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ORIGINAL PAPER

Isolation and characterization of *Pseudomonas* sp. DX7 capable of degrading sulfadoxine

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Abstract Given that the intensive application of sulfonamides in aquaculture, animal husbandry and malaria treatment has lead to an increase in sulfonamide discharge into the environment, there is an increasing need to find a way to remediate sulfonamide-contaminated sites. The bacterial strain DX7 was isolated from a marine environment and is capable of degrading sulfadoxine. DX7 was identified as a *Pseudomonas* sp. based on 16S rRNA gene sequencing. Approximately 30% of sulfadoxine was degraded after *Pseudomonas* sp. DX7 was inoculated into mineral salt plus tryptone media containing 10 mg l^{-1} sulfadoxine for 2 days. The degradation efficiency under different environmental conditions was characterized using HPLC. The optimal temperature and pH for sulfadoxine biodegradation were around 30°C and 6.0, respectively. The optimal concentrations of sulfadoxine and tryptone for sulfadoxine biodegradation were determined to be approximately 30 mg l^{-1} and between 2.0 and 8.0 g l^{-1}, respectively. Cytotoxicity analysis indicated that the metabolites of sulfadoxine generated by *Pseudomonas* sp. DX7 showed significantly reduced cytotoxicity to Hela cells. These results suggest that *Pseudomonas* sp. DX7 is a new bacterial resource for degrading sulfadoxine and indicate the potential of the isolated strain in the bioremediation of sulfadoxine-contaminated environments.

Keywords Sulfadoxine · *Pseudomonas* sp. · Degradation · Cytotoxicity

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Introduction

Sulfadoxine, 4, 5-dimethoxy-6-sulfanilamidopyrimidine, is a long-acting sulfonamide that was used alone for the treatment of various infections in the past but is now almost exclusively used in combination with pyrimethamine to treat or prevent uncomplicated

malaria (Mulenga et al. 1999; Van Dillen et al. 1999; Kofoed et al. 2006; Kolaczinski et al. 2007; Obonyo et al. 2007; Conteh et al. 2010; Aziken et al. 2011). Sulfadoxine provides a bacteriostatic effect by inhibiting the production of folic acid, which is required for bacterial growth (Craig and Stitzel 1994).

The presence of sulfonamides in surface and ground water has become an issue (Khetan and Collins 2007; Kostopoulou and Nikolaou 2008). These compounds are continually released into the environment by several pathways, of which patient excretion following therapy is considered the primary pathway (Heberer 2002). These substances can enter the environment in its original form or as metabolites via urine or feces (Heberer 2002; Loffler and Ternes 2003; Kümmerer 2004). Increasing concern regarding sulfonamides in the past few years stems from the fact that the improper and untreated disposal of sulfonamide drugs in animal feed or medical wastes has resulted in the contamination of water or soil, thus leading to the generation of resistant bacterial strains (Piddock 1996; Halling et al. 1998; Fent et al. 2006; Ye et al. 2007; Barnes et al. 2008; Focazio et al. 2008).

The biodegradation of sulfonamides present in wastewater treatment plants (WWTPs), surface water, manure, and soils was summarized by Jesús García-Galán et al. (2008). Sulfonamides appeared to resist natural biodegradation rather strongly, which was reflected in the high frequency of detection in streams and rivers (Kim et al. 2007; Vieno et al. 2007; Barnes et al. 2008; Focazio et al. 2008). Various studies have indicated that the removal of sulfonamides by adapted microorganisms or microbial communities during sewage treatment is incomplete (Richter et al. 2007, 2008a, b; Jesús García-Galán et al. 2008; Li and Zhang 2010). In addition to aerobic biodegradation, anaerobic biodegradation of para-toluenesulfonamide and benzenesulfonamide has been observed in natural aquifer slurries (Kuhn and Suflita 1989). In recent years, one specific strain, *Staphylococcus epidermidis*, has been studied for its better activity against tetracycline, ampicillin and sulfathiazole (Park and Choung 2007 and 2010). In addition, strains of *Escherichia* sp. and *Acinetobacter* sp. have been shown to degrade sulfapyridine and sulfathiazole (Zhang et al. 2011). Until now, no specific bacterial strain had been isolated with the capacity to degrade sulfadoxine.

