Degradation of furazolidone by bacteria *Acinetobacter calcoaceticus* T32, *Pseudomonas putida* SP1 and *Proteus mirabilis* V7

Weiwei Zhang [a,b], Zongliang Niu [a,b], Kun Yin [a,b,c], Feng Liu [d], Lingxin Chen [a,b,*]

[a] *Key Laboratory of Coastal Zone Environmental Processes, Yantai Institute of Coastal Zone Research(YIC), Chinese Academy of Sciences(CAS), 17 Chunhui Road, Yantai 264003, PR China*
[b] *Shandong Provincial Key Laboratory of Coastal Zone Environmental Processes, YICCAS, Yantai Shandong 264003, PR China*
[c] *Graduate University of the Chinese Academy of Sciences, Beijing 100049, China*
[d] *Department of Aquaculture, China Agriculture University (Yantai), 2006 Binhai Road, Yantai 264670, China*

ARTICLE INFO

ABSTRACT

Furazolidone (FZD) has been widely used as an antibacterial and antiprotozoal feed additive for poultry, cattle and farmed fish. Since FZD has been shown to have mutagenic, genotoxic and potentially carcinogenic properties when tested in a variety of systems, there is an increasing need to find a way to remove FZD from contaminated environments. In this report, three bacterial strains *Acinetobacter calcoaceticus* T32, *Pseudomonas putida* SP1 and *Proteus mirabilis* V7 capable of degrading FZD effectively were isolated, identified and characterized. The reduced FZD concentration after degradation was determined by HPLC. After bacterial cells were grown in the media containing 5 mg l^{-1} FZD for 5 days, almost all FZD was degraded by *A. calcoaceticus* T32, and more than 50% of FZD was degraded by *P. putida* SP1 and *P. mirabilis* V7, respectively. Bacterial GST activity of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 was determined to be influenced by different FZD concentrations. Cytotoxicity analysis showed that FZD was degraded to the metabolites with far less cytotoxicity compared to FZD. The inoculation of bacterial strains *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 into FZD-contained media resulted in a higher degradation efficiency than natural degradation, which indicated the potential application of these strains in treatment of FZD-polluted freshwater or seawater environments.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Furazolidone (FZD), namely 3-(5-nitrofurfurylideneamino)-2-oxazolidinone, has been used for more than 30 years as an anti-protozoal and antibacterial medicine for animal and human (DrugBank, 2008). FZD can also protect the animals against the protozoiasis, bacterial gill-rot disease and red skin disease in aquaculture (Meng et al., 1998). FZD was also identified as an important human medicine during World Health Organization (WHO) Expert Consultations on "Critically Important Antimicrobials for Human Medicine" in Canberra, Australia, in 2005 and in Copenhagen, Denmark, in 2007 (WHO, 2005, 2007). FZD is believed to be a valuable alternative for patients who need retreatment for *Helicobacter pylori* eradication (Eisig et al., 2005; Machado et al.,

2008; O'Connor et al., 2009). Recently, some studies have been carried out to determine whether FZD can be used to treat leishmaniasis in human (Reimão et al., 2010). FZD works depending on its ability to crosslink DNA, especially bacterial DNA which is particularly susceptible to this drug, and leads to high levels of mutations (DrugBank, 2008). However, FZD and its metabolites 3-Amino-2-oxazolidinone (AOZ) have shown some toxic effects on the animals and induced oxidative DNA damage (Ali, 1999; Karamanakos, 2008; Jin et al., 2011). Due to the carcinogenic and mutagenic properties and its retention in the animal tissue, FDA had withdrew the approval of FZD in 1991 and later in 2002 FZD was prohibited in the field of food-producing animals (Andrew and von Eschenbach, 2008). In China, from the year 2002, usage of FZD in the aquaculture has also been banned (Wu and Yang, 2006).

Since the assimilation of FZD by animal and human was at a very low rate, the major part of the drug used in farms was ultimately released into the environments (Lunestad, 1992). Removal of FZD from environments by microorganisms was studied previously (Samuelsen et al., 1991; Lunestad et al., 1995; Halling-Sørensen et al., 1998). FZD was actively metabolized by microorganisms and the T$_{1/2}$

* Corresponding author. Key Laboratory of Coastal Zone Environmental Processes, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, 17 Chunhui Road, Yantai 264003, PR China. Tel.: +86 535 2109130; fax: +86 535 2109000.

E-mail addresses: wwzhang@yic.ac.cn (W. Zhang), nzl5423@163.com (Z. Niu), hanmiltion@163.com (K. Yin), liufeng0324@hotmail.com (F. Liu), lxchen@yic.ac.cn (L. Chen).

of FZD in both the sediment and seawater environments was between 50 h and 2 months influenced by the biodegradation process. For example, FZD was reported to lose its antibacterial activity during the experimental period of 21 days in seawater. Although microorganisms played an important role in FZD degradation, only one specific bacterial strain *Pseudomonas aeruginosa* F5 capable of degrading FZD had been isolated, identified and characterized. The isolated strain was expected to remove FZD effectively from the environments (Jiang et al., 2007).

