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Research article

Biodegradation of sulfamethoxazole in bacteria from three different origins

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A B S T R A C T

Sulfamethoxazole (SMX) is a common medicine prescribed to treat infections. Due to vast use, SMX has been detected in different parts of the world. Hence, it has become a high risk because of its long term persistence with high biological activity in the ecosystem. Therefore, it is necessary to understand the mechanism of SMX degradation in different genus of bacteria, which is presently unclear. In the present study, degradation of 5 mg L^{-1} SMX was studied in three isolated pure bacterial cultures, *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 and results showed up to 45.2%, 62.2% and 51.4% degradation, respectively within 288 h. Additionally, strain SA1 and strain SCD14 showed up to 66.2% and 69.2% of 4-aminophenol degradation at an initial concentration of 5 mg L^{-1} within 216 h whereas *Labrys* sp. SMX-W1-SC11 completely degraded 4-aminophenol at the same concentration within 120 h. Moreover, all three pure bacteria also completely degraded 3-amino-5-methylisoxazole at initial concentration of 4 mg L^{-1} within 120 h. Furthermore, gas chromatography-mass spectrometry and quadrupole time-of-flight mass spectrometry analysis results revealed that 3-amino-5-methylisoxazole, 4-aminophenol and hydroquinone were the three main by-products of SMX catabolism. In addition, cell free extracts of both *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 showed hydroquinone dioxygenase activity. Besides, all three bacterial strains showed resistance to different heavy metals. Moreover, all three pure bacterial cultures also showed positive chemotactic response toward 3-amino-5-methylisoxazole and hydroquinone based on the drop plate assay. The results of this study recommend these microorganisms for bioremediation of SMX contaminated sites.

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1. Introduction

Sulfamethoxazole [4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide; SMX] is a commonly used medicine used to cure venereal infections (uncomplicated gonorrhea and chlamydia), gastrointestinal infections, central nervous system infections, respiratory infections and genitourinary tract infections (Kielhofner, 2005). Additionally, SMX is also used as herbicide in agriculture and aquaculture system (Boreen et al., 2004). Because of continuous usage, SMX has been observed in most part of world. For example, SMX was detected up to 113 ng L^{-1} in drinking water supply wells, 38–450 ng L^{-1} in ground water, 7.9–1900 ng L^{-1} in surface water (Lv et al., 2014; Wang et al., 2015) and up to 95.2 ng L^{-1} in the wastewater (Sun et al., 2016). Its long-term persistence in the environment might pose a high risk to the aquatic and neighboring living systems (Akhtar et al., 2011) and possibilities to enhance antibiotic resistant bacteria in the surrounding field (Ricken et al., 2015). Even at a lower concentration, it can induce genetic mutations and chronic effects (Zhang et al.,

Abbreviations: SMX, sulfamethoxazole; W1, wastewater; W2, activated sludge of a wastewater treatment plant; PM1, pig manure; AMS, ammonium mineral salts; MeOH, methanol; NCBI, national center for biotechnology information; ESI, electrospray ionization; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; Q/TOF-MS, quadrupole time-of-flight mass spectrometry; MIC, minimum inhibitory concentration; Cu, Copper; Cd, Cadmium; Cr, Chromium; Co, Cobalt; Pb, Lead; Ni, Nickel; Zn, Zinc.

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2010) and therefore, it is necessary to remove and/or detoxify SMX from the contaminated sites.

Previous studies revealed that the chemical treatment methods like ozonation and electro-Fenton treatment generally cause the creation of toxic intermediates (Dantas et al., 2008; Dirany et al., 2011; Yargeau et al., 2008). Advanced oxidation and adsorption by activated carbon or membrane reactors can eliminate micropollutants including SMX from water (Dirany et al., 2011; Michael et al., 2013; Trovo et al., 2009). However, advanced oxidation and adsorption processes are costly, and hence, not fit in certain urban WWTPs. On the other hand, conventional techniques were used to remove micropollutants; however, certain micropollutants could not be completely removed from wastewater treatment plants (WWTPs), which represent serious challenge to the researchers.

Biological remediation is therefore expected to be an eco-friendly and cost-effective method for SMX removal from contaminated sites especially from wastewater. Till date, removal of SMX in pure bacteria, mixed cultures as well as sulfate reducing bacteria under aerobic conditions or anaerobic conditions was reported by researchers (Jia et al., 2017; Jiang et al., 2014; Kassotaki et al., 2016; Larcher and Yargeau, 2011; Muller et al., 2013; Reis et al., 2014; Ricken et al., 2015; Wang et al., 2015; Xu et al., 2011). However, degradation mechanism of SMX and its main by-products in different pure bacteria is still unclear. Hence, it is essential to isolate and identify different genus of potential pure bacteria having the ability to degrade SMX and its catabolic by-products simultaneously in the environment and also to characterize their ability to resist various heavy metals as well as their chemotaxic behavior towards SMX by-products. Generally, microbes have certain kinds of mechanisms which help them to adjust their cellular functional properties in response towards variations in its environmental system. Chemotaxis is a type of property present in the microbial cell which helps microorganisms to adjust their migration property under the influence of a substance gradient (Pandey and Jain, 2002). For example, the microorganism moves towards chemical substrate(s), which implies positive chemotaxis. Whereas the microorganism moves away from the substrate(s), the process is considered as negative chemotaxis (Arora et al., 2015; Pandey and Jain, 2002).

