

# *Kocuria rosea* HN01, a newly alkaliphilic humus-reducing bacterium isolated from cassava dreg compost

Chun-Yuan Wu · Nan Chen · Hong Li · Qin-Fen Li

Received: 31 October 2012 / Accepted: 28 February 2013 / Published online: 4 April 2013  
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## Abstract

**Purpose** The aims of this study were to isolate an alkaliphilic humus-reducing bacterium, investigate the fastest microbial reduction of humus analog as affected by different cultivation, and examine its ability for iron(III) oxide reduction and organochlorine pollutants (OCPs) degradation.

**Materials and methods** A strain of pure culture, designated as HN01, was isolated from cassava dreg compost using anaerobic enrichment procedure with glucose as the electron donor and anthraquinone-2,6-disulphonate (AQDS) as the sole terminal electron acceptor. The isolate strain was identified using phenotypic and phylogenetic analysis. Iron(III) oxides and OCPs were chosen as potential electron acceptors. Strict anaerobic techniques and sterile conditions were applied throughout the incubation experiments, purged with O<sub>2</sub>-free N<sub>2</sub> for 15 min. The concentration of reduced AQDS and Fe(II) was then quantified using a UV–vis spectrophotometer. The concentration of OCPs was analyzed by gas chromatography with a micro-electron capture detector. Cell number was determined by direct plate counting on aerobic Luria–Bertani medium agar medium at pH 9.

**Results and discussion** (1) Strain HN01 was identified as *Kocuria rosea*, and the AQDS reduction by HN01 was observed in NaCl concentrations below 12 % (w/v) (optimum, 10 %) and pH ranges of 6.0–10.0 (optimum, 9.0) with sucrose as electron donor at 30 °C; (2) glucose, sucrose, methanol, ethanol, glycerol, and acetate were the favorable electron donors for AQDS reduction by strain HN01; (3) the strain had the ability of reducing iron(III) oxides in the presence of sucrose at pH 9.0 and its Fe(III)-reducing capacity ranked as goethite ( $\alpha$ -FeOOH) > lepidocrocite ( $\gamma$ -FeOOH) > haematite ( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>); and (4) the strain could effectively dechlorinate *p,p'*-DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane), and the dechlorination rate reached 71.3 %.

**Conclusions** This is the first report of a strain of *K. rosea* capable of reducing AQDS, iron (III) oxides, and *p,p'*-DDT, which extends the diversity of the alkaliphilic and halotolerant humus/Fe(III)-reducing bacterium associated with dechlorination. The strain may have the potential to be used for bioremediation of an anoxic alkaline wastewater or site contaminated with OCPs.

**Keywords** Dechlorination · Humus-reducing bacterium · Iron(III) oxides · *Kocuria rosea* · Organochlorine pollutants · Reductive transformation

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Responsible editor: Jizheng He

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Chun-Yuan Wu and Nan Chen contributed equally to this work.

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C.-Y. Wu · N. Chen · H. Li · Q.-F. Li (✉)  
Environment and Plant Protection Institute,  
Chinese Academy of Tropical Agricultural Sciences,  
Haikou 571101, People's Republic of China  
e-mail: qinfenli2005@163.com

N. Chen  
College of Environment and Plant Protection, Hainan University,  
Haikou 570228, People's Republic of China

H. Li  
Danzhou Scientific Observing and Experimental Station  
of Agro-Environment, Ministry of Agriculture,  
Danzhou 571737, People's Republic of China

## 1 Introduction

The decay of soil and sedimentary organic matter yields organic compounds with a high molecular weight, termed humic substances (humus) (Stevenson 1994). Humus can serve as the terminal electron acceptor for microbial respiration coupling with organic compound oxidation, and this biochemical process driven by microbes has been recognized as the main pathway for humus reduction in soils and sedimentary environments (Gralnick and Newman

2007; Lovley et al. 1996; Roden et al. 2010; Scott et al. 1998). The microbe-reduced humus can transfer electrons to insoluble minerals and refractory organics, such as iron(III) oxides (Liu et al. 2007; Wolf et al. 2009), azo dyes (Hong et al. 2007; Rodrigues da Silva et al. 2012), nitroaromatic compounds (Bhushan et al. 2006), and chlorinated organic contaminants (Cao et al. 2012; Wang et al. 2009). Therefore, there is a growing interest in humic-reducing microorganisms (HRMs) and humus-mediated reduction of extracellular substrates in recent years (Lovley et al. 1996; Gralnick and Newman 2007; Roden et al. 2010; Wu et al. 2012).

It is well recognized that HRMs are widespread in nature; more than a hundred HRMs have been isolated from a broad diversity of environments, mainly with circumneutral pH (Lovley et al. 2004; Wu et al. 2009). However, there are few reports on alkaliphilic HRM and the process that it can mediate substrate transformation under alkaline conditions. Only relatively recently have researchers turned their attention to alkaliphilic HRM, and several alkaliphilic humus-reducing bacteria were isolated, including *Alkaliphilus peptidofermans*, *Bacillus pseudofirmus*, *Corynebacterium humireducens*, *Natronincola ferrireducens*, and *Natronincola peptidovoranshava* (Ma et al. 2012; Wu et al. 2011; Zhilina et al. 2009a, b).