The main objective of this study was to isolate, identify and characterize bacterial strains capable of degrading FZD, and to explore their potential application in the elimination of FZD. Changes of bacterial glutathione-S-transferase (GST) activity induced by the presence of FZD were also investigated. Furthermore, cytotoxicity analysis was also carried out to determine whether there was any reduction in the cytotoxicity of the metabolites of FZD that was generated by the bacterial strains.

2. Materials and methods

2.1. Chemicals, media and strains

Analytical-grade FZD and chromatographic grade acetonitrile were purchased from J&K Chemical Inc. (Beijing, China). All other chemicals used were at the highest purity available commercially. The mineral salt plus tryptone media (MTM) used in this study 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} and tryptone 2.5 g l^{-1}, and the media was adjusted to pH 7.0. The mineral salt media used in this study was prepared as described by Moreira et al. (2012). ZoBell 2216E media contained tryptone 5 g l^{-1}, yeast extract 1 g l^{-1}, $FePO_4$ 0.01 g l^{-1} and aged seawater 1 l.

2.2. Isolation and identification of bacterial strains

The collected water samples were plated onto MTM amended with 1.2% agar and 5 mg l^{-1} FZD. The emerged colonies were picked out to exam the FZD degradation ability. Genomic DNA of the isolated strains was extracted according to the method described by Syn and Swarup (2000). The 16S rRNA genes of bacterial strains were amplified by PCR with primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) according to the method described by Lane et al. (1985) respectively. Sequencing was carried out by Genomics Institute (Beijing, China).

2.3. FZD degradation by bacterial strains

The bacterial cells of *Acinetobacter calcoaceticus* T32, *Pseudomonas putida* SP1 and *Proteus mirabilis* V7 were collected by centrifugation at 10,000 × g for 5 min at room temperature after cultured in MTM at 28 °C till OD_{600} of 1.0, respectively. The cell pellets were washed twice with sterilized phosphate buffer (PBS), and adjusted to OD_{600} of approximately 1.0. Then 1.0×10^7 CFU ml^{-1} cells were grown in MTM containing 5 mg l^{-1} FZD at 28 °C. The same media containing 5 mg l^{-1} FZD without inoculation of bacterial cells was used as control. Supernatants from the culture and control media were collected, filtered and stored for HPLC analysis. Relative FZD concentration was expressed as percentage of 5 mg l^{-1} FZD that was initially added into MTM. To determine the effect of initial FZD concentration on FZD degradation, bacterial cells were grown in MTM containing 1, 5, 10, 20 and 40 mg l^{-1} FZD respectively for 2 days. Relative FZD concentration was expressed as percentage of the each FZD concentration that was initially added into the MTM. To determine whether the decreased FZD concentration in the supernatant was due to accumulated by bacterial cells,

FZD associated with and in the bacterial cells was detected as described by Zhang et al. (2012). Briefly, bacterial cells were cultured in 10 ml MTM containing 5 mg l^{-1} FZD at 28 °C for 2 days, and cells were pelleted by centrifugation at 10,000 × g for 5 min. The cells were resuspended in 200 μl 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 water and the mixture was sonicated using a sonicator equipped with a microtip with sixty 2 s bursts at 200 W and a 15 s cooling period between each burst. The cell lysate was centrifuged at 10,000 × g for 10 min to pellet the cellular debris and the supernatant was then stored for HPLC analysis.

2.4. Determination of FZD using HPLC

Detection of FZD using HPLC was carried out as the description of Kuhne et al. (1992). Briefly, HPLC was performed using Waters 600 equipped with a 4.6 mm × 250 mm reverse-phase C18 column (Waters, USA) in conjunction with a UV detector monitoring at 365 nm. The mobile phase consisted acetonitrile:acetate solution (acetate acid:pure water = 1:1000) at a ratio of 55:45. The flow rate was maintained at 1.0 ml min^{-1}. Supernatant was filtered through 0.22 μm nylon membrane prior to analysis. The retention time of FZD by HPLC analysis under these conditions was 4.06 min. Relative FZD concentration was quantified based on the peak area and was expressed as the percentage of peak area of initial FZD concentration in control media, i.e. the FZD contained MTM without inoculation of bacterial cells.