In this study, we isolated three different genera of pure bacteria, *Ochrobactrum* sp. SMX-PM1-SA1 (henceforth referenced as strain SA1), *Labrys* sp. SMX-W1-SC11 (henceforth referenced as strain SC11) and *Gordonia* sp. SMX-W2-SCD14 (henceforth referenced as strain SCD14) from different sources by enrichment technique on SMX. All three isolated pure bacterial strains showed the ability to degrade SMX and also its transformation by-products such as 4-aminophenol as well as 3-amino-5-methylisoxazole. The degradation pathway of SMX in all three bacteria were compared by GC-MS as well as Q/TOF-MS analysis and also by hydroquinone enzymatic study. Moreover, bacterial resistivity against different heavy metals were studied and reported here. Finally, chemotaxis of all three bacterial strains towards 3-amino-5-methylisoxazole and hydroquinone was also assessed by drop plate method. The results of this study might be helpful to enhance our understanding of SMX biodegradation.

2. Materials and methods

2.1. Chemicals and culture media

SMX was purchased from Sigma-Aldrich Co., USA. Acetone, acetonitrile and methanol were purchased from Merck, Germany. All other chemicals were of pure analytical grade and available commercially. Stock solutions of SMX, 4-aminophenol as well as 3-amino-5-methylisoxazole at 1 g L^{-1} were prepared in methanol and stored in amber bottles at −20 °C before use. The ammonium mineral salts (AMS) medium supplemented with yeast extract (0.04%) was prepared by method described previously (Mulla et al., 2016a) and the medium was set to pH 7.00 (using 2M NaOH or 2M HCl). The AMS medium was then distributed in 100 mL quantities into 250 mL Erlenmeyer flasks and sterilized by autoclaving for 20 min at 15 psi. For degradation study, specific substrates like SMX (5 mg L^{-1}), 4-aminophenol (5 mg L^{-1}) and 3-amino-5-methylisoxazole (4 mg L^{-1}) were added to the autoclaved AMS medium just before inoculation. Solid media contained 1.7–1.8% agar in AMS.

2.2. Enrichment of cultures and isolation of organisms

For enrichment technique, samples like wastewater (W1) and activated sludge (W2) of WWTPs (Mulla et al., 2016b) were collected from Xiamen whereas pig manure (PM1) was collected from Gaozhou District, Maoming City, China. The collected samples were used for the isolation of indigenous bacterial cultures by enrichment with SMX (6 mg L^{-1}) as a sole source of carbon and energy as described previously (Mulla et al., 2016b). In brief, 5 g and/or 5 mL of samples were dispensed in 100 mL of sterile milliQ water, mixed and filtered. The 5 mL of filtrates were transferred into 95 mL sterile AMS medium in 250 mL Erlenmeyer flasks supplemented with SMX. The enrichment cultures were then incubated aerobically under dark condition at 30 °C on a rotary shaker at 150 rpm. After one month, 5 mL of the inocula were transferred into fresh medium supplemented with SMX (6 mg L^{-1}). The flasks were again incubated for one month (until turbid, $O.D_{600}$ between 0.50 and 0.70). After 5 transfers, good growth was observed. Finally, the bacterial cultures were purified by serial dilution between 10^{-4} to 10^{-6} using 0.85% of sodium chloride (Reis et al., 2014) and were used for spread plate method. Cultured SMX (6 mg L^{-1})-AMS agar plates were kept in incubator at 30 °C for 48–72 h. Different colonies obtained from mixed microbial cultures were further grown on AMS agar plates containing SMX (6 mg L^{-1}). This process was repeated for several times to get pure individual bacteria. Based on growth response on SMX-AMS agar plates, three isolates designated as strain SA1, strain SC11 and strain SCD14 were identified and then preserved for further studies.

2.3. 16S rRNA gene sequence analysis for the identification of bacteria

Genomic DNA of each isolate was extracted using a TIANamp Bacteria DNA kit (Tiangen Biotech, Beijing, China). The 16S rRNA gene was amplified by PCR using forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTTGTTACGACTT-3′). PCR amplification was performed with a GenePro Thermal Cycler (Bioer, Hercules, China) (Mulla et al., 2016b). The 16S rRNA gene sequences of closely related taxa were obtained from the EzBioCloud database (Yoon et al., 2017). Multiple sequence alignment and phylogenetic analysis were performed using MEGA v7.0 (Kumar et al., 2016). Phylogenetic trees were constructed using the maximum-likelihood algorithm (Felsenstein, 1981) and tree topologies were evaluated using the bootstrap analysis of 500 replications.