The solubility of humus is strongly pH dependent, and a high pH would increase the fraction of dissolved humus that is accessible to bacteria. However, in alkaline environments, iron species are generally insoluble, solid-phase minerals; humus-mediated reduction of iron(III) oxides will become significantly higher compared with circumneutral pH levels. The mineralogy and geochemistry of alkaline systems are complex and cannot be simply deduced from those studies conducted under near or below neutral pH conditions. For instance, Fe(III) reduction had not been recognized to occur beyond a pH of 9.0 before alkaliphilic bacteria capable of Fe(III) reduction was isolated from alkaline environments (Gorlenko et al. 2004; Ma et al. 2012; Ye et al. 2004). Therefore, the finding of alkaliphilic HRM is helpful in thoroughly understanding the mechanisms of electron transferring between cells and extracellular electron acceptors. Moreover, these strains might play an important role in organic matter oxidation, element biogeochemical cycles, and pollutant detoxification in alkaline environments (Hobbie et al. 2012; Wu et al. 2012).

We conducted a study on an isolate from a cassava dreg composting reactor that was continuously reacting for 35 days and in mesophilic and maturation phase of pH 8.5–9.0 to determine the isolate's possible humus-reducing activity, optimum humus reduction conditions, and alternative electron acceptors for its anaerobic metabolism. The humus analog anthraquinone-2,6-disulfonate (AQDS) was used in the enrichment and reduction experiments. The specific objectives of this study were to: (1) characterize

and identify the new isolate, alkaliphilic strain HN01; (2) determine its humus-reducing activity and optimum reducing conditions; (3) explore its potential capacity of reducing iron(III) oxides (goethite ( $\alpha$ -FeOOH), lepidocrocite ( $\gamma$ -FeOOH) or hematite( $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>)) and degrading organochlorine pollutants (OCPs) under alkaline conditions. To our knowledge, this is a new research on describing the capacity of *Kocuria rosea* to reduce humus, Fe(III), and OCPs.

## 2 Materials and methods

### 2.1 Chemicals

$\alpha$ -FeOOH was synthesized according to the procedures of Li et al. (2008). Preparation of  $\gamma$ -FeOOH and  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub> followed the method described by Li et al. (2007a). The hexane of high-performance liquid chromatography grade and the anhydrous sodium sulfate of analytical grade were purchased from Guangzhou Chemical Reagent Co. (China). AQDS of 98.6 % purity was purchased from Sigma-Aldrich (Tokyo, Japan).  $\gamma$ -Hexachlorohexanes ( $\gamma$ -HCH; 98.2 %), 1,1-(2,2,2-trichloroethylidene)bis[4-chloro-benzene] (*p,p'*-DDT; 98.6 %), and 1-chloro-4-[2,2,2-trichloro-1-(2-chlorophenyl)ethyl]benzene (*o,p'*-DDT; 98.6 %) of analytical grade were obtained from Accu Stangard (New Haven, USA). A mixture of standard solution containing *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, and *o,p'*-DDD, with 10.0 mg l<sup>-1</sup> per compound was purchased from Labor Dr. Ehrenstorfer, Germany. All of the chemicals were used as received, without further purification.

### 2.2 Enrichment and isolation

According to previous studies, quinones serve as electron-accepting moieties when microorganisms transfer electrons to humus, and AQDS has been used extensively as a humus analog in studies on humus for microbial respiration (Lovley et al. 1996; Scott et al. 1998). Thus, we selected AQDS to replace humus in the enrichment and reduction experiments. The inoculum source for enrichment was the cassava dreg compost in mesophilic and maturation phase of pH 8.5–9.0. Primary enrichments were initiated in anaerobic culture serum bottles (purged with O<sub>2</sub>-free N<sub>2</sub> for 15 min, sealed with a butyl-rubber stopper and aluminum cap) containing mineral salts medium (MSM) added with 5 mmol l<sup>-1</sup> sucrose (electron donor) and 0.5 mmol l<sup>-1</sup> AQDS (electron acceptor). The preparation of MSM was performed according to Wu et al. (2011). The bottles were cultured at 30 °C in the dark. Positive enrichments for AQDS reduction were qualitatively determined by the color change of the aqueous solution from transparent to red. The primary enrichments were then successively transferred to fresh

medium three times. Finally, the mixed solutions were serially diluted and plated onto aerobic Luria–Bertani medium (LB medium: peptone, 10 g l<sup>-1</sup>; NaCl, 10 g l<sup>-1</sup>; yeast extract, 5 g l<sup>-1</sup>; pH 9.0). Afterwards, distinct colonies were picked and streaked three times on agar plates, and each colony was tested for AQDS reduction in sterilized anaerobic medium. The single colony of interest was stored in a glycerol tube at -20 °C.

### 2.3 Phenotypic and 16S rRNA analysis

The motility of cells was tested by the hanging drop method. Physiological–biochemical characteristics were determined by the standard methods (Buchanan and Gibbons 1974). The salt tolerance of strain HN01 was determined on LB medium (pH 9.0) by varying NaCl concentrations from 0 to 15 % (w/v). The 16S rRNA gene of the isolate was amplified by PCR as described in Li et al. (2007b). The obtained sequences were then aligned with related 16S rRNA sequences from GenBank data libraries (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Ribosomal Database Project (<http://rdp.cme.msu.edu/seqmatch>). Corresponding nucleotide sequences of representatives of the genus *Kocuria* were aligned using the program CLUSTAL X (Thompson et al. 1997). The phylogenetic tree was constructed with the software package MEGA version 4.0 using the maximum-parsimony and neighbor-joining method according to Kimura 2-parameter model and bootstrap analyses based on 1,000 replicates.

