figure 6. The influence of extra carbon and organic matter on the biodegradation of UDMH.

(a) Organic matter: In Figure 6, the final degrading percentage of *n*OM sample was only 66.5%, and the final degrading percentage of the SS sample was 81%. It could be inferred that the difference between the two samples was caused by chemical absorption of soil organic matter. The soil samples collected from 5–10 cm below the ground surface contain abundant dead plant matter, which forms the humic substances. The organic matter content in various soils is very different, so the natural degradation ability of UDMH in different areas is variable.

(b) Indigenous microorganisms: The samples in *n*SS had a higher final degrading percentage than those in SS, and the *n*SS sample could even have a final degrading percentage of 92%, which was almost the same as the P4 + SS sample. Those proved that autochthonous microorganisms could have some biodegrading effect on UDMH in the soil. Microbiological studies also showed there were certain bacteria that could biodegrade hydrazine fuels in natural environment [49]. The relationship between exogenous bacteria (P4, M12) and indigenous microorganisms was competitive. In the first two days, the degrading percentage of UDMH for the P4 + SS sample was higher than the P4 + *n*SS sample (Figure 3). The biodegradation capacity of P4 was influenced in *n*SS soil by the autochthonous microorganisms with the competition of the nutrient. When P4 gained the competitive advantage in *n*SS soil after two days, the degrading percentage of the P4 + *n*SS + broth sample began to exceed that of the P4 + SS sample. In the initial degrading period, the M12 + *n*SS + broth sample had an even lower final degrading percentage than the *n*SS + broth sample. M12 decreased the biodegrading ability of the autochthonous microorganisms in the M12 + *n*SS sample.

(c) Metal ions: Following the entry into the soil environment, UDMH rapidly bound to the mineral and soil particle (solid phase) via a combination of physical and chemical processes. Sorption, complexation, and precipitation constituted the pollutant–soil interaction. Many transition metals in the soil could catalyze the process, acting as a one- or two-electron acceptor [51].

4.3. The Influencing Factors on Microbial Degradation of UDMH

The two strains in solution and soil environment displayed different degradation ability. P4 could not biodegrade UDMH as a sole carbon source in aqueous MSM, and M12 could not biodegrade UDMH in soil. Three important factors—initial UDMH concentration, oxygen content,

and co-metabolism—were chosen to be discussed, which may help with finding the biodegradation pathway of UDMH. The results are shown in Figures 7 and 8.

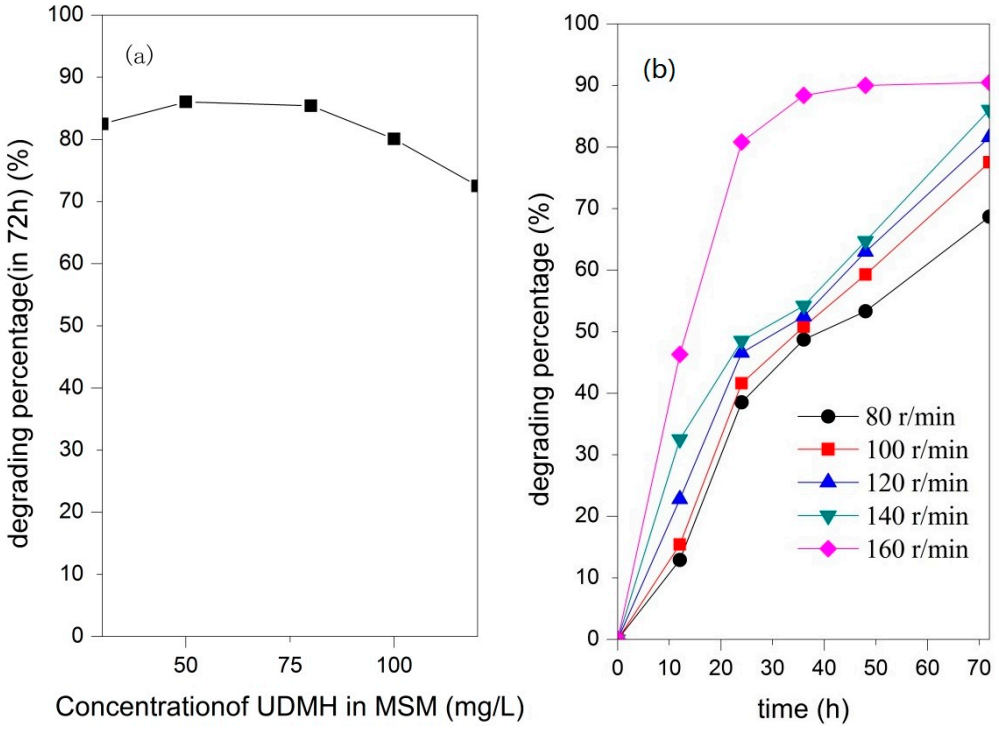

Figure 7. The influencing factors on UDMH degradation for M12 in liquid MSM: (**a**) the concentration of UDMH; (**b**) the shaking speed.