2.4. Bacterial growth and degradation of SMX, 4-aminophenol as well as 3-amino-5-methylisoxazole

To assess the effect of various concentrations of SMX, 4-aminophenol and 3-amino-5-methylisoxazole on the growth of *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 as well as *Gordonia* sp. SMX-W2-SCD14 and their degradation, organisms

were individually determined by measurement of growth in yeast extract (0.04%)-AMS (100 mL) medium supplemented with appropriate concentration of SMX, 4-aminophenol and 3-amino-5-methylisoxazole (2–7 mg L^{-1}). The growth of strain SA1, strain SC11 and strain SCD14 was individually measured at 600 nm by UV-spectrophotometer (UV-5200 Spectrophotometer). For SMX (5 mg L^{-1}), 4-aminophenol (5 mg L^{-1}) and 3-amino-5-methylisoxazole (4 mg L^{-1}) degradation studies, 2 mL aliquots collected from each experimental flask at various intervals were centrifuged (8000 × *g*). The supernatant was further filtered using 5 mL sterile syringe with PVDF filter (0.22 μm) and 1 mL of filtrate was dispensed directly in sterile chromatographic vial for high performance liquid chromatography (HPLC) analysis. Un-inoculated culture flasks with the same concentrations of SMX, 4-aminophenol and 3-amino-5-methylisoxazole were used as control.

2.5. Identification of metabolites

The metabolic products of SMX (5 mg L^{-1}) in cell-free filtrates of individual microorganisms (strain SA1, strain SC11 and strain SCD14) were identified by gas chromatography-mass spectrometry (GC-MS) and quadrupole time-of-flight (Q-TOF) mass spectrometry (MS). The cell-free filtrates were collected at a regular interval. The same bacterial culture supernatant lacking SMX was used as a negative control and un-inoculated control containing SMX (5 mg L^{-1}) was used too. The collected samples were centrifuged (8000 × *g*) for 15 min, adjusted to pH 2.0 with 2 M HCl and then extracted with ethyl acetate. In brief, supernatant was mixed with equal volume of ethyl acetate in a brown bottle and shaken for 8 h at 150 rpm on shaker. The sample was then transferred to separating funnel and allowed for 20 min to separate aqueous and organic (ethyl acetate) layer. The procedure was repeated one more time for aqueous layer. Both organic layers (ethyl acetate) were combined together and evaporated at room temperature on anhydrous sodium sulfate. The dried samples were dissolved in acetone (460 μL) and derivatized with 40 μL N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Mulla et al., 2016c). The samples (derivatized and non-derivatized) were then analyzed by GC-MS. In addition, the extracted samples were dissolved in methanol (500 μL), filtered and then analyzed by Q/TOF-MS. The metabolites identified by GC-MS and Q/TOF-MS analysis were matched with authentic standard compounds as well as reported data.

2.6. Enzyme assays

The cell-free extracts were prepared from washed cells of *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 individually grown on SMX by ultrasonication (KQ-500DE) for 5 min followed by centrifugation at 12,000 × *g* for 45 min at 4 °C. The supernatant obtained was used as crude enzyme for enzyme assays (Mulla et al., 2016c).

Hydroquinone dioxygenase activity was determined as described previously (Arora and Jain, 2012) with little modification. The enzyme activity was measured spectrophotometrically by measuring the increase in absorbance at 320 nm due to the formation of γ(gamma)-hydroxymuconic semialdehyde. The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.0), 1 mM hydroquinone, 0.1 mM manganese sulfate and cell-free extract of protein (0.7–0.9 mg). Protein content was determined by BCA protein assay kit (Thermo Scientific, USA) (Mulla et al., 2016c). One unit of enzyme activity was defined as the amount required to catalyze the formation or consumption of 1 μmol of product or substrate per minute (Hoskeri et al., 2011).

2.7. Analytical methods

The residual concentrations of SMX, 4-aminophenol and 3-amino-5-methylisoxazole were determined using HPLC (Dionex Ultimate 3000, USA) equipped with a UV detector. The separation was executed on Luna C_{18} (5 μm, 4.6 mm × 250 mm, phenomenex, Torrance, CA, USA) column using a mixture of water-methanol (49:1) buffered with 1 mL L^{-1} phosphoric acid (25%) (A) and methanol (MeOH) (B) as the mobile phase at a flow rate of 1.2 mL min^{-1} (Mulla et al., 2016b).

Intermediates of SMX metabolism were analyzed by GC-MS as described previously (Mulla et al., 2016a). The oven temperature was programmed from 60 °C (5 min) to 310 °C at 12 °C min^{-1} followed by a 5 min hold at 310 °C. The injector temperature was kept at 250 °C and 1 μl sample was injected. Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. MS was operated under electron ionization mode at 70 eV with mass scan range of 40–800 amu. In addition, intermediates of SMX metabolism were also analyzed by Q/TOF-MS (Bruker, Germany). MS was operated in electrospray ionization (ESI) positive mode, with a capillary voltage of 4.5 kV and collision energy of 8 eV (Mulla et al., 2016b).

2.8. Chemotaxis assay

The chemotactic response of strain SA1, strain SC11 and strain SCD14 towards 3-amino-5-methylisoxazole and hydroquinone were tested qualitatively using drop plate assay according to the procedure described earlier (Arora and Bae, 2014; Mulla et al., 2016b). The individual pure bacterial cultures were grown in LB medium supplemented with SMX (5 mg L^{-1}). The bacterial cultures were harvested at mid-log phase ($O.D_{600\ nm}$ between 0.60 and 0.80) by centrifugation at 4000 × *g* for 8 min and pellet was washed two time with phosphate buffered saline (PBS), The washed individual pure bacterial pellet was re-suspended in drop plate assay medium (AMS with 0.3% bacto agar) and transferred into petri-plates (96 mm). Pinch of respective substrates were placed in the middle of plates and then incubated at 25 °C under dark conditions. The chemotactic response was observed between 4 and 7 h of incubation.