### 2.4 Batch anaerobic incubation experiments

All reduction and degradation experiments were conducted in 25.2-ml serum bottles under anaerobic and sterile conditions (Wu et al. 2011), performed in three sets, and all the bottles were incubated in the dark. Resting cell suspension of HN01 was used throughout the experiments. The suspension was aerobically prepared in LB medium at 30 °C and then harvested at late log phase by centrifugation (8,000×g at 4 °C for 10 min). Pellets were then washed twice and resuspended in sterile fresh MSM to an optical density of about 1.5 ( $\lambda=600$  nm).

To test the pH range for AQDS reduction, each bottle containing 20 ml MSM with 1 ml cell suspension, 0.5 mmol l<sup>-1</sup> AQDS, and 5 mmol l<sup>-1</sup> sucrose was adjusted to pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0. The pH was maintained using 20-mmol l<sup>-1</sup> phosphate buffer (for pH 5.0, 6.0, 7.0, 8.0) or carbonate buffer (for pH 9.0, 10.0, 11.0) (Wu et al. 2011). The medium without cells served as the control set.

For testing the effect of NaCl% (m/v) on AQDS reduction by HN01, mixed cultures containing AQDS (1 mmol l<sup>-1</sup>), cell suspension (1 ml), sucrose (5 mmol l<sup>-1</sup>), and carbonate buffer (20 mmol l<sup>-1</sup>; pH 9.0) were supplied with NaCl% (m/v) of 0.5, 1, 2, 3, 5, 7, 10, 12, 15, or 20 %.

To investigate the effect of alternative electron donors on AQDS reduction, seven possible organic substrates were tested, including acetate, pyruvate, glycerol, methanol, ethanol, glucose, and sucrose, with pH 9.0 and without NaCl%. The medium without cells served as the control set.

Under favorable conditions (i.e., pH, NaCl%, and electron donor) for AQDS (1 mmol l<sup>-1</sup>) microbial reduction by strain HN01, we investigated the cell growth coupling with AQDS reduction by the variation of active cell numbers.

The ability of strain HN01 to use different electron acceptors was studied in the serum bottles containing 1 ml cell suspension, 20 ml MSM, 5 mmol l<sup>-1</sup> of sucrose (electron donor), carbonate buffer (20 mmol l<sup>-1</sup>; pH 9.0), and one of the following substrates as an electron acceptor: 5 mmol l<sup>-1</sup> of Fe(III) oxides ( $\alpha$ -FeOOH,  $\gamma$ -FeOOH, or  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>) and 1 mg l<sup>-1</sup> of organochlorine pollutants ( $\gamma$ -HCH, *o,p'*-DDT, or *p,p'*-DDT). Two control assays were performed under the same conditions: an abiotic set without bacterial cells and a biotic set without the addition of sucrose.

### 2.5 Chemical analysis

Triplicate bottles were used for chemical analysis at each sampling interval. The concentration of the reduced AQDS (AH<sub>2</sub>QDS) was quantified with a UV–vis spectrophotometer (UV-3600, Japan) at a wavelength of 386 nm (pH 5.0–6.0 sets) or 408 nm (pH 7.0–11.0 sets), which was due to the different AH<sub>2</sub>QDS UV–vis spectra at different pH levels (Liu et al. 2007; Wu et al. 2012). The total Fe(II), including dissolved and sorbed Fe(II), was quantified photometrically at 510 nm after being extracted using 0.5 mol l<sup>-1</sup> HCl for 1.5 h and reaction with 1,10-phenanthroline. Dissolved Fe(II) was determined by removing the mineral and sorbed Fe(II) from the aqueous phase using a 0.22-mm syringe filter and then assaying the filtrate by colorimetric method (Li et al. 2010).

For analysis of organochlorine compounds ( $\gamma$ -HCH, *o,p'*-DDT, *p,p'*-DDT, and their intermediates), the sample bottles were immediately added with 20 ml hexane, and the mixtures were extracted using the ultrasonic bath as described in Cao et al. (2010). The quantification of the organochlorine compounds was carried out with an Agilent 6890 GC/mECD gas chromatograph and AT7693 auto sampler. The separation was performed on a fused silica capillary column (Aligent 1909/J-413; 30 m×320  $\mu$ m×0.25  $\mu$ m). The carrier gas was the high pure N<sub>2</sub> (99.999 %) with a flow of 1.0 ml min<sup>-1</sup> and the make-up gas at 60 ml min<sup>-1</sup>. The injector and detector temperatures were 250 and 300 °C, respectively. The GC oven temperature was programmed as follows: initial temperature of 100 °C held for 1 min, increased to 160 °C at a rate of 100 °C min<sup>-1</sup>, then increased to 270 °C at a rate of 10 °C min<sup>-1</sup>. Samples (1  $\mu$ l) were injected in splitless mode. GC peaks were identified by

accurate assignment of retention times for each standard; quantitative calculation was conducted with the external standard method. The organic chlorine compounds were quantitatively determined by comparing the area under each peak with the area under the standard peak. The resulting correlation coefficients for the calibration curves of the compounds were all greater than 0.995. The detection limits of quantification of target compound ranged from 0.01 to 0.04  $\mu\text{g l}^{-1}$ .