2.5. Measurement of bacterial GST activity

A. calcoaceticus T32, *P. putida* SP1 and *P. mirabilis* V7 was separately cultured at 28 °C untill OD_{600} reached 1.0, and bacterial cells were inoculated into MTM containing 0, 1, 5, 10, 20 and 40 mg l^{-1} FZD to a concentration of 1.0×10^7 CFU ml^{-1} respectively. After cultured at 28 °C for 12 h, cell density was monitored by measuring the OD_{600} with the UV–Vis recording spectrophotometer (Beckman, USA). Relative absorbance was expressed as the percentage of the absorbance of culture that was grown in MTM without FZD. Two milliliters cell culture was mixed with 4 ml 100 mM potassium phosphate buffer with 1.0 mM ethylenediaminetetraacetic acid and 1 mg ml^{-1} lysozyme (pH 6.5; 25 °C), and incubated on ice for 30 min. Sonication of the mixture sixty 2 s bursts at 200 W with a 15 s cooling period between each burst on ice using a sonicator equipped with a microtip (Xinzhi, Ningbo, China). Then the GST activity of the cell lysate was measured according to the description of Jiancheng kit (Nanjing, China) using glutathione (GSH) and 1-chloro-2,4-m-Dinitrobenzene (CDNB) as the substrates. One unit of GST was defined as the decrease of 1 μmol l^{-1} GSH per minute at 37 °C. Relative GST activity was expressed as the percentage of the GST activity of bacterial cells cultured in MTM without FZD.

2.6. Cytotoxicity analysis of FZD and its metabolites generated by bacterial strains

Cytotoxicity analysis was performed according to the method described by Zhang et al. (2009). Briefly, supernatants containing FZD and/or the metabolites from bacterial culture and control media were collected and were ultrafiltered with Amicon Ultra-4 centrifugal filter devices (Millipore) to remove any molecules larger than 10 kDa. Then the treated supernatants and MTM was separately added to the wells containing confluent Hela cells. After incubation at 37 °C for 8 h and 48 h, the cells were used for microscopic observation under inverted microscope and for the determination of viability using the MTT Cell Proliferation. Relative

cytotoxicity of the supernatants from bacterial cultures grown in MTM containing FZD was expressed as percentage of supernatant from control media, i.e. FZD contained MTM without inoculation of bacterial cells.

2.7. Nucleotide sequence numbers, strain accession numbers and statistical analysis

The nucleotide sequences of the 16S rRNA genes of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 have been deposited in the GenBank database under accession numbers HQ902142, HM217131 and HM217132, respectively. *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 isolates were deposited with the China General Microbiological Culture Collection (CGMCC, Beijing, China) with accession number were CGMCC No. 4311, 3887 and 4312 respectively. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc., USA). Differences were analyzed by Student's *t*-test.

3. Results

3.1. Screening, isolation and identification of bacterial strains capable of decreasing FZD concentration

HPLC analysis showed that the FZD concentration in the supernatants from the cultures of three bacterial isolates T32, SP1 and V7 decreased compared to FZD in the supernatant from FZD contained MTM without inoculation of bacterial cells. To examine whether the isolated bacterial strains could use FZD as sole carbon source, T32, SP1 and V7 was separately grown in mineral salt media amended with 5 mg l^{-1} FZD, but no growth of the three isolated bacterial strains was observed. This result suggested that all the isolated strains could not use FZD as the sole carbon source to propagate. In order to genetically position the isolates within the genus, the 16S rRNA genes of T32, SP1 and V7 were amplified by PCR respectively and the PCR products were purified and submitted directly for sequencing. Comparison with the known 16S rRNA gene sequence data indicated that the best matches for the 16S rRNA gene sequence of T32, SP1 and V7 were those of *A. calcoaceticus*, *P. putida* and *P. mirabilis*, respectively. In order to determine whether *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 could reduce FZD in marine environments, bacterial cells were inoculated into 2216E media containing 5 mg l^{-1} FZD. HPLC analysis showed that FZD concentration in 2216E media also significantly decreased after inoculation of bacterial cells, which suggested the potential application of the three strains in removal of FZD in the marine environments.

3.2. Degradation of FZD by bacterial strains

FZD degradation by bacterial strains with time increasing was investigated. Bacterial cells were inoculated into MTM containing 5 mg l^{-1} FZD and grown at 28 °C in the dark for the selected time points. The result of HPLC analysis showed that the differences between the FZD concentrations at different time points in the control media were between 1- and 0.9-fold for a five-day observation, suggesting that the tested environmental factors had minor effect on stability of FZD; however, inoculation of bacterial cells into the media caused FZD concentration significantly decreased with time increasing. After incubated with bacterial cells for 3 days, almost all FZD was degraded by *A. calcoaceticus* T32, 88.7% FZD was degraded by *P. putida* SP1, and 82% FZD was degraded by *P. mirabilis* V7, respectively. *A. calcoaceticus* T32 possessed the highest FZD-degradation ability, and 95% of total FZD was degraded after it was cultured for 24 h (Fig. 1). To confirm that the reduced FZD in the
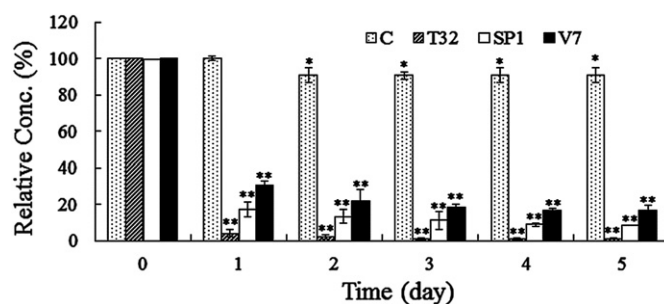