2.9. SMX degrading microorganism's resistance to heavy metals and their minimum inhibition concentration (MIC)

The heavy metals like $(CdCl_2)_2 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $NiCl_2 \cdot 6H_2O$, $Pb(NO_3)_2$ and $K_2Cr_2O_7$ stock solutions (1000 mg L^{-1}) were prepared in sterilized Milli-Q water (Mulla et al., 2016b) and stored at 4 °C until further study.

The SMX degrading bacterial cultures such as *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14 were tested for their resistance to different heavy metals including copper, cadmium, cobalt, chromium, lead, zinc and nickel using sucrose-minimal salts low-phosphate (SLP) medium [sucrose (0.9%), $(NH_4)_2SO_4$ (0.1%), K_2HPO_4 (0.05%), $MgSO_4$ (0.05%), NaCl (0.01%), yeast extract (0.04%) and $CaCO_3$ (0.05%)]. The SLP medium was set to pH 7.00 (using 2M NaOH or $2H_2SO_4$) (Mulla et al., 2016b). The bacterial cultures grown on SMX were placed onto SLP agar plates supplemented with different concentrations of individual heavy metals. The cell concentration of strain SA1, strain SC11 and strain SCD14 were 2.1×10^7, 2.4×10^7 and 2.3×10^7 cfu mL^{-1}, respectively. The organism cell concentrations were determined by plate-count method (Mulla et al., 2012). Inoculated plates were incubated at 30 °C for 72 h whereas SLP agar plate without heavy metal was taken as a positive control. The lowest heavy metal concentration that inhibits the growth of organism was recorded as the minimum inhibitory concentration