The main objective of this study was to isolate, identify and characterize a bacterial strain capable of degrading sulfadoxine. HPLC was used to characterize the optimal temperature, pH, and initial concentrations of sulfadoxine and tryptone for sulfadoxine biodegradation. Cytotoxicity analysis was also carried out to determine whether the metabolites of sulfadoxine generated by the isolated strain (DX7) exhibited reduced cytotoxicity.

Materials and methods

Chemicals, bacterial strains and growth conditions

Analytical-grade sulfadoxine and chromatographic grade methanol were purchased from J&K Chemical Inc. (Beijing, China). All other chemicals were used at the highest commercially available purity. The mineral salt plus tryptone media (MTM) contained $Na_2HPO_4{\cdot}12H_2O$ 3 g l^{-1}, KH_2PO_4 1 g l^{-1}, NaCl 3 g l^{-1}, $MgSO_4$ 0.3 g l^{-1}, Tryptone 2.5 g l^{-1}, and the media was adjusted to pH 6.0–8.0. The mineral salt media used in this study was the same as that described by Wang et al. (2010). Growth of bacteria was monitored by measuring OD_{600} of culture using an ultraviolet–visible spectrophotometry (Beckman). Seawater collected from Yantai coastal zone near a fish farm was concentrated and plated onto MTM amended with 1.2% agar. After incubation at 30°C for 2 days, colonies that emerged were tested for sulfadoxine degradation activity using HPLC.

Determination of sulfadoxine concentration using HPLC

HPLC analysis was performed using a Waters 600 HPLC equipped with a 4.6 × 250 mm reverse-phase C18 column (Waters, USA) in conjunction with a UV detector monitoring at 280 nm. Cell-free supernatants were filtered through 0.45 μm nylon membranes prior to analysis. The mobile phase consisted of a methanol: acetate solution (acetic acid: H_2O, 1:1,000) at a ratio of 50:50. The flow rate was maintained at 1.0 ml min^{-1}. Quantification of samples was based on peak areas. The new peak that appeared on HPLC was detected by HPLC/MS, LCQ Fleet high sensitivity multistage ion trap mass spectrometer system (Thermo Fisher Scientific Corporation, USA). MS was operated in the electron spray ionization mode with a positive polarity and scanned by normal mass range from 100 to 2,000 m/z.

Identification of a bacterial strain capable of degrading sulfadoxine

Genomic DNA of the bacterial strain capable of degrading sulfadoxine was extracted according to the method described by Syn and Swarup (2000). The 16S rRNA gene of the isolated bacterium was amplified by PCR with primers 8F (5′-AGAGTTTGATCCTGG CTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGA CTT-3′) according to the method described by Lane et al. (1985). Sequencing was carried out by the Genomics Institute (Beijing, China).

Sulfadoxine biodegradation

DX7 cells were collected by centrifugation at 10,000×*g* for 5 min at room temperature after it was cultured in MTM at 30°C until reaching an OD_{600} of 1.0. The cell pellet was washed twice with sterilized phosphate buffer (PBS), and adjusted to an OD_{600} of approximately 1.0. Then, 10^7 CFU ml^{-1} cells were inoculated into the MTM containing 10 mg l^{-1} sulfadoxine and incubated at 30°C for 2 days. The same media containing 10 mg l^{-1} sulfadoxine without bacteria was used as control. The supernatants of the culture and control media were collected, filtered and stored for further analysis. To determine whether the reduced sulfadoxine concentration in the supernatant was due to absorption to the bacterial cells, the sulfadoxine associated with or in the bacterial cells was detected. DX7 was cultured in 10 ml MTM with sulfadoxine at 30°C for 2 days, and cells were pelleted by centrifugation at 10,000×*g* for 5 min. The cells were resuspended in 200 µl of buffer B (100 mM NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, adjusted pH to 8.0 using NaOH) and lysed for 1 h by gently shaking. Then, cell lysate was diluted with 1.8 ml of water and the mixture was sonicated using a sonicator equipped with a microtip with six 10 s bursts at 200–300 W and a 10 s cooling period between each burst. The lysate was centrifuged at 10,000×*g* for 10 min to pellet the cellular debris and the supernatant was then stored for HPLC analysis.