### 3 Results

#### 3.1 Identification of the strain

The vegetative cells of isolated strain HN01 were coccus, 0.5–0.7  $\mu\text{m}$  in diameter. The cells occurred singly or in pairs, were nonmotile, and stained Gram positive in both the exponential and stationary growth phases. After 48-h incubation on an LB agar plate, the colonies were uniformly round, 0.5–1.0 mm in diameter, red, wet, and with a distinct smooth morphology.

The optimum growth temperature for the strain ranged from 15 to 30  $^{\circ}\text{C}$ , and no growth was observed at 45 and below 5  $^{\circ}\text{C}$ . Microbial growth was observed between pH 6.0 and 10.0, and the optimum pH was 8.5–9.0. The strain can survive at NaCl concentrations of 0–12 % (optimum, 0–7 %), but not in 13 % NaCl. Strain HN01 had more similar characteristics with *K. rosea* ATCC 186<sup>T</sup> compared with other closely related species, was oxidase-positive and gelatin negative, and produced acid from glucose and fructose, but not from glycerol and lactose. No activity was detected for nitrate reduction, lysine decarboxylase, and arginine dihydrolase (Table 1). In terms of overall phenotype characteristics, the isolate should resemble *Kocuria*. Furthermore, chemotaxonomic characteristics of the strain and the topology of the 16S-rRNA-based phylogenetic tree (Fig. 1) clearly indicated that the nearest phylogenetic neighbors (sequence similarity values ranged from 96.0 to 99.7 %) of strain HN01 were members of the genus *Kocuria*. The strain had the highest similarity of 99.6 % with *K. rosea* D40 (JN192402). Consequently, the strain was identified as *K. rosea* strain HN01, which was deposited in the China General Microbiological Culture Collection Center (CGMCC no. 5810).

#### 3.2 AQDS reduction

Here, we investigated the optimal cultivating conditions for AQDS reduction by the isolate HN01 and if it can grow during anaerobic metabolism. The color change of the culture liquid from transparent to yellow or red was a good indicator for AQDS reduction.

**Table 1** Characteristics that can be used to differentiate strain HN01 from type strains of closely related species

Characteristic	1	2	3	4	5
Catalase activity	+	–	+	ND	+
Oxidase activity	+	+	+	–	–
Hydrolysis of gelatin	–	–	–	ND	–
Nitrate reduction	+	+	+	–	+
Lysine decarboxylase	–	–	–	ND	+
Arginine dihydrolase	–	–	+	ND	ND
Carbon source utilization					
Citrate utility	–	–	–	ND	ND
Glucose	+	+	+	–	+
Fructose	+	+	+	ND	+
Glycerol	–	–	+	ND	+
Lactose	–	–	+	–	+
Aesculin	+	–	–	–	ND

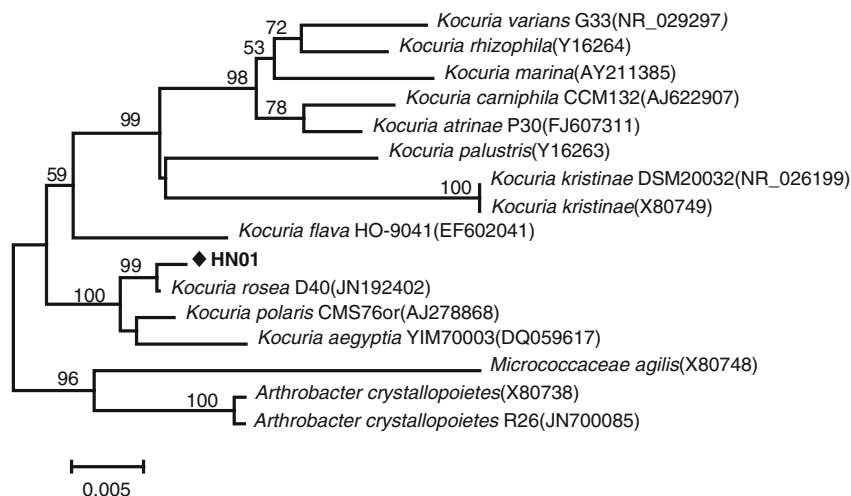
1 *K. rosea* HN01, 2 *K. rosea* ATCC 186<sup>T</sup> (data from Reddy et al. 2003), 3 *K. polaris* CMS76or<sup>T</sup> (Reddy et al. 2003), 4 *K. aegyptia* YIM70003 (Zhou et al. 2008), 5 *K. flava* HO-9041<sup>T</sup> (Zhou et al. 2008), + positive, – negative, ND not determined

The AQDS reduction by HN01 using sucrose as the electron donor varied at different pH levels (Fig. 2). After 5 days of incubation, as the color of the solution changed, the AH<sub>2</sub>QDS concentrations in the treatments of AQDS + HN01 + sucrose at pH 7.0, 8.0, 9.0, and 10.0 increased to 0.10, 0.15, 0.18, and 0.13  $\text{mmol l}^{-1}$ , respectively. In contrast, only less than 0.038  $\text{mmol l}^{-1}$  AH<sub>2</sub>QDS was formed in the treatments at pH 5.0, 6.0, and 11.0, respectively. The reduction in the abiotic control without cells was negligible (data not shown). The results indicated that the pH ranges for AQDS reduction by strain HN01 were within 7.0–10.0, and the optimum pH was 9.0. To the best of our knowledge, only few alkaliphilic bacteria capable of reducing humus and quinone have been reported. The identification of strain HN01 expands the species that can rapidly reduce AQDS at high pH levels.