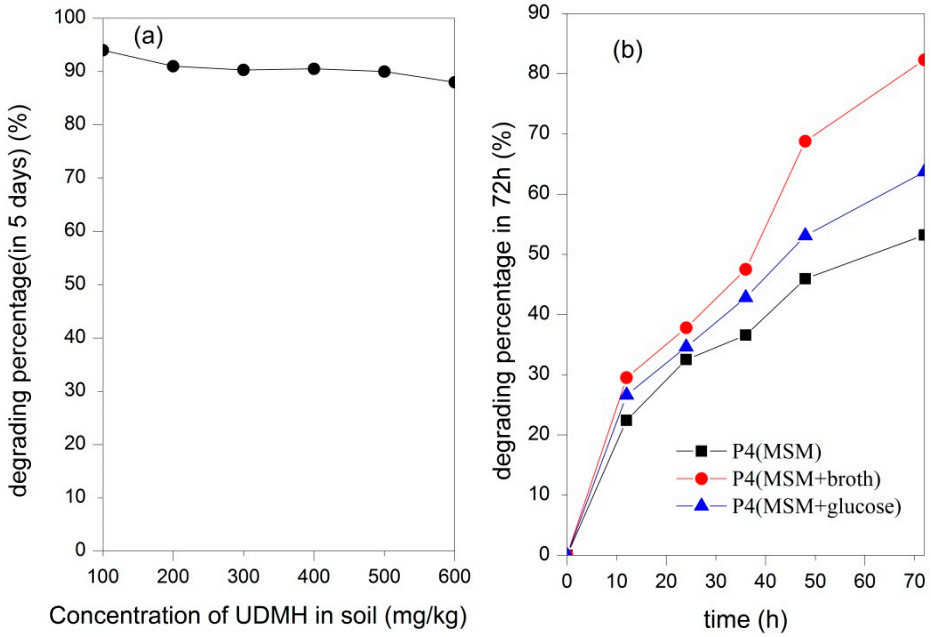

Figure 8. The influencing factors on UDMH degradation for P4: (**a**) the concentration of UDMH in soil; (**b**) the extra carbon source in MSM.

According to the results of the influences of different initial UDMH concentrations given in Figures 7a and 8a, UDMH was proven to inhibit metabolism of the strains, as had been found early

in 1979 [32,43]. It could carry the double mutation of *rec*A$^-$ and *vr*A$^-$ by interacting with different compounds of the cell, including lipids, proteins, and nucleic acids [52]. The inhibition influence was more severe in aqueous MSM than in soil. This was because the biodegradation of UDMH in the soil was complex, which was affected by organic matter, autochthonous microorganisms, and metal ions. P4 had high tolerance for UDMH in soil, and could degrade 100–600 mg· kg^{-1} UDMH, reflecting a good soil degradation capability.

Although M12 could biodegrade UDMH as a sole carbon source, the oxygen concentration in solution might influence this strain to biodegrade UDMH effectively. The content of oxygen in solution was mainly controlled by the shaking speed. To prove that oxygen was an important influencing factor for M12, the effect of the shaking speed is discussed in this study. The degrading percentages of UDMH in aqueous MSM with different shaking speeds are listed in Figure 7b. The faster the shaking speed, the higher the UDMH degrading percentage was. When the shaking speed was 80 r· min^{-1}, the degrading percentage of UDMH was close to that of the control sample. When the shaking speed was 160 r· min^{-1}, the degradation of UDMH was greatly improved in the initial 20 h. Then the degradation of UDMH trended to be stable. The great improvement of the degrading capacity was probably due to the air oxidation of UDMH [53]. Oxygen could help M12 resist the toxicity of UDMH by reducing the concentration of UDMH, and it was also essential for the growth and metabolism of this strain. After UDMH was air oxidized, small molecules of carbonaceous and nitrogenous compounds could provide the carbon and nitrogen needed for the growth of the bacterium (Figure 9). Based on the above, UDMH in soil could not be degraded by the M12 strain, most likely due to low oxygen levels in the soil, which caused M12 to reduce the capacity of biodegradation for UDMH.