Effects of temperature, pH, and concentrations of sulfadoxine and tryptone on sulfadoxine biodegradation

The effect of temperature on sulfadoxine biodegradation by DX7 was determined under standard conditions, except that DX7 was cultured at temperatures ranging from 20 to 40°C. To determine the effect of pH, DX7 was inoculated into MTM of different pHs ranging from 5.0 to 9.0. To determine the effect of the initial sulfadoxine concentration, DX7 was inoculated into MTM containing different sulfadoxine concentrations ranging from 10 to 70 mg l^{-1}. To determine the effect of other nutrients, DX7 was inoculated into media, which was amended from MTM containing different tryptone concentrations ranging from 0.5 to 16 g l^{-1}. After culturing at 30°C for 2 days under all the above conditions, supernatants were collected by centrifugation at 10,000×*g* for 5 min and then filtered through 0.45 µm filter membranes and stored at −20°C for HPLC analysis. Degradation efficiency was expressed as the decrease of the sulfadoxine concentration in the supernatant from the bacterial culture relative to the sulfadoxine concentration in the supernatant from the sterile control.

Cytotoxicity analysis of sulfadoxine and its metabolites

Cytotoxicity analysis was performed according to the method described by Zhang et al. (2009). Briefly, Hela cells were cultured to confluence in 96-well plates. Supernatants from cultures of DX7 containing sulfadoxine and/or its metabolites were collected and ultrafiltered with Amicon Ultra-4 centrifugal filter devices (Millipore) to remove any molecules larger than 10 kDa. The supernatants from cultures of DX7 grown in MTM without sulfadoxine and from sulfadoxine containing MTM without inoculated bacteria were also collected. The treated supernatants were added to the HeLa cells; MTM was also added to the Hela cells and used as a control. After incubation at 37°C for 48 or 8 h, the cells were used either for the determination of viability using the MTT Cell Proliferation assay or for microscopic observation (Olympus, Japan). The cytotoxicity of the metabolites of sulfadoxine that were generated by the isolated strain (DX7) was expressed as the percentage of the cytotoxicity of sulfadoxine.

Nucleotide sequence accession number and statistical analysis

The nucleotide sequence of the 16S rRNA gene of DX7 has been deposited in the GenBank database

under the accession number JF742596. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc., USA). Differences were analyzed by Student's t-test. In all cases, the significance level was defined as $P < 0.05$.

Results and discussion

Isolation and identification of bacterium capable of decreasing sulfadoxine concentration

In order to isolate sulfadoxine degrading microorganisms, seawater samples were concentrated and plated onto sulfadoxine-containing MTM. Approximately ten colonies emerged and each was used to prepare a liquid culture. The sulfadoxine concentrations in the supernatants from the ten cultures were quantified using HPLC. The sulfadoxine concentration only decreased in the supernatant from the culture of DX7; however, the sulfadoxine concentration in the supernatants from the other nine cultures was the same as the sulfadoxine concentration in the supernatant from the sterile controls. From this, one strain (DX7), capable of decreasing the sulfadoxine concentration in MTM, was isolated (Fig. 1a, b). To examine whether DX7 could use sulfadoxine as a sole carbon source, DX7 was grown in mineral salt media broth with

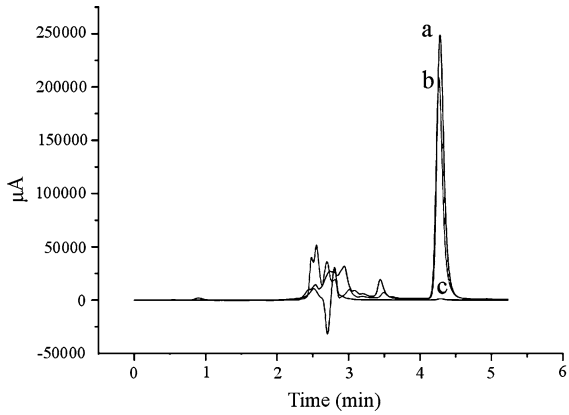

Fig. 1 HPLC analysis of the sulfadoxine concentration in the supernatant from sulfadoxine contained MTM without bacteria *a* , supernatant from *Pseudomonas* sp. DX7 culture grown in the sulfadoxine contained MTM *b*, and supernatant from the cell lysate of *Pseudomonas* sp. DX7 cultured in the sulfadoxine contained MTM *c*. Supernatants were collected and filtered through 0.45 μm membranes. Cell lysates of *Pseudomonas* sp. DX7 were prepared by cell lysis followed by sonication

10 mg l^{-1} sulfadoxine. However, no growth was observed under these conditions. This suggested that DX7 could not use sulfadoxine as the sole carbon source to propagate, and the growth of DX7 needed the presence of another carbon source, such as tryptone.