Additionally, the reduction of AQDS was studied at various increasing NaCl% (v/w) of the cultivation medium (i.e., from 0.5, 1, 2, 3, 5, 7, 10, 12, 15, and 20 %). We found that the concentration of AH<sub>2</sub>QDS was increased with the increasing of NaCl% content from 0 to 12 %. However, the reduction content was decreased while the NaCl level greater than 12 %, and no reduction was measured at the 20 % NaCl level (Fig. 3). These results showed that: NaCl% was also a main factor influencing the reduction of AQDS by strain HN01, and the optimum NaCl concentrations for AQDS reduction were between 7 and 12 %.

Seven types of organic substrates were tested as the alternative electron donors for AQDS reduction. As shown in Fig. 4, the production of AH<sub>2</sub>QDS increased with time in the incubations with acetate, glycerol, methanol, ethanol,

**Fig. 1** Phylogenetic tree obtained by neighbor-joining analysis based on 16S rRNA gene sequences, showing the position of strain HN01 among its phylogenetic neighbor. Bar, 0.005 sequence divergence



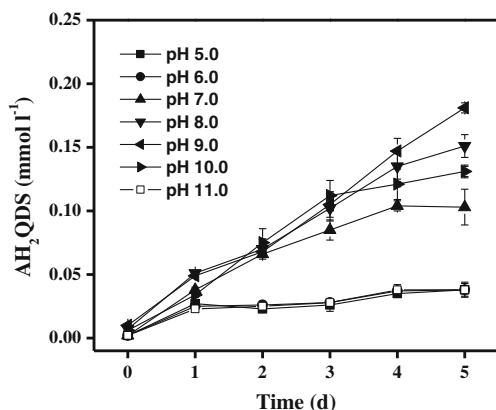
glucose, and sucrose, while no AH<sub>2</sub>QDS was detected in the incubations with pyruvate. The concentration of produced AH<sub>2</sub>QDS ranked as an order of glucose > sucrose > ethanol > methanol > glycerol > acetate. In the biotic (HN01 + AQDS) and abiotic (AQDS + organic substrates) controls, nearly no AH<sub>2</sub>QDS was detected (data not shown). These results indicated that glucose, sucrose, ethanol, methanol, glycerol, and acetate could serve as favorable electron donors for AQDS reduction by strain HN01, and the reduction rate depended on the type of organic substrates. Organic substrate such as pyruvate was not available as an electron donor with respect to AQDS reduction.

In the sucrose-fed incubation (pH 9.0; NaCl, 10 %), significant microbial growth and AQDS reduction were observed over 5 days. From Table 2, there was no obvious increase in active cell number of HN01 in both AQDS + HN01 + sucrose and HN01 + sucrose treatments, which indicated that HN01 cannot ferment sucrose and conserve

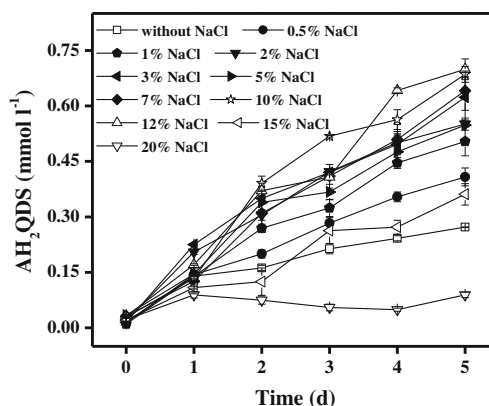
energy to support microbial growth coupling AQDS reduction.

### 3.3 Iron(III) oxides reduction

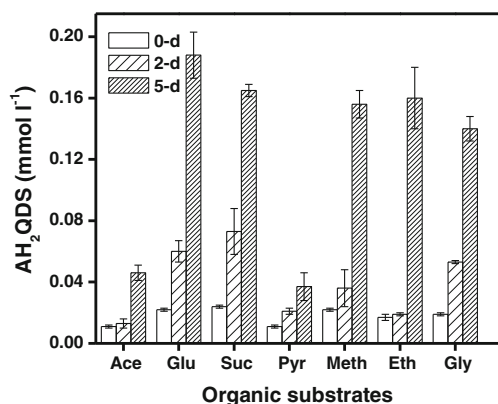
The Fe(III)-reducing activity of strain HN01 was explored with three types of iron(III) oxides: α-FeOOH, γ-FeOOH, and α-Fe<sub>2</sub>O<sub>3</sub>. After 25 days (Fig. 5), no Fe(II) was formed in the abiotic control without active cells, and less than 0.06 mmol l<sup>-1</sup> of Fe(II) was observed in the two biotic controls (without sucrose and with dead cells). In contrast, the total Fe(II) concentration in the active tests using α-FeOOH, γ-FeOOH, or α-Fe<sub>2</sub>O<sub>3</sub> as the electron acceptor reached 0.16, 0.087, and 0.066 mmol l<sup>-1</sup>, respectively. The results suggested that (1) Fe(III) reduction by HN01 was a biological process because it required both active cells and sucrose, (2) all the three types of iron(III) oxides can be reduced by the strain with sucrose as the electron donor,