Fig. 1. FZD degradation with time increasing. Bacterial cells of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 were separately inoculated into 5 mg l^{-1} FZD contained MTM at concentration of 1.0×10^7 CFU ml^{-1}. MTM containing 5 mg l^{-1} FZD without inoculation of bacterial cells was used as control. Supernatants were collected at intervals of 1 day and analyzed for remaining FZD using HPLC. Relative FZD concentration was expressed as the percentage of 5 mg l^{-1} FZD that was initially added into MTM. Data are the means for three independent experiments and are presented as the means ± SE. *, $P < 0.05$; **, $P < 0.01$.

supernatants from bacterial cultures was due to degradation by bacterial strains, HPLC analysis was carried out to determine whether there was any FZD in and associated with the bacterial cells. The result showed that cell lysate contained scarcely any FZD, which confirmed the fact that the reduced FZD in the culture was due to degradation by bacterial cells rather than simple accumulation. Meanwhile, the colour of FZD in the supernatants from bacterial culture weakened or changed to be colorless after FZD was degraded, while the colour of the supernatant from control media still remained to be yellow.

3.3. Effect of FZD concentration on FZD degradation

To determine the effect of initial FZD concentration on FZD degradation, bacterial cells were grown in MTM amended with different FZD concentrations. After the bacterial cells were cultured at 28 °C for 2 days, supernatants were collected and applied for HPLC analysis. The results showed that the three bacterial strains possessed highest FZD degradation efficiency at relative lower concentration (1 mg l^{-1}) and the degradation efficiency gradually decreased as FZD concentration in the media increased (Fig. 2). *A. calcoaceticus* T32 possessed the highest ability to degrade FZD at every selected concentration. After cultured for 24 h, *A. calcoaceticus*
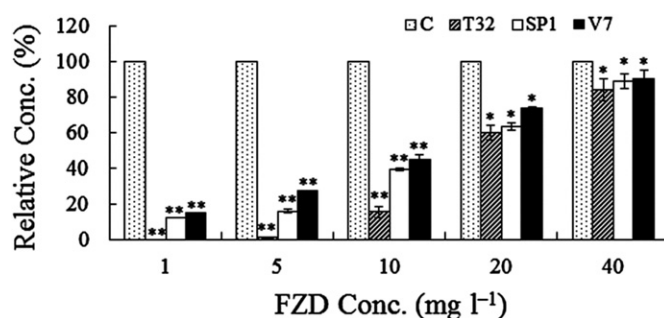

Fig. 2. Effect of initial FZD concentrations on FZD degradation. 1.0×10^7 CFU ml^{-1} bacterial cells of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 were cultured in MTM containing 1, 5, 10, 20 and 40 mg l^{-1} FZD, respectively. MTM containing 1, 5, 10, 20 and 40 mg l^{-1} FZD without inoculation of bacterial cells was used as control. After cultured at 28 °C for 2 days, supernatants were collected, filtered and analyzed for the remaining FZD using HPLC. Relative FZD concentration was expressed as the percentage of 1, 5, 10, 20 and 40 mg l^{-1} FZD that was initially added into MTM respectively. Data are the means for three independent experiments and are presented as the means ± SE. *, $P < 0.05$; **, $P < 0.01$.

T32 degraded almost all of the FZD that was initially added into MTM at concentrations of 1 mg l^{-1} and 5 mg l^{-1}.

3.4. Bacterial GST activity response to FZD

To investigate the bacterial GST activity response to FZD, *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 was cultured at 28 °C in MTM containing different FZD concentrations for 12 h respectively, and then OD_{600}, HPLC and GST activity was analyzed. OD_{600} of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures grown in MTM without FZD was 0.63, 0.60 and 0.42 respectively and these values were considered to be 100% in the analyses of growth state under different FZD concentrations. Fig. 3a indicated that FZD showed no significant effect on the growth of bacterial strains at lower concentrations, and showed inhibitory effect on the growth of bacterial strains at relative higher concentrations. The inhibitory FZD concentration showed certain strain specific. When FZD concentration rose up to 40 mg l^{-1}, it showed inhibitory effect on the growth of *A. calcoaceticus* T32 and *P. putida* SP1, while 5 mg l^{-1} FZD showed obviously inhibitory effect on the growth of *P. mirabilis* V7. Meanwhile, FZD degradation in the media by bacterial strains *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 was the same as that described in Fig. 2.