To identify DX7, the 16S rRNA gene of DX7 was amplified by PCR and sequenced. Comparison with known 16S rRNA gene sequence data indicated that the best match was to *Pseudomonas putida* strain SKG-1, *P. fluorescens* strain BHUJY29, and *Pseudomonas* sp. RBP1, all of which showed 99% identity to the 16S rRNA gene sequence of DX7.

Degradation of sulfadoxine by *Pseudomonas* sp. DX7

To confirm that the decrease of sulfadoxine concentration in the supernatant from the culture of *Pseudomonas* sp. DX7 was not due to bacterial adsorption, HPLC analysis was carried out to determine whether there was any sulfadoxine in and/or associated with the bacterial cells. The supernatant from the culture of *Pseudomonas* sp. DX7 contained 77% of the sulfadoxine concentration in the control supernatant (Fig. 1a, b). The cell lysate contained scarcely any sulfadoxine (Fig. 1c). This result confirmed the fact that the reduced sulfadoxine concentration in the culture was due to biodegradation by *Pseudomonas* sp. DX7 rather than simple biosorption. Combined with the fact that *Pseudomonas* sp. DX7 could not use sulfadoxine as the sole carbon source, it suggested that the sulfadoxine metabolism in *Pseudomonas* sp. DX7 may be co-metabolic pathway.

After incubation of *Pseudomonas* sp. DX7 for 2 days in MTM containing sulfadoxine, approximately 20–30% of the sulfadoxine was degraded. Moreover, a new peak at a retention time of approximately 6.3 min appeared on the HPLC that increased in size with increasing sulfadoxine concentration added to MTM (Fig. 2c, d). The new peak had a molecular ion at m/z of 446.11 (Fig. 3). However, HPLC analysis of both the supernatants from non-inoculated MTM containing sulfadoxine and from the *Pseudomonas* sp. DX7 culture grown in MTM without sulfadoxine showed that there was no peak at the a retention time of 6.3 min (Fig. 2a, b). The new peak appearing on the HPLC was postulated to be the secretion of *Pseudomonas* sp. DX7 that was stimulated by the presence of sulfadoxine in MTM or to be a