**Fig. 2** The production of AH<sub>2</sub>QDS over a 5-day incubation in the AQDS + sucrose + HN01 treatment at pH 5.0 to 11.0. The experiments used 5 mmol l<sup>-1</sup> sucrose and 0.5 mmol l<sup>-1</sup> AQDS, and were performed under anaerobic conditions at 30 °C in the dark. Error bars represent standard deviation of the mean (n=3)



**Fig. 3** The production of AH<sub>2</sub>QDS over a 5-day incubation in the AQDS + sucrose + HN01 treatment with different NaCl% (v/w). The experiments used 5 mmol l<sup>-1</sup> sucrose and 1.0 mmol l<sup>-1</sup> AQDS, and were performed under anaerobic conditions at 30 °C in the dark. Error bars represent standard deviation of the mean (n=3)

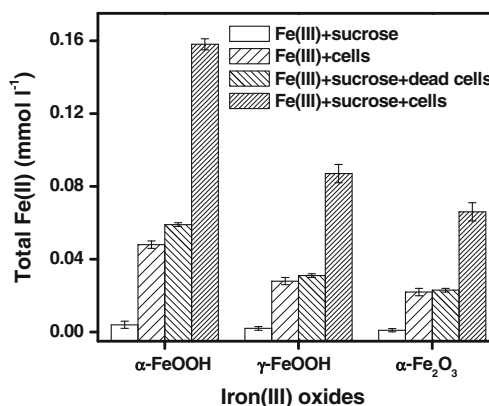


**Fig. 4** The effect of electron donors ( $5 \text{ mmol l}^{-1}$ ) on AQDS ( $0.5 \text{ mmol l}^{-1}$ ) reduction by strain HN01 at 2- and 5-day intervals. Electron donors: acetate (*Ace*), glucose (*Glu*), sucrose (*Suc*), pyruvate (*Pyr*), methanol (*Meth*), ethanol (*Eth*), and glycerol (*Gly*). The experiments were conducted in the medium not added with NaCl and at pH 9.0, and were performed under anaerobic conditions at  $30 \text{ }^{\circ}\text{C}$  in the dark. Error bars represent standard deviation of the mean ( $n=3$ )

and (3) the reduction rate of  $\alpha\text{-FeOOH}$  was the highest, followed by  $\gamma\text{-FeOOH}$  and  $\alpha\text{-Fe}_2\text{O}_3$ .

### 3.4 *p,p'*-DDT biodegradation

Figure 6 shows the anaerobic degradation of three types of organochlorine pollutants by strain HN01 with sucrose as the electron donor at pH 9.0. After 25-day incubation, the concentration of *p,p'*-DDT in the controls lacking sucrose (biotic control) or active cells (abiotic control) remained almost unchanged, demonstrating that *p,p'*-DDT was persistent in the absence of microbial activity of HN01, and the chemical reduction of *p,p'*-DDT by sucrose was negligible. In contrast, *p,p'*-DDT was significantly degraded in the active treatments, and the degradation rate reached 71.3 %. These results indicated the overall success of the biodegradation process of *p,p'*-DDT by HN01. However, as there was no decrease of  $\gamma\text{-HCH}$  and *o,p'*-DDT in all related treatments, it was suggested that strain HN01 cannot



**Fig. 5** The production of total Fe(II) from the reduction of  $1 \text{ mmol l}^{-1}$   $\alpha\text{-FeOOH}$ ,  $\gamma\text{-FeOOH}$ , or  $\alpha\text{-Fe}_2\text{O}_3$  by strain HN01 using  $5 \text{ mmol l}^{-1}$  sucrose as the electron donor after 25-day incubation. Error bars represent standard deviation of the mean ( $n=3$ )

perform  $\gamma\text{-HCH}/o,p'\text{-DDT}$  transformation involving anaerobic metabolism.

It was reported that DDT firstly degrades to DDD (anaerobic biotransformation product) or DDE (Cao et al. 2012). The GC analysis during the incubation period showed no *p,p'*-DDE was detected, while a variable amount of *p,p'*-DDD was quantified as  $0.34 \text{ mg l}^{-1}$  at 10 days, and the concentration decreased to  $0.21 \text{ mg l}^{-1}$  at the end of incubation (Fig. 7). This indicated that *p,p'*-DDT was firstly dechlorinated to *p,p'*-DDD but not *p,p'*-DDE in the treatments of *p,p'*-DDT + HN01 + sucrose. The total priority pollutants (*p,p'*-DDTs; i.e., *p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD) decreased more than 60 % after 25 days of incubation, suggesting the overall success of the bio-dechlorination process of *p,p'*-DDT by HN01.

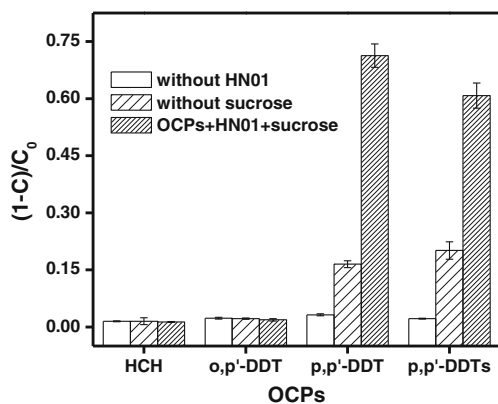
## 4 Discussion

Microorganism-mediated electron transfer between electron donors inside the cells and extracellular substances has been