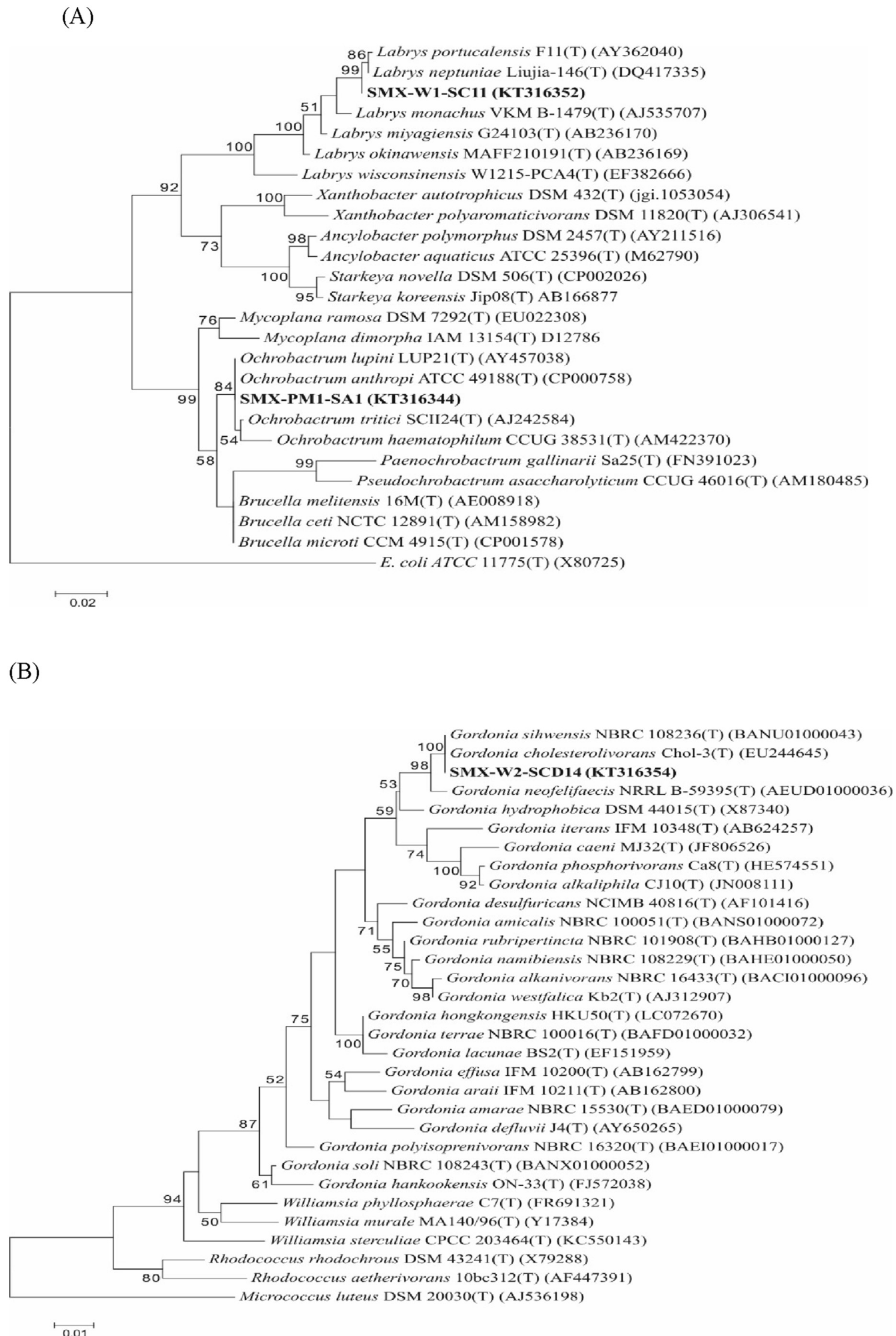

Fig. 1. Phylogenetic relationships established by the neighbor-joining method based on 16S rRNA gene sequences of isolated bacterial strains [**SMX-PM1-SA1** (A), **SMX-W1-SC11** (A) **and SMX-W2-SCD14** (B)]. Scale bar, no. of nucleotide changes/sequence position. The number at nodes shows the bootstrap values obtained with 500 resampling analyses. *E. coli* ATCC 11775 and *Micrococcus luteus* DSM 20030 were served as outgroup for the analysis of A and B, respectively.

(MIC).

3. Results and discussion

3.1. Isolation and characterization of organisms

As the results of various samples like PM1, W1 and W2 enrichment method, three different strains (one from each sample) capable to grow on ASM agar plates supplemented with SMX at 6 mg L^{-1} were attained by streaking plate method. On the basis of 16S rRNA gene sequence analysis, the pure bacterial cultures such as strain SA1, strain SC11 and strain SCD14 were identified and belonged to genus *Ochrobactrum*, *Labrys* and *Gordonia*, respectively. They were further used to construct phylogenetic tree by 16S rRNA gene sequences in comparison with other related bacteria as shown in Fig. 1. The bacterial isolates such as *Ochrobactrum* sp. SMX-PM1-SA1 and *Labrys* sp. SMX-W1-SC11 were Gram-negative whereas *Gordonia* sp. SMX-W2-SCD14 was Gram-positive. The 16S rRNA gene sequences of strain SA1, strain SC11 and strain SCD14 have been deposited in GeneBank (National Center for Biotechnology Information, NCBI) under accession number KT316344, KT316352 and KT316354, respectively. Previously, SMX degradation and/or transformation in different genus of microorganisms such as *Rhodococcus*, *Achromobacter*, *Ralstonia*, *Pseudomonas*, *Brevundimonas*, *Variovorax* and *Microbacterium* was studied and reported (Jiang et al., 2014; Larcher and Yargeau, 2011; Reis et al., 2014; Ricken et al., 2015; Wang et al., 2015).

3.2. Degradation of SMX, 4-aminophenol and 3-amino-5-methylisoxazole

The effect of initial concentration of SMX on strain SA1, strain SC11 and strain SCD14 showed maximum growth at 5 mg L^{-1}. The growth of *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 as well as *Gordonia* sp. SMX-W2-SCD14 in yeast extract (0.04%)-AMS medium supplemented with SMX (5 mg L^{-1}) are shown in Fig. 2. All three bacteria were able to degrade SMX (5 mg L^{-1}) up to 45.2%, 62.2% and 51.4%, respectively within 288 h. No further SMX degradation was observed even after incubating for prolonged periods (>310 h). The bacterial strain SC11 showed higher degradation of SMX than other two microorganisms. In addition, all three bacteria were able to grow in yeast extract (0.04%)-AMS medium supplemented with 4-aminophenol, 3-amino-5-methylisoxazole and hydroquinone at 5 mg L^{-1}.

The individual bacterial cultures such as strain SA1 and strain SCD14 were capable to degrade 4-aminophenol (5 mg L^{-1}) up to 66.2% and 69.2%, respectively within 216 h whereas strain SC11 completely degraded 4-aminophenol at 5 mg L^{-1} within 120 h (Fig. 3). Furthermore, all three pure bacteria completely degraded 3-amino-5-methylisoxazole at 4 mg L^{-1} within 120 h (Fig. 4). The low degradation rate of SMX might be due to the toxic effect of parent compound or its by-product(s). Previous studies have described only the SMX degradation in microorganism (Larcher and Yargeau, 2011; Reis et al., 2014). Larcher and Yargeau (2011), studied the removal of SMX in individual and mixed consortia and reported that *Rhodococcus equi* alone had the maximum capacity to remove SMX up to 29% elimination with glucose as a co-substrate and the formation of a metabolite. However, this elimination was unaffected in the absence of glucose, without any intermediate formation. In another study, Reis et al. (2014) reported that the SMX was degraded in mixed cultures with the accumulation of 3-amino-5-methylisoxazole only. Moreover, they also observed that the addition of co-substrate (succinate) enhanced greater yields of bacterial biomass, which helps quicker reduction of SMX (28.5 h) than SMX alone as a carbon source. However, these studies mainly focused on the removal of SMX. In this study, we provided the results of SMX degradation in all three pure individual bacteria as well as the degradation/removal of more toxic transformation by-products (SMX by-products) such as 3-amino-5-methylisoxazole and 4-aminophenol.