Enzymatic analyses showed that cell lysate of *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 grown in MTM without FZD exhibited GST activity of 4.24 μmol min^{-1} ml^{-1}, 4.48 μmol min^{-1} ml^{-1} and 6.72 μmol min^{-1} ml^{-1} respectively. Different bacterial strains showed varied GST activity with the changes of FZD concentrations. Within the range of the tested concentrations, GST activity of
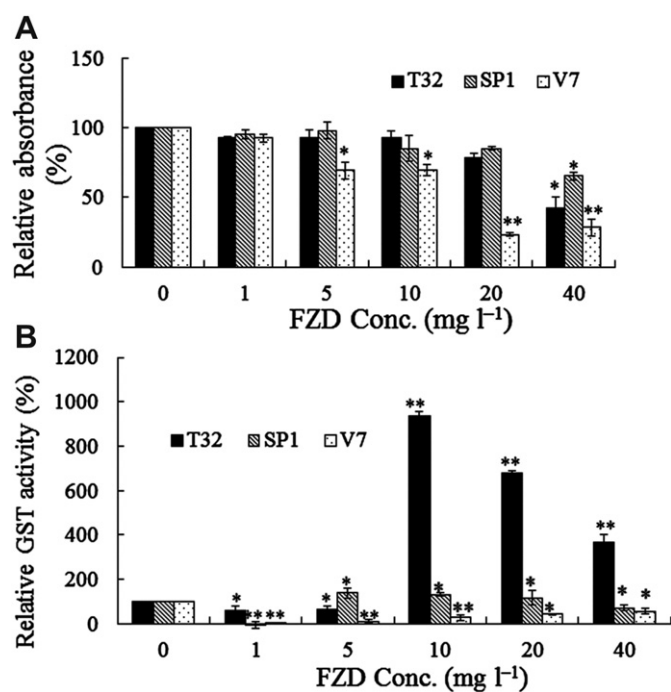

Fig. 3. Effects of FZD on bacterial growth (A) and bacterial GST activity (B). Bacterial cells were cultured in MTM containing 0, 1, 5, 10, 20 and 40 mg l^{-1} FZD respectively at 28 °C for 12 h. (A) Aliquots of cell culture were collected and measured for OD_{600}. Relative absorbance was expressed as the percentage of absorbance (OD_{600}) of cultures grown in MTM without FZD, which was 0.63, 0.60 and 0.42 for *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 respectively. (B) Cells were broken and cell lysate was measured for GST activity. Relative GST activity was expressed as the percentage of GST activity of bacterial cells grown in MTM without FZD, which was 4.24, 4.48 and 6.72 μmol min^{-1} ml^{-1} for *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 respectively. Data are the means for three independent experiments and are presented as the means ± SE. *, $P < 0.05$; **, $P < 0.01$.

A. calcoaceticus T32, strain with the highest FZD degradation efficiency, was significantly stimulated as the FZD concentrations in the media rose to and above 10 mg l^{-1}. GST activity of *P. putida* SP1 response to different FZD concentrations exhibited the same trend as that of *A. calcoaceticus* T32, but reached maximum at the concentration of 5 and 10 mg l^{-1}. GST activity of *P. mirabilis* V7 was inhibited by FZD at all the tested concentrations, but showed stimulated effect as the concentration increasing (Fig. 3b).

3.5. Cytotoxicity analysis of the metabolites of FZD generated by bacterial strains

Cytotoxicity analysis of FZD and its metabolites was carried out to determine whether the metabolites of FZD generated by bacterial strains had any reduction in the cytotoxic effects. The supernatants from *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures grown in 5 mg l^{-1} FZD contained MTM remained 0.96%, 27% and 36.7% of FZD compared to the FZD concentration in the control media (Fig. 4a). The MTM and supernatants from control media and from *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures were applied to Hela cells. MTT Cell Proliferation analysis showed that treatment with supernatant from control media significantly reduced the viability of Hela cells, with 35% of the cell viability reduced compared to the MTM treated cells, and the cytotoxicity of the supernatant from control media was considered to be 100% in the following cytotoxicity analyses. However, treatment with supernatants from the *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures grown in 5 mg l^{-1} FZD contained media showed significantly reduced cytotoxicity to the Hela cells, with 6.4%, 27% and 36% cytotoxicity remained respectively (Fig. 4b). Consistently, microscopic observation showed that incubation with supernatant from control media imposed a profound damaging effect on Hela cells and led to complete changes of cell shape at 8 h after the incubation; however, supernatants from the *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures grown in the FZD contained media showed far less negative effect on the cells and most of the cells remained the normal shape (Fig. 4c). Considering the fact that there was still FZD remaining in the cultures after degraded by bacterial strains, the much less negative effect, as shown in Fig. 4c 3–5, was postulated to be the effect caused by FZD remained in the supernatants. Together these results it was demonstrated that the isolated strains *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 degraded FZD to far less toxic metabolites that were harmless at cellular level compared to FZD.