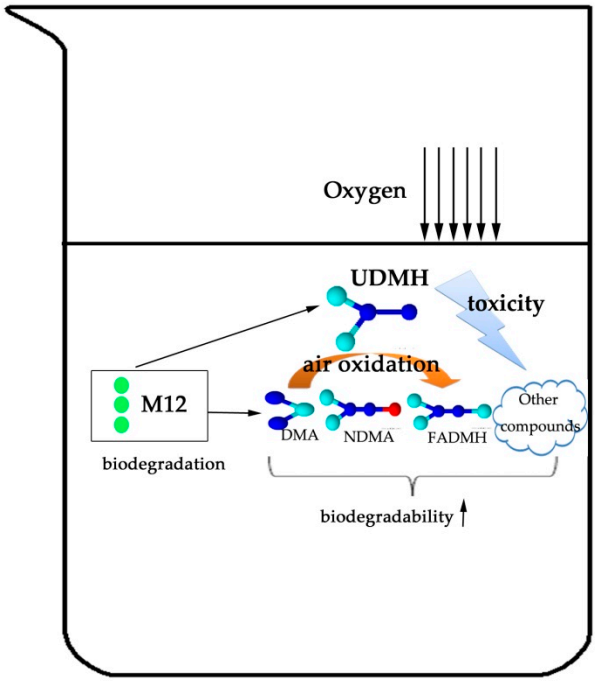

Figure 9. The influence of air oxidation on UDMH degradation for M12 strain in solution.

Co-metabolism with a substrate might cause the P4 strain to biodegrade UDMH more effectively. To resist the toxic inhibition of UDMH, co-metabolism with a substrate was adopted to improve the biodegradation rate of UDMH. Figure 8b shows the co-metabolism effect of two kinds of medium in aqueous solution. It can be seen that the co-metabolism medium is very useful for degrading UDMH for P4, but a broth medium is a more effective choice.

The key enzyme should be the basis of UDMH biodegradation for P4 (Figure 10). When UDMH was the sole carbon source, the toxicity effect of UDMH was embodied in the cells of P4. Fortunately,

the microorganism had a self-detoxification mechanism and a self-recovery function [54]. As soon as the microorganism was supplied with enough energy, the self-recovery would be carried out after a lag period. The new enzyme might also be synthesized to decompose the toxic UDMH in the course of self-detoxification of the microorganism with the extra carbon sources added. The broth medium provided more reasonable carbon–nitrogen percentages and sufficient energy for the bacteria to grow. In this process, the *de novo* related protein synthesis, especially the appearance of the key enzyme, had an important role. So the addition of broth medium alleviated the toxic effect of UDMH on the cell and the key enzyme, possibly because it shortened the lag time for the P4 strain [55].

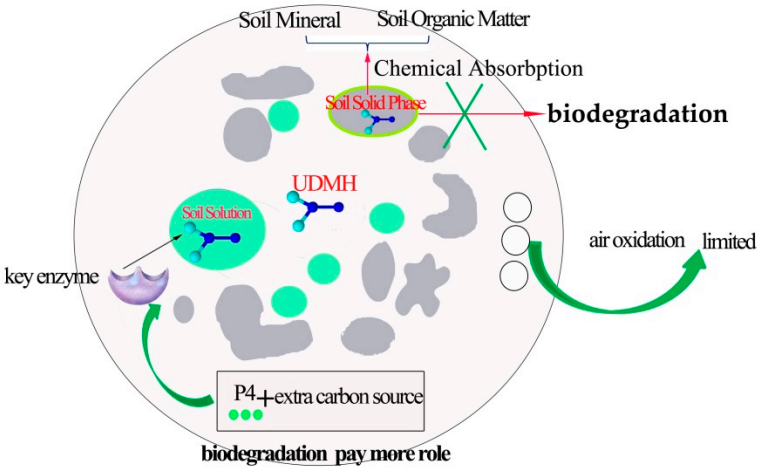

Figure 10. The influence of extra carbon source on UDMH degradation for P4 strain in soil.

Altogether, biodegradation of both strains relied somewhat on the environmental conditions. If the proper conditions were lacking, the toxicity effect of UDMH would be enlarged. The ability to degrade UDMH would be seriously constrained.