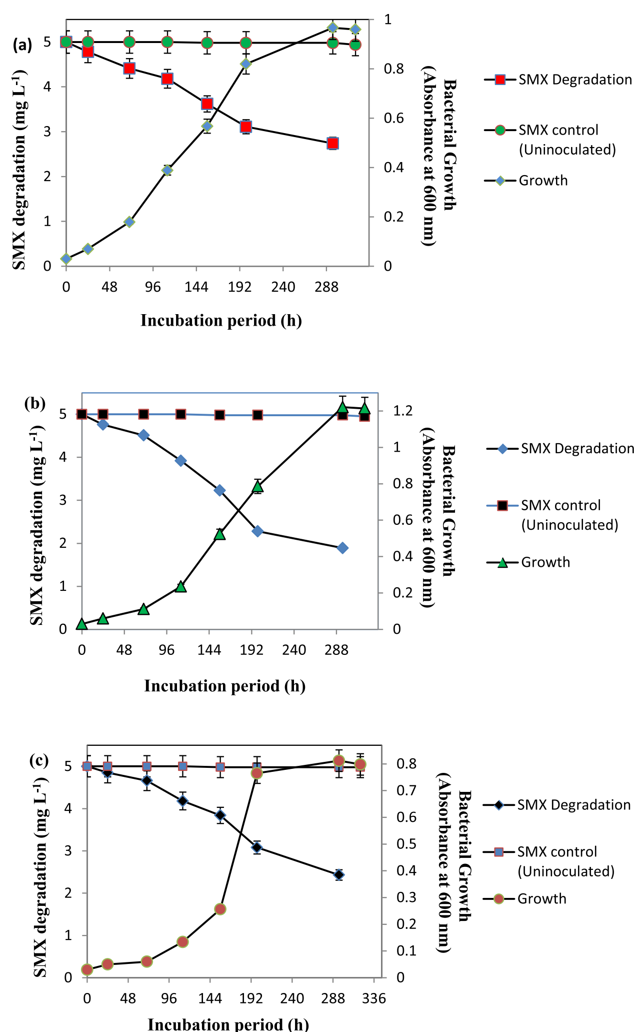

Fig. 2. Degradation of SMX (5 mg L^{-1}) in ***Ochrobactrum* sp. SMX-PM1-SA1** (a), ***Labrys* sp. SMX-W1-SC11** (b) and ***Gordonia* sp. SMX-W2-SCD14** (c). Error bar indicates the standard deviation of the triplicates.

3.3. Identification of metabolites, enzyme assays and SMX degradation mechanism

The analysis of culture supernatants of all three bacterial strains grown on the yeast extract (0.04%)-AMS medium supplemented with SMX (5 mg L^{-1}) revealed that the presence of three metabolites (Isolate I, Isolate II, and Isolate III). The mass spectra (GC-MS) of isolated compound II (Fig. 5a) was identified as 4-aminophenol by comparing with authentic compound (Supplementary information, Fig. S1), whereas compound III (Fig. 5b) was identified as hydroquinone by comparing with previously published data (Mulla et al., 2016c). In addition, Isolate I and Isolate II were identified as 3-amino-5-methylisoxazole and 4-aminophenol, respectively from Q/TOF results (Fig. S2). The 3-amino-5-methylisoxazole mass was confirmed by previously reported data (Wang et al., 2015). In all three microorganisms, the isolated metabolites (I, II and III) were same, and therefore, GC-MS and Q/TOF-MS spectral data of SMX

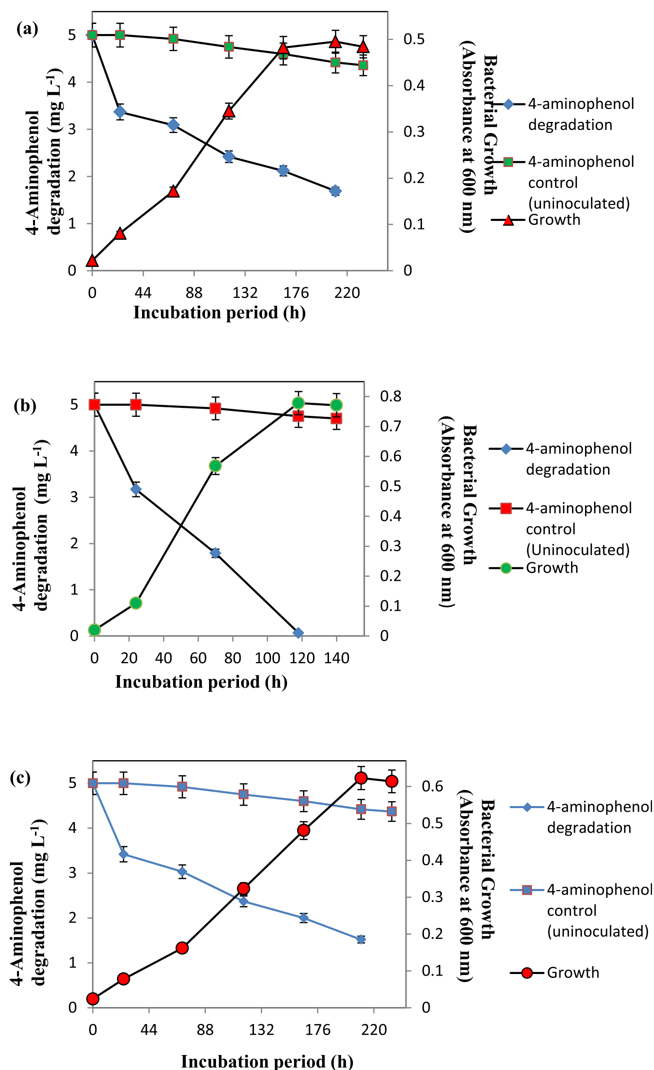

Fig. 3. 4-Aminophenol (5 mg L^{-1}) degradation in ***Ochrobactrum* sp. SMX-PM1-SA1** (a), ***Labrys* sp. SMX-W1-SC11** (b) and ***Gordonia* sp. SMX-W2-SCD14** (c). Error bar indicates the standard deviation of the triplicates.