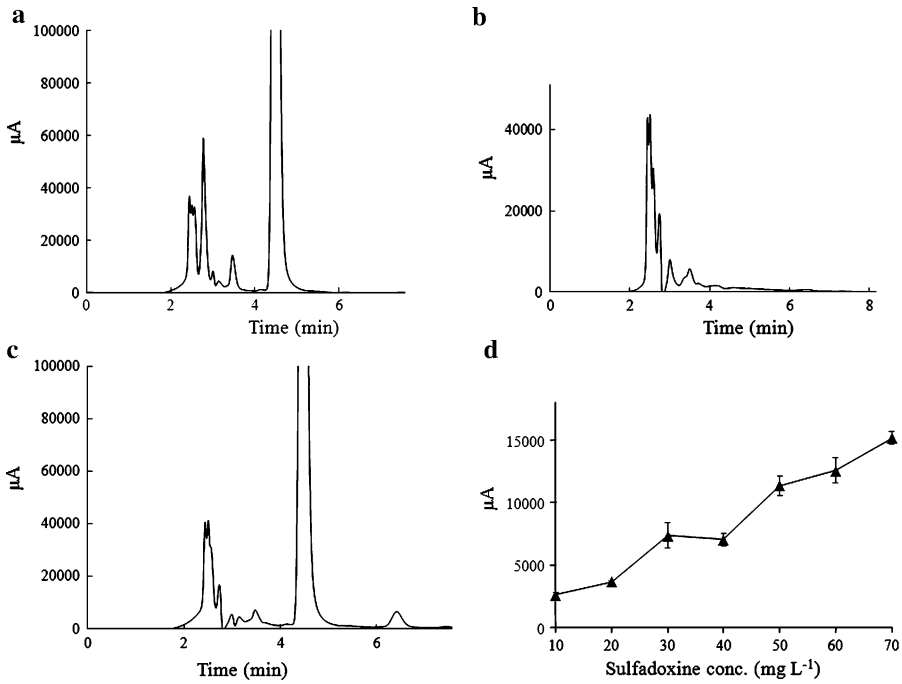

Fig. 2 HPLC analysis of supernatant from non-bacteria inoculated MTM containing 10 mg l^{-1} sulfadoxine (**a**), supernatant from *Pseudomonas* sp. DX7 culture grown in MTM without sulfadoxine (**b**), supernatant from *Pseudomonas* sp. DX7 culture grown in MTM containing 10 mg l^{-1} sulfadoxine (**c**). All of the supernatants were collected and filtered through 0.45 μm membranes. Peak area of the new emerged peak increased as the sulfadoxine concentration increased in MTM (**d**). Data are the means for three independent experiments and are presented as the means ±SE

sulfadoxine metabolite generated by *Pseudomonas* sp. DX7. As research focusing on the biodegradation of

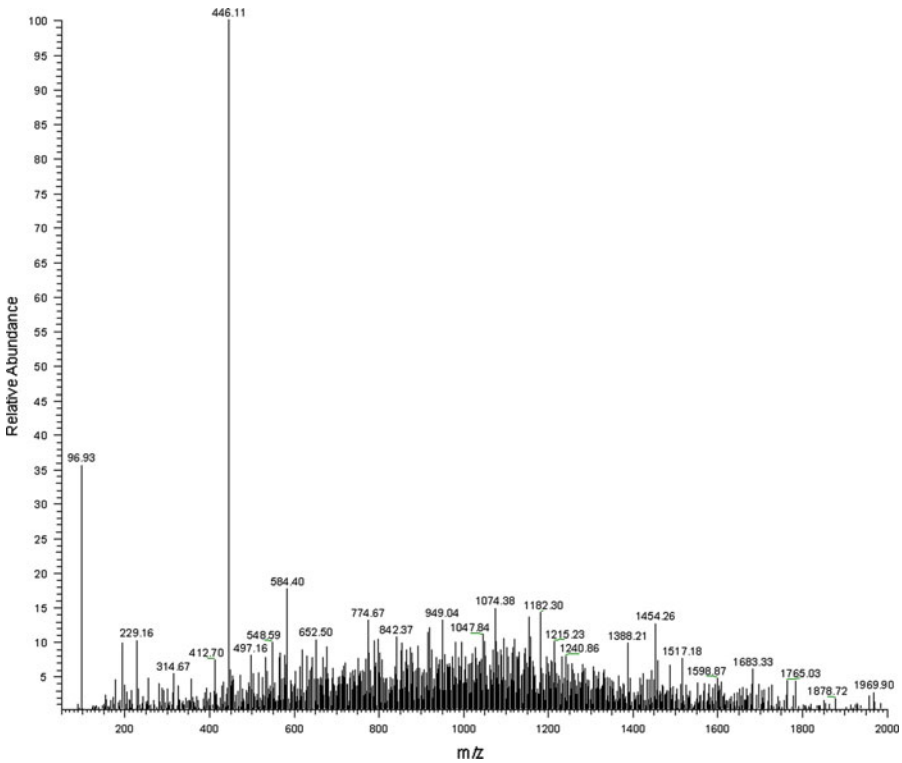

Fig. 3 MS detection of the new peak that appeared on HPLC. *Pseudomonas* sp. DX7 was inoculated into MTM containing sulfadoxine and cultured at 30°C for 2 days. Ultrafiltrated supernatant was subjected to LCQ Fleet high sensitivity multistage ion trap mass spectrometer system. MS was operated in the electron spray ionization mode with a positive polarity and scanned by normal mass range from 100 to 2,000 m/z

antibiotics and sulfonamides increases (Park and Choung 2007, 2010; Li and Zhang 2010; Zhang et al. 2011), the degradation of sulfadoxine by *Pseudomonas* sp. DX7 may be potentially exploited to eliminate sulfadoxine contamination from the environment.