**Table 2** Active cell numbers in the AQDS + sucrose + HN01 treatments during a 5-day incubation period; biotic sets without AQDS served as control. The initial concentration of AQDS and cells were  $1.0 \text{ mmol l}^{-1}$  and  $1.8 \times 10^7 \text{ cells ml}^{-1}$

Incubation period (days)	AQDS + sucrose + HN01		Sucrose + HN01
	AH <sub>2</sub> QDS ( $\text{mmol l}^{-1}$ )	Cell number ( $10^7 \text{ cells ml}^{-1}$ )	Cell number ( $10^7 \text{ cells ml}^{-1}$ )
0	$0.026 \pm 0.004$	$1.80 \pm 0.009$	$1.79 \pm 0.010$
1	$0.131 \pm 0.029$	$1.54 \pm 0.009$	$1.54 \pm 0.006$
2	$0.381 \pm 0.019$	$1.38 \pm 0.009$	$1.38 \pm 0.005$
3	$0.509 \pm 0.015$	$1.33 \pm 0.011$	$1.33 \pm 0.008$
4	$0.558 \pm 0.026$	$1.21 \pm 0.002$	$1.22 \pm 0.005$
5	$0.679 \pm 0.022$	$1.16 \pm 0.005$	$1.16 \pm 0.004$

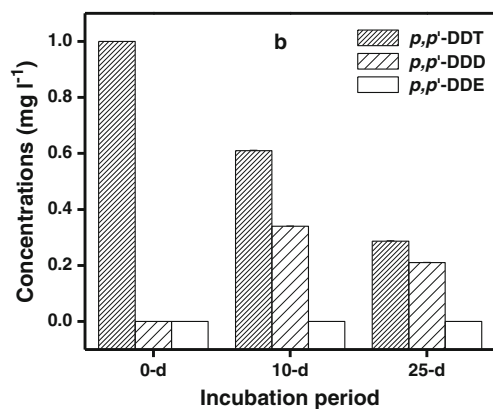
Values are means of three experiments  $\pm$  standard deviation



**Fig. 6** Anaerobic degradation of OCPs ( $\gamma$ -HCH,  $o,p'$ -DDT, and  $p,p'$ -DDT) by strain HN01 over 25-day incubation. The experiments used  $5 \text{ mmol l}^{-1}$  sucrose and  $1.0 \text{ mmol l}^{-1}$   $\gamma$ -HCH,  $o,p'$ -DDT, or  $p,p'$ -DDT, and were performed under anaerobic conditions at  $30^\circ\text{C}$  in the dark. Error bars represent standard deviation of the mean ( $n=3$ )

recognized as a significant biochemical process in anoxic soils or sediments (Lovley et al. 1996, 2004; Hernandez and Newman 2001; Jiang and Kappler 2008). Various substances, including minerals, electrodes, humus, soluble metal ions, and organic pollutants, can serve as the terminal electron acceptor for microbial anaerobic metabolism (Gralnick and Newman 2007). To date, the subject of extracellular reduction in neutral environments has been most thoroughly explored through studies concerning the reduction of humus and iron minerals. Only relatively recently, however, have researchers turned their attention to alkaliphilic humus/Fe(III)-reducing microorganisms and related process under alkaline conditions, and only a few strains have been isolated from natural or artificial alkaline environments (Hobbie et al. 2012; Zhilina et al. 2009ab).

According to Ulukanli and Diğrak (2002), alkaline-adapted microorganisms can be classified into two groups,



**Fig. 7** The major biotransformation products of  $p,p'$ -DDT by strain HN01 at 10- and 25-day incubation periods in the  $p,p'$ -DDT + HN01 + sucrose treatments. The experiments used  $5 \text{ mmol l}^{-1}$  sucrose and  $1.0 \text{ mmol l}^{-1}$   $p,p'$ -DDT, and were performed under anaerobic conditions at  $30^\circ\text{C}$  in the dark. Error bars represent SD of the mean ( $n=3$ )

alkaliphiles and alkalitolerants, the former requiring actually alkaline media for growth and the optimum growth above pH9; the latter can grow at pH values more than 9 or 10 but the optimum pH at around 7 or less. Strain HN01 could get growth at pH of 6–10 and obtain optimum growth at pH 9; thus, it was classified as alkaliphiles. Less than five alkaliphilic strains (alkaliphiles) capable of reducing extracellular substances have been reported, and the strains include *A. peptidofermantans* (Zhilina et al. 2009a), *Anaerobranca californiensis* (Gorlenko et al. 2004), *B. pseudofirmus* (Ma et al. 2012), *Bacillus* sp (Pollock et al. 2007), *Corynebacterium humireducens* (Wu et al. 2011).