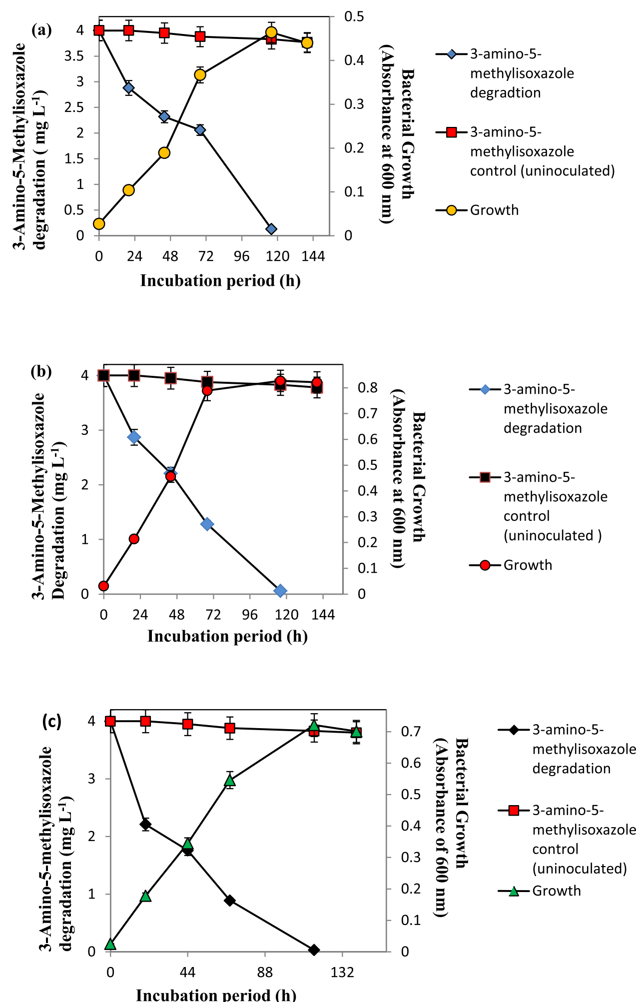

Fig. 4. 3-Amino-5-methylisoxazole (4 mg L^{-1}) degradation in ***Ochrobactrum* sp. SMX-PM1-SA1** (a), ***Labrys* sp. SMX-W1-SC11** (b) ***Gordonia* sp. SMX-W2-SCD14** (c). Error bar indicates the standard deviation of the triplicates.

metabolites from only one microorganism (*Labrys* sp. SMX-W1-SC11) were provided. Literatures revealed that hydroquinone was commonly identified as the intermediate during the degradation of various chemicals like triclosan, 2-chloro-4-nitrophenol, SMX, 4-nitrophenol, gamma-hexachlorocyclohexane, pentachlorophenol and 2,4,6-trichlorophenol (Arora and Jain, 2012; Ju and Parales, 2010; Mulla et al., 2016c; Ricken et al., 2015).

Additionally, cell-free extracts prepared individually from strain SC11 and strain SCD14 grown on SMX contained the activity of hydroquinone dioxygenase. The specific activity of hydroquinone dioxygenase for hydroquinone was found to be 0.292 ± 0.03 (strain SC11) and 0.227 ± 0.02 (strain SCD14). Similarly, hydroquinone dioxygenase activity was also observed in other bacteria like *Arthrobacter* sp. SPG, *Burkholderia* sp. RKJ 800, *Moraxella* sp., and *Pseudomonas* sp. strain WBC-3 (Arora, 2012; Arora and Jain, 2012; Spain and Gibson, 1991; Zhang et al., 2009), which proceeds through similar hydroquinone ring-cleavage pathway like in strain SC11 and strain SCD14. Generally, hydroquinone dioxygenase assay was performed to determine ring-cleavage pathway. On the other hand, cell-free extract of strain SA1 grown on SMX did not show hydroquinone dioxygenase activity.

Here, we propose a pathway for the degradation of SMX in bacteria (Fig. 6). In all three pure bacteria, SMX was initially transformed into 4-aminophenol and 3-amino-5-methylisoxazole by ipso-hydrolysis. Previously, 4-aminophenol degradation in *Pseudomonas* sp. strain ST-4 was studied and reported (Khan et al., 2006). However, there is no information on accumulation of intermediates during 4-aminophenol degradation in strain ST-4 (Khan et al., 2006). In all three bacteria, 4-aminophenol was further transformed into hydroquinone. However, no further degradation by-products were detected in all three bacterial culture supernatants. On the other hand, in strain BR1, hydroquinone was further transformed into 1,2,4-trihydroxybenzene (Ricken et al., 2015). In another study, SMX was sequentially transformed into aniline, 3-amino-5-methylisoxazole, 4-aminothiophenol and sulfanilamide by *Pseudomonas psychrophila* HA-4 (Jiang et al., 2014).