4. Discussion

Although the usage of FZD is prohibited in numerous countries for its genotoxic carcinogen, there are indications of its illegal use in the world. The half life of FZD in the environments varied from hours to months in specific circumstances (Samuelsen et al., 1991; Lunestad et al., 1995; Halling-Sørensen et al., 1998). In this study, three bacterial strains were isolated and characterized to degrade FZD effectively both in the fresh and seawater environments, which was consistent with the fact that these isolates were from the interaction region of river and sea. The investigation of FZD degradation by the isolated bacterial strains *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 showed that FZD degradation significantly increased after inoculation of bacterial cells compared to the natural FZD degradation in simulate water environment and in natural seawater, in which condition FZD degradation needed almost 5–20 days (Lunestad, 1991; Choo, 1998; Lin et al., 2007). Compared to the FZD degrading strain *P. aeruginosa* F5 isolated by Jiang et al. (2007), *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 degraded 1 mg l^{-1} FZD at higher efficiency, with 99.6% FZD degraded by
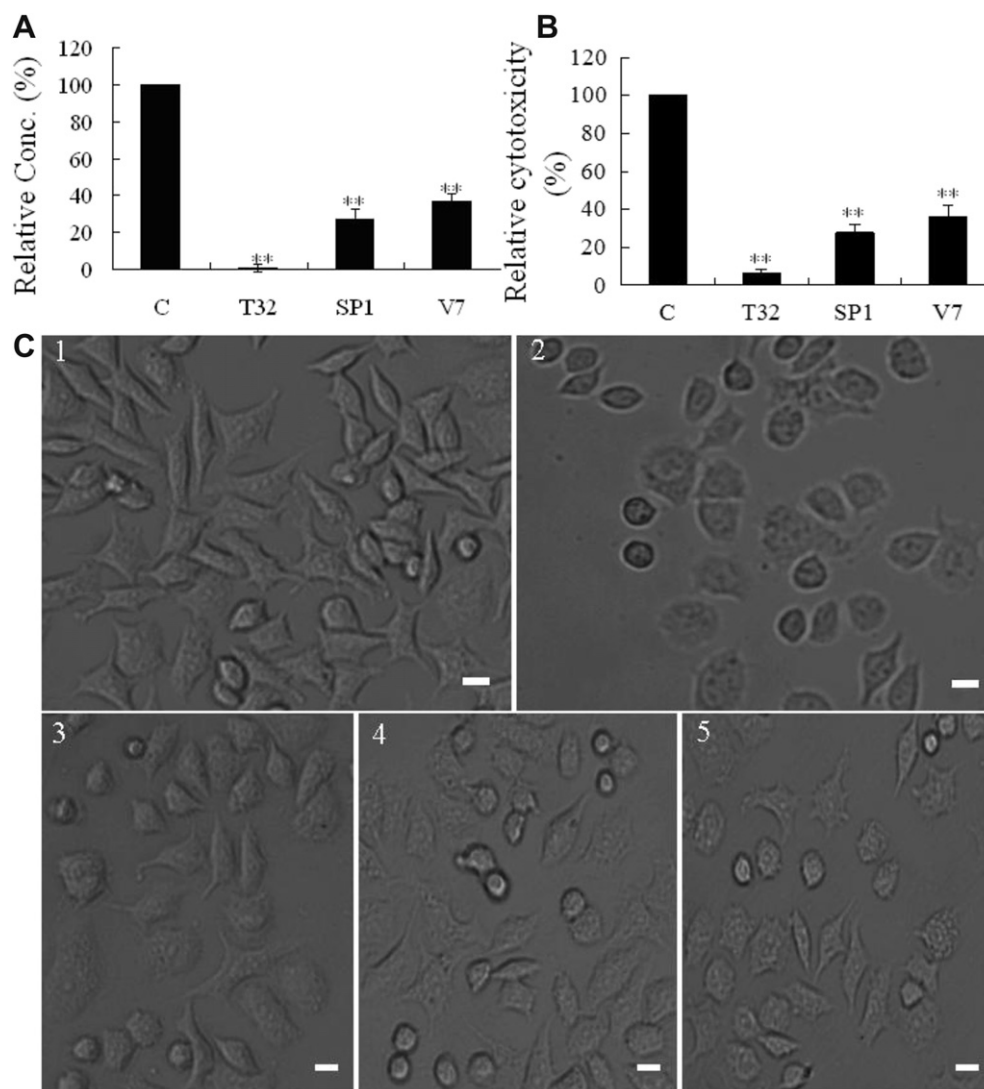
Fig. 4. (A) HPLC analysis of FZD concentration in supernatants. Relative FZD concentration was expressed as the percentage of FZD in MTM without inoculation of bacterial cells, which was added in an initial concentration of 5 mg l^{-1} and was used as control. (B) Examination of the cytotoxic effect of FZD and its metabolites on cultured Hela cells by cell viability analysis. Viabilities of the cell after treatment were determined by MTT method. The relative cytotoxicity was expressed as the percentage of cytotoxicity of the supernatant from FZD contained MTM without inoculation of bacterial cells, which reduced 35% of the cell viability and was used as control. Data are the means of three independent assays and presented as the means ± S.E. *, $P < 0.05$; **, $P < 0.01$. (C) Examination of the cytotoxic effect of FZD and its metabolites on cultured Hela cells by microscopy observation. Hela cells were incubated with (1) MTM, (2) supernatant from control media containing 5 mg l^{-1} FZD, (3–5) supernatants from *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 cultures grown in MTM containing 5 mg l^{-1} FZD. The scale bars represent 20 μm.