Effects of temperature and sulfadoxine concentration on sulfadoxine biodegradation

To determine the effect of temperature on sulfadoxine biodegradation, 1×10^7 CFU ml^{-1} *Pseudomonas* sp. DX7 was inoculated into MTM containing 10 mg l^{-1} sulfadoxine and cultured under different temperatures ranging from 20 to 40°C. Temperature was found to have a strong impact on sulfadoxine biodegradation. As shown in Fig. 4, within the range of the tested temperatures, the degradation efficiency increased with the temperature up to 30°C, at which *Pseudomonas* sp. DX7 exhibited the maximum degradation efficiency. Once the temperature rose above 30°C, the degradation efficiency decreased precipitously and was barely detectable at 40°C. The effect of sulfadoxine concentration on sulfadoxine biodegradation was examined in MTM containing sulfadoxine at different concentrations. HPLC analysis showed that the degradation efficiency enhanced with increasing concentrations from 10 to 20 mg l^{-1}, and maintained at almost the same high efficiency state at the concentrations of 20–30 mg l^{-1} (Fig. 5). Once the initial

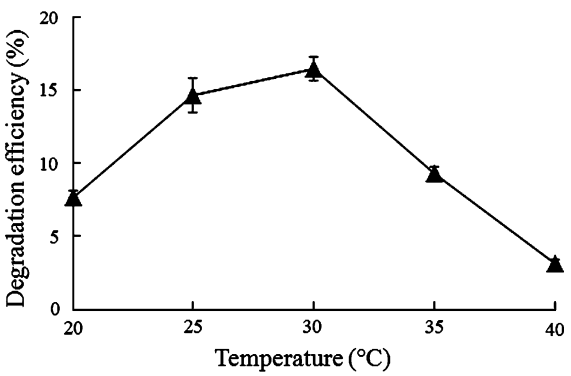

Fig. 4 Effect of temperature on sulfadoxine biodegradation. *Pseudomonas* sp. DX7 was cultured in MTM containing 10 mg l^{-1} sulfadoxine at various temperatures ranging from 20 to 40°C for 2 days. Supernatants were collected, filtered and analyzed for the remaining sulfadoxine concentration using HPLC. Data are the means for three independent experiments and are presented as the means ±SE

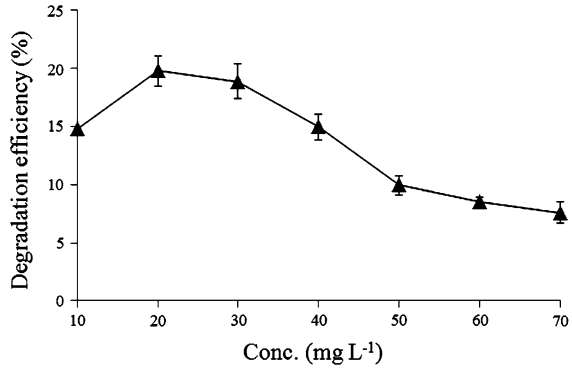

Fig. 5 Effect of initial sulfadoxine concentration on sulfadoxine biodegradation. *Pseudomonas* sp. DX7 was cultured at 30°C for 2 days in MTM containing 10, 20, 30, 40, 50, 60 and 70 mg l^{-1} sulfadoxine, respectively. Supernatants were collected, filtered and analyzed for the remaining sulfadoxine concentration using HPLC. Data are the means for three independent experiments and are presented as the means ±SE

sulfadoxine concentration in MTM was over 30 mg l^{-1}, the degradation efficiency of *Pseudomonas* sp. DX7 decreased. Thus, the optimal sulfadoxine concentration for the degradation by *Pseudomonas* sp. DX7 was at 30 mg l^{-1}. This phenomenon corresponded with the biomass of *Pseudomonas* sp. DX7 grown under different sulfadoxine concentrations, with 10–30 mg l^{-1} sulfadoxine showing no inhibitory effects, but above 30 mg l^{-1} sulfadoxine showing certain inhibitory effects. Thus it was speculated that the decreased degradation efficiency at concentrations over 30 mg l^{-1} was probably due to the reduced biomass under higher sulfadoxine concentrations. It also can be calculated from this result that when the initial sulfadoxine concentration added into MTM was over 30 mg l^{-1}, the converted sulfadoxine concentration remained at almost the same level. Combined with the result obtained in Fig. 2d, it was concluded that the new peak was likely the secretion of *Pseudomonas* sp. DX7 stimulated by the presence of sulfadoxine in MTM.