Among the factors influencing the microbial reduction of humus or Fe(III), types of electron donors are considered to be one of the most important factors (Lovley et al. 2004; Ma et al. 2011). Organic acids and sugars are usually used to study the microbial reduction of humus. Among them, acetate is the most important electron donor in many sedimentary environments (Lovley et al. 2004), but only partial humus-reducing bacteria can utilize it, while sugars might be the most effective electron donors according to our recent studies (Ma et al. 2012; Wu et al. 2011). For example, Ma et al. (2012) tested the effect of electron donors on AQDS reduction by *B. pseudofirmus* MC02, and the experimental results showed that the extent to which the AQDS reduction varied as a sequence of glucose > sucrose > lactate > glycerol > citrate, and acetate could not be utilized for AQDS reduction by the strain. The concentration of produced  $\text{AH}_2\text{QDS}$  by *C. humireducens* ranked as an order of sucrose > acetate > lactate > ethanol > formate, and citrate, propionate, and glycerol were not available electron donors with respect to AQDS reduction (Wu et al. 2011). Seven possible organic substrates could serve as alternative electron donors for AQDS reduction by *K. rosea* HN01 in this study with a sequent effect in the order of glucose > sucrose > ethanol > methanol > glycerol > acetate, and pyruvate was not associated with AQDS reduction. The physical and chemical characteristics of Fe(III) are the main factors which influence Fe(III) reduction rate by Fe(III)-reducing microbes (Liu et al. 2001; Roden and Zachara 1996; Wu et al. 2010). Consistent with the previous reports, the reduction of iron(III) oxides by *K. rosea* HN01 was consistent with their surface area and crystalline property, ranking as  $\alpha\text{-FeOOH} > \gamma\text{-FeOOH} > \alpha\text{-Fe}_2\text{O}_3$ ; Among these Fe(III),  $\alpha\text{-Fe}_2\text{O}_3$  reduction proceeded with the most difficulty, probably due to its smallest surface area ( $29.4 \text{ m}^2 \text{ g}^{-1}$ );  $\alpha\text{-FeOOH}$  and  $\gamma\text{-FeOOH}$  are with similar surface areas ( $120.93$  and  $115.44 \text{ m}^2 \text{ g}^{-1}$ ), whereas  $\alpha\text{-FeOOH}$ 's crystal size ( $41.9 \text{ nm}$ ) is extremely larger than  $\gamma\text{-FeOOH}$  ( $13.7 \text{ nm}$ ), and this is likely the reason for the different Fe(III) microbial reduction rates.

The newly isolated strain HN01 belonged to the genus *Kocuria*, which is taxonomically dissected from the genus *Micrococcus* to accommodate phylogenetically distinct

actinobacteria (Stackebrandt et al. 1995) and composed of 18 species: *K. rosea* (the type species), *Kocuria aegyptia*, *Kocuria atrinae*, *Kocuria carniphila*, *Kocuria flava*, *Kocuria gwangalliensis*, *Kocuria halotolerans*, *Kocuria himachalensis*, *Kocuria koreensis*, *Kocuria kristinae*, *Kocuria marina*, *Kocuria palustris*, *Kocuria polaris*, *Kocuria rhizophila*, *Kocuria salsiccia*, *Kocuria sediminis*, *Kocuria turfanensis*, and *Kocuria varians* (Bala et al. 2012; Li et al. 2006; Liu 2011). Among them, there are more than five strains capable of growing under alkaline and saline conditions, including *K. aegyptia*, *K. atrinae*, *K. flava*, *K. turfanensis* (Zhou et al. 2008), *K. halotolerans* (Tang et al. 2009), *K. koreensis* (Park et al. 2010), *K. marina*, *K. polaris*, and *K. sediminis* (Bala et al. 2012). Compared with these alkaline-adapted microorganisms, *K. rosea* has much more potential applications in the field of environment protection. As a strain of environmental origin, *K. rosea* has been reported to be capable of decolorization of malachite green, azo, triphenylmethane, and industrial dyes (Parshetti et al. 2006, 2010) and also active in feather degradation (Coello and Vidal 2002). In our study, we found the new anaerobic metabolism of *K. rosea*, which was capable of effectively reducing extracellular soluble substrates (AQDS; *p,p'*-DDT) and insoluble iron(III) oxides under extreme alkaline conditions. The reducing pathway was proposed as: electrons transferred from organic substrates to extracellular substrates by a series of electron carriers inside the cells; in this biochemical process, extracellular substrates (AQDS, iron(III) oxides, *p,p'*-DDT), as the terminal electron acceptors, were reductively transformed or degraded into reducible form, coupling with the oxidation of organic substrates, which served as the electron donor.

## 5 Conclusions

A novel halotolerant, alkaliphilic humus-reducing bacterium, designated as HN01, was isolated from a cassava dreg composting reactor in mesophilic and maturation phase of pH 8.5–9.0. The strain was a Gram-positive, facultatively anaerobic, nonfermentative, nonmotile, coccus-shaped bacterium and was identified as *K. rosea* HN01 according to phylogenetic analysis based on 16S rRNA gene sequences. The AQDS reduction by strain HN01 was observed at NaCl concentrations below 12 % (w/v) (optimum, 10 %) and pH levels of 6.0–10.0 (optimum, 9.0) with sucrose as electron donor at 30 °C. Glucose, sucrose, methanol, ethanol, glycerol, or acetate could also serve as favorable electron donors for AQDS reduction. Coupling with sucrose as the electron donor, the strain could effectively transfer electrons from active cells to extracellular iron(III) oxides and *p,p'*-DDT. This was the first report that a strain of the *Kocuria* genus was involved in AQDS/Fe(III) reduction and *p,p'*-DDT

dechlorination under anaerobic alkaline conditions. Such findings explore the new environmental functions of the *Kocuria* genus and also provide opportunity for the development of bioremediation of alkaline wastewater and other organic-contaminated environments containing humus.

**Acknowledgments** The authors thank the National Natural Science Foundations of China (no. 41101477, 21007091) and the Fundamental Research Funds for Environment and Plant Protection Institute, CATAS (no. 2011Hzs1J006, 2010hzsZDZX001) for financial support for this study.

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