4.4. Degradation Pathway

The types of biodegradation transformation products differed very little compared with air oxidation in Figure 5, and the pathway of UDMH oxidation did not change in biodegradable participation. A universally applicable degradation pathway is proposed in Figure 11. In the earlier study, $(CH_3)_2NN{\cdot}H$ was thought to be an important intermediate in the degradation mechanism of UDMH, which was the source of redox reactions [45,53,56]. According to the quantum chemical calculation, the first stage forming $(CH_3)_2NN{\cdot}H$ was an exothermic process releasing energy of 181.96 $KJ{\cdot}mol^{-1}$ [57]. This process was a no barrier process with a reaction rate constant of 5×10^7 $L{\cdot}mol^{-1}{\cdot}s^{-1}$ [17]. Then $(CH_3)_2NN{\cdot}H$ lost an H atom and rearranged into $(CH_3)_2N^+{=}N$, releasing energy of 2896.4 $KJ{\cdot}mol^{-1}$ [57]. FADMH, which was found in high concentration in Figure 5, could also prove that this process was proceeding easily. Therefore, the production of $(CH_3)_2NN{\cdot}H$ was crucial for accelerating the degradation rate of the UDMH oxidation process. According to the mechanism inferred by Sierka, $(CH_3)_2NN{\cdot}H$ was the precursor of a series of degradation intermediates [56]. Consequently, $(CH_3)_2NN{\cdot}H$ was very important for the processes of biodegradation and air oxidation. It could be further oxidized into different compounds with lower toxicity that could provide the necessary carbon and nitrogen for bacteria to grow. In solution, it had been found that the oxygen content was positively correlated with the oxidation rate of UDMH. In soil, the oxygen content was lacking so that the air oxidation of UDMH was slower than in an aqueous solution. At this time, biodegradation played bigger role than air oxidation. It could be deduced that the biodegradation and the air oxidation of UDMH were synchronous, and had a mutually reinforcing relationship.

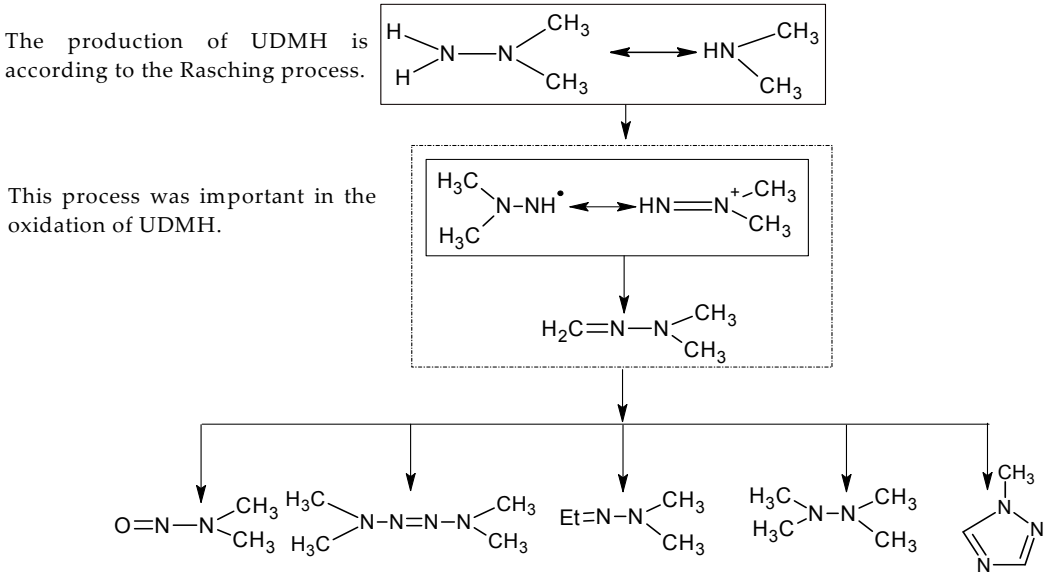

Figure 11. The degradation pathway of UDMH.

5. Conclusions

In this study, bacteria belonging to *Stenotrophomonas* sp. and *Comamonas* sp. were isolated and had the ability to degrade UDMH in an aqueous solution and in soil. Moreover, they may also have the potential to metabolize other degradation intermediates.

In fact, M12 was originally isolated for the purpose of degradation in the soil. However, this strain can biodegrade UDMH in an aqueous solution, but cannot do so in soil. In a natural soil environment, a co-metabolism medium also cannot enhance its ability. So reselecting a new strain is a good choice for reducing the concentration of UDMH in the isolation medium. Then P4 was chosen for this situation, and it sufficiently revealed the influence of co-metabolism on biodegradation. The degradation pathway of UDMH was also discussed in this study to lay a foundation for some future research. Maybe the addition of hydrogen peroxide or aeration in the soil could help M12 degrade UDMH; this deduction should be verified in our next study.

Although the related studies for biodegradation of UDMH are very limited, the isolated strain may still be useful for a bioremediation of UDMH-polluted environment. There is a need for further research on the biochemical and genetic aspects of UDMH-degrading bacteria. In the future, a combination of different methods could increase the degrading efficiency of UDMH.

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Author Contributions: Qili Liao designed the experiments; Qili Liao and Li Wang carried out the experiments; Qili Liao accomplished the data analysis and wrote the paper; Changgen Feng reviewed and improved the manuscript. Li Wang and Changgen Feng supervised the project.

Conflicts of Interest: The authors declare no conflict of interest.

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