Effects of pH and tryptone concentration on sulfadoxine biodegradation

To determine the effect of pH on sulfadoxine biodegradation, a combination of MTM of different pHs was used. pH was found to have a strong impact on sulfadoxine biodegradation. As shown in Fig. 6, the degradation efficiency was highly pH dependent. The

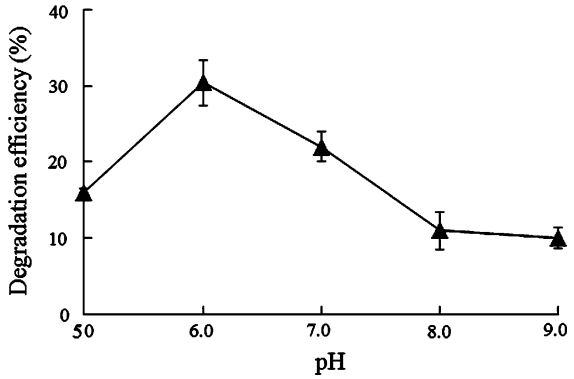

Fig. 6 Effect of pH on sulfadoxine biodegradation. *Pseudomonas* sp. DX7 was cultured at 30°C for 2 days in MTM of different pHs ranging from 5.0 to 9.0 containing 30 mg l^{-1} sulfadoxine. Supernatants were collected, filtered and analyzed for the remaining sulfadoxine concentration using HPLC. Data are the means for three independent experiments and are presented as the means ±SE

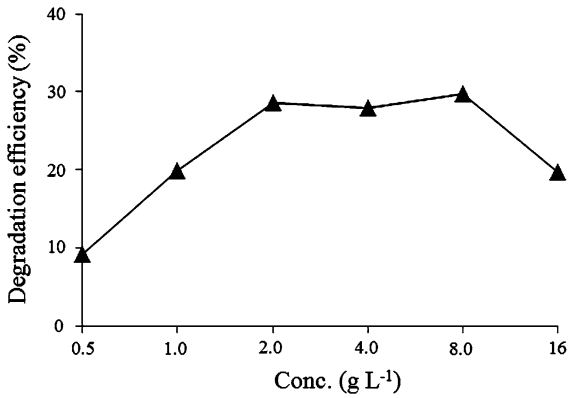

Fig. 7 Effect of initial tryptone concentration on sulfadoxine biodegradation. *Pseudomonas* sp. DX7 was cultured at 30°C for 2 days in media containing different tryptone concentrations ranging from 0.5 to 16 g l^{-1}. Supernatants were collected, filtered and analyzed for the remaining sulfadoxine concentration using HPLC. Data are the means for three independent experiments and are presented as the means ±SE

optimal pH for *Pseudomonas* sp. DX7 to degrade sulfadoxine was centered approximately 6.0; deviations from this optimum led to drastic diminutions in degradation efficiency. To determine the effect of tryptone concentration on sulfadoxine biodegradation, a combination of media with different tryptone concentrations was used. As shown in Fig. 7, tryptone was found to have a strong impact on sulfadoxine biodegradation. The degradation efficiency was also highly dependent on the tryptone concentration. *Pseudomonas* sp. DX7 degraded sulfadoxine at the highest efficiency when the tryptone concentration in the media was between 2.0 and 8.0 g l^{-1}. *Pseudomonas* sp. DX7 did not grow well in media containing 0.5 g l^{-1} tryptone, and perhaps the low biomass led to the poor degradation efficiency. However, the degradation efficiency was not solely dependent on the biomass of *Pseudomonas* sp. DX7, as the degradation efficiency decreased when the tryptone concentration in the media increased from 8.0 g to 16.0 g l^{-1}.