A. calcoaceticus T32, 87.5% FZD degraded by *P. putida* SP1 and 85% FZD degraded by *P. mirabilis* V7 in 1 day, while *P. aeruginosa* F5 degraded 64.1% FZD in 30 days. FZD degradation by *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 strains needed the presence of other carbon source, because of their defective growth using FZD as the sole carbon source. So it was postulated that the FZD degradation occurred in the isolated bacterial strains belonged to the co-metabolic pathway, which was consist with that in the bacterial strain *P. aeruginosa* F5 (Jense, 1963; Jiang et al., 2007).

GSTs are part of a superfamily of enzymes that play a key role in cellular detoxification. GSTs are widely distributed in prokaryotes and bacterial GSTs are implicated in a variety of distinct processes such as the biodegradation of xenobiotics, protection against chemical and oxidative stresses and antimicrobial drug resistance (Piccolomini et al., 1989; Vuilleumier, 1997; Allocati et al., 2009). The result obtained in this study indicated that bacterial GST activity was influenced by the presence of different FZD concentrations and thus GST might be involved in FZD degradation by bacterial strains. Since bacterial GST was known to form pesticide/herbicides-glutathione conjugates, bacterial GST contributed to the detoxification of FZD in the bacterial strains, perhaps by the way of forming FZD-glutathione conjugates. Moreover, FZD was degraded to the metabolites with far less cytotoxicity. To our knowledge, this was the first time that the metabolites of FZD were subjected to cytotoxicity test and also showed much attenuated cytotoxic effects compared to FZD. But whether FZD degradation pathway in bacterial strains was the same as that in the body of the target organisms, in where FZD was degraded to AOZ, still remains to be investigated (Hu et al., 2007; Jin et al., 2011).

5. Conclusions

Three bacterial strains *A. calcoaceticus* T32, *P. putida* SP1 and *P. mirabilis* V7 were isolated, identified and were characterized to be

capable of degrading FZD. The isolated bacterial strains degraded FZD with higher efficiency, especially *A. calcoaceticus* T32 which could degrade 99% FZD that was initially added into media at a concentration of 5 mg l^{-1} in 3 days. Bacterial GSTs activity was determined to be varied with FZD concentration and possessed strain specificity. FZD was degraded by bacterial strains into the metabolites with far less cytotoxicity. The results obtained in this research help in understanding the characteristics of FZD degrading bacterial strains and exploring application potential of bacterial strains in removal of FZD from contaminated environments.

Acknowledgments

References

Ali, B.H., 1999. Pharmocological therapeutic and toxicological properties of furazolidone. Veterinary Research Communications 23, 343–360.

Allocati, N., Federici, L., Masulli, M., Di Ilio, C., 2009. Glutathione transferases in bacteria. FEBS Journal 276, 58–75.

Andrew, C., von Eschenbach, M.D., 2008. Enhanced Aquaculture and Seafood Inspection-report to Congress. Seafood Regulations & Guidance. Online at. http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/SeafoodRegulatoryProgram/ucm150954.htm.

Choo, P.S., 1998. Degradation of furazolidone in fresh- and seawater. Asian Fisheries Science 11, 295–301.

DrugBank: Showing Furazolidone (DB00614). Online at http://www.drugbank.ca/drugs/DB00614

Eisig, J.N., Silva, F.M., Rodriguez, T.N., Hashimoto, C.L., Barbuti, R.C., 2005. A furazolidone-based quadruple therapy for *Helicobacter pylori* retreatment in patients with pepticulcer disease. Clinics 60, 485–488.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-A review. Chemosphere 36, 357–393.

Hu, X.Z., Xu, Y., Yediler, A., 2007. Determinations of residual furazolidone and its metabolite, 3-Amino-2-oxazolidinone (AOZ), in fish feeds by HPLC-UV and LC-MS/MS, respectively. Journal of Agricultural and Food Chemistry 55, 1144–1149.

Jense, H.L., 1963. Carbon nutrition if some microorganisms decomposting halogen-substituted aliphatic acids. Acta Agriculturae Scandinavica 13, 404–412.

Jiang, L.J., Zhang, T.Q., Yang, L.Y., Xiao, L., Pan, J.L., Yin, D.Q., 2007. The isolation and identification of furazolidone degrading bacteria F5 and optimization of its degradation condition. Freshwater Fisheries 37, 36–40.