Cytotoxicity analysis of the metabolites of sulfadoxine generated by *Pseudomonas* sp. DX7

To investigate whether the metabolites of sulfadoxine generated by *Pseudomonas* sp. DX7 had cytotoxic effects, cytotoxicity analysis of sulfadoxine and its metabolites was carried out. The result of MTT method showed that the cytotoxicity of the supernatant from the *Pseudomonas* sp. DX7 culture grown in sulfadoxine-contained MTM remained only 50% of the cytotoxicity of supernatant from sulfadoxine-contained MTM without inoculated bacteria. Microscopic observations showed that incubation with sulfadoxine had a profound damaging effect on Hela cells and led to cell lysis at 8 h after the incubation (Fig. 8b). Supernatant from *Pseudomonas* sp. DX7 cultured in MTM containing sulfadoxine showed attenuated cytotoxicity to Hela cells according to the MTT Cell Proliferation assay, and the cell morphology was the same as that incubated with the supernatant from *Pseudomonas* sp. DX7 cultured in MTM without sulfadoxine (Fig. 8c, d). Because there was still 70% sulfadoxine remaining in the culture after *Pseudomonas* sp. DX7 degradation, it was postulated that the metabolites of sulfadoxine generated by *Pseudomonas* sp. DX7 possessed less or even negligible cytotoxicity to Hela cells.

Conclusions

One *Pseudomonas* sp. strain DX7 capable of degrading sulfadoxine was isolated, identified and characterized. The optimal temperature, pH, and concentrations of sulfadoxine and tryptone in the media for *Pseudomonas* sp. DX7 to degrade sulfadoxine were determined to be 30°C, 6.0, 30 mg l^{-1}, and between 2.0 and 8.0 g l^{-1}, respectively. The metabolites of sulfadoxine

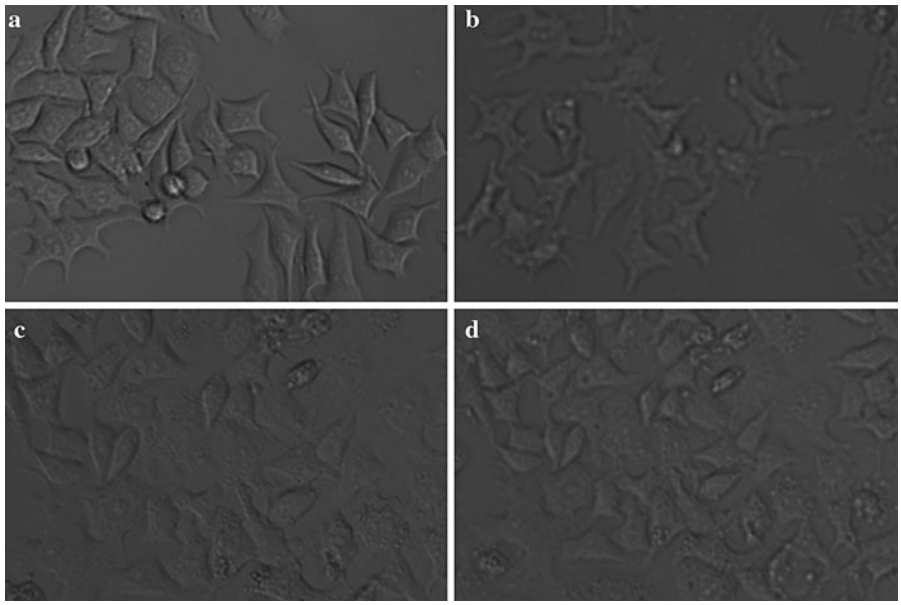

Fig. 8 Examination of the cytotoxic effect of sulfadoxine and its metabolites on cultured Hela cells by inverted microscopy. Hela cells were treated for 8 h with supernatants from different cultures and observed under an inverted microscope. Hela cells were incubated with MTM (**a**), supernatant from non-bacteria inoculated MTM containing 20 mg l^{-1} sulfadoxine (**b**), supernatant from *Pseudomonas* sp. DX7 culture grown in MTM containing 20 mg l^{-1} sulfadoxine (**c**), and supernatant from *Pseudomonas* sp. DX7 culture grown in MTM without sulfadoxine (**d**). Images are taken at 400 × magnification

that were generated by *Pseudomonas* sp. DX7 showed significantly less cytotoxicity to Hela cells compared to the sulfadoxine. This study suggested that *Pseudomonas* sp. DX7 might be useful for the bioremediation of sulfadoxine-contaminated environments.

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