Jin, X., Tang, S.S., Chen, Q., Zou, J.J., Zhang, T., Liu, F.Y., Zhang, S., Sun, C.D., Xiao, X.L., 2011. Furazolidone induced oxidative DNA damage via up-regulating ROS that caused cell cycle arrest in human hepatoma G2 cells. Toxicology Letters 201, 205–212.

Karamanakos, P.N., 2008. Furazolidone and serotonin syndrome: is there any association? Clinics 63, 553–554.

Kuhne, M., Kobe, A., Fries, R., Ebrecht, A., 1992. Detection of oxytetracycline and furazolidone in poultry by HPLC. Archivfür Lebensmittelhygiene 43, 59–63.

Lane, D., Pace, B., Olsen, G., Stahl, D., Sogin, M., Pace, N., 1985. Rapid determination of 16S ribosomal sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences 82, 6955–6959.

Lin, H., Zhang, T.Q., Zhou, G., Zhu, Q.S., Li, Q., 2007. A preliminary study on the nature degradation rule of furazolidone in stimulant water environment. Jiangsu Agricultural Sciences 4, 239–241.

Lunestad, B.T., 1991. Fate and effects of antibacterial agents in aquatic environment. In: Alderman, J., Michel, C. (Eds.), Chemotherapy in Aaquaculture: From Theory to Reality. Office International Des Epizooties, Paris, pp. 151–161.

Lunestad, B.T., 1992. Environmental Effects of Antibacterial Agents Used in Aquaculture. PhD thesis, Department of Microbiology and Plant Physiology, University of Bergen, Norway.

Lunestad, B.T., Samuelsen, O.B., Fjelde, S., Ervik, A., 1995. Photostability of eight antibacterial agents in seawater. Aquaculture 134, 217–225.

Machado, R.S., Silva, M.R., Viriato, A., 2008. Furazolidone, tetracycline and omeprazole: a low-cost alternative for *Helicobacter pylori* eradication in children. Jornal de Pediatria 84, 160–165.

Meng, J., Mangat, S.S., Grudzinski, I.P., Law, F.C., 1998. Evidence of 14C-furazolidone metabolite binding to the hepatic DNA of trout. Drug Metabolism and Drug Interaction 14, 209–219.

Moreira, I.S., Amorim, C.L., Carvalho, M.F., Castro, P.M.L., 2012. Co-metabolic degradation of chlorobenzene by the fluorobenzene degrading wild strain *Labrys portucalensis*. International Biodeterioration & Biodegradation 72, 76–81.

O'Connor, A., Gisbert, J., O'Morain, C., 2009. Treatment of *Helicobacter pylori* infection. Helicobacter 14, 46–51.

Piccolomini, R., Di Ilio, C., Aceto, A., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G., Federici, G., 1989. Glutathione transferase in bacteria: subunit composition and antigenic characterization. Journal of General Microbiology 135, 3119–3125.

Reimão, J.Q., Taniwaki, N.N., Tempone, A.G., 2010. Furazolidone is a selective in vitro candidate against *Leishmania (L.) chagasi*: an ultrastructural study. Parasitology Research 106, 1465–1469.

Samuelsen, O.B., Solheim, E., Lunestad, B.T., 1991. Fate and microbiological effects of furazolidone in a marine aquaculture sediment. Science of the Total Environment 108, 275–283.

Syn, C.K., Swarup, S., 2000. A scalable protocol for the isolation of large-sized genomic DNA with in an hour from several bacteria. Analytical Biochemistry 278, 86–90.

Vuilleumier, S., 1997. Bacterial glutathione S-transferases: what are they good for? Journal of Bacteriology 179, 1431–1441.

World Health Organization (WHO), 2005. Critically Important Antibacterial Agents for Human Medicine: For Risk Management Strategies of Nonhuman Use. Report of a WHO working group consultation, Canberra, Australia, 15–18 February. WHO, Geneva.

World Health Organization (WHO), 2007. Critically Important Antimicrobials for Human Medicine: Categorization for the Development of Risk Management Strategies to Contain Antimicrobial Resistance Due to Non-human Use. Report of the Second WHO Expert Meeting, Copenhagen, Denmark, 29–31 May. WHO, Geneva.

Wu, F.Z., Ou Yang, L.Q., 2006. Determination of furazolidone, furacilin in aquatic product by HPLC. Chinese Journal of Health Laboratory Technology 16, 812–813.

Zhang, W.W., Hu, Y.H., Wang, H.L., Sun, L., 2009. Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain. Veterinary Microbiology 139, 183–188.

Zhang, W.W., Xu, D.X., Niu, Z.L., Yin, K., Liu, P., Chen, L.X., 2012. Isolation and characterization of *Pseudomonas* sp. DX7 capable of degrading sulfadoxine. Biodegradation 23, 431–439.