Moreover, in all three pure bacteria, 3-amino-5-methylisoxazole was completely degraded. However, no further degradation by-products were observed. Meanwhile, there are reports on SMX transformation into 3-amino-5-methylisoxazole in other bacteria and also in activated sludge (Muller et al., 2013; Ricken et al., 2013, 2015; Jiang et al., 2014; Wang et al., 2015). These results indicate that 3-amino-5-methylisoxazole is a common by-product of SMX catabolism. However, there is no information on further

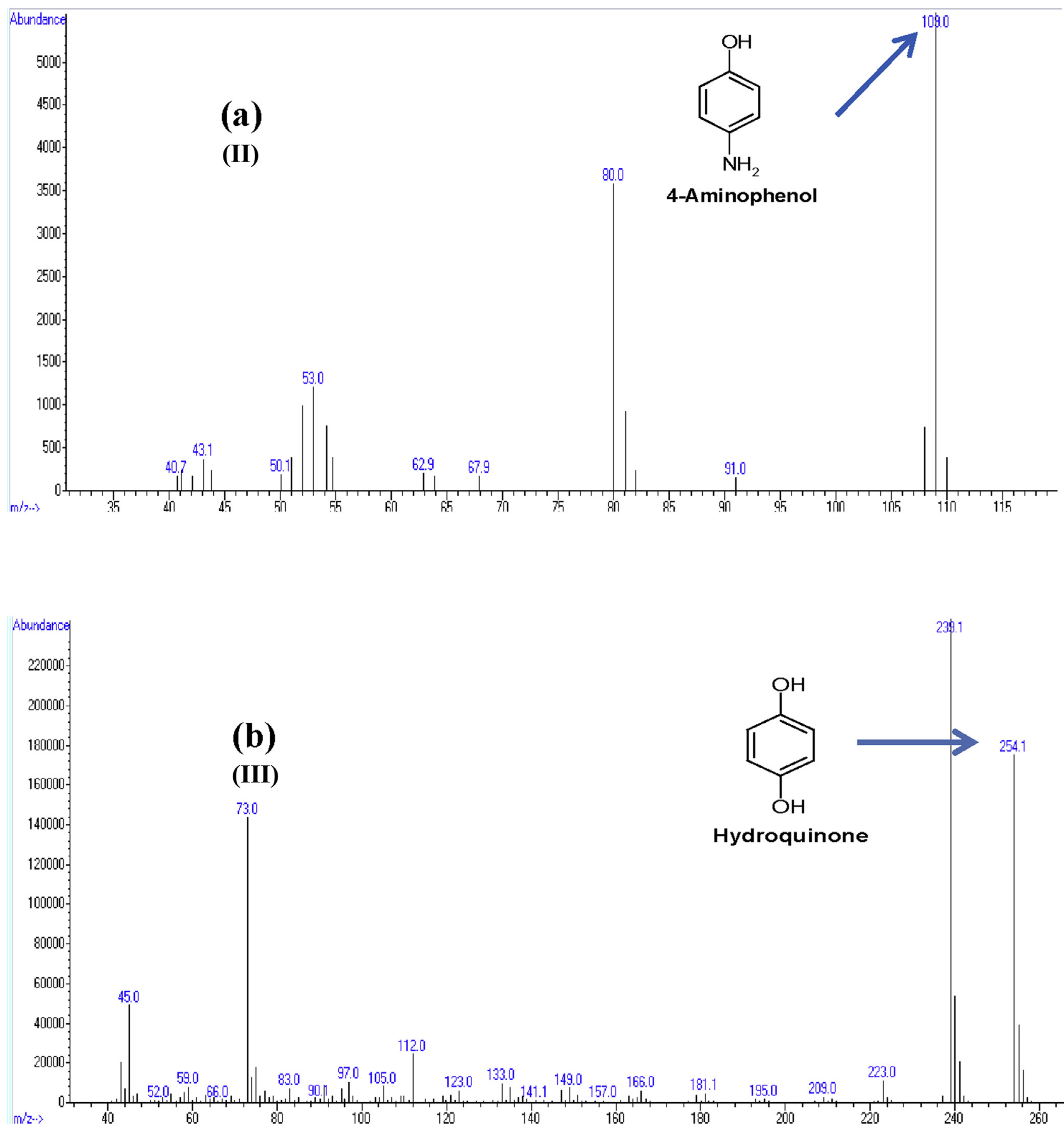

Fig. 5. Mass spectra of isolated metabolites (a and b) of SMX degradation by bacteria.

degradation of 3-amino-5-methylisoxazole in microorganism.

Additionally, the presence of high activities of hydroquinone dioxygenase in the cell-free extract of both strain SC11 and strain SCD14 individually grown on SMX suggested that hydroquinone would be further oxidized into γ(gamma)-hydroxymuconic semialdehyde as shown in Fig. 6. These results suggest that in both bacteria, SMX degradation pathway proceeds in the same direction (Fig. 6). On the other hand, cell-free extract of strain SA1 did not show hydroquinone dioxygenase activity, which suggests that in this microorganism, the later pathway (after hydroquinone) of SMX proceeds in different direction from strain SC11 and strain SCD14 (Fig. 6). The bacterial strains reported in this study were not previously reported for the degradation of SMX. In addition, these pure bacterial cultures were also able to degrade SMX toxic by-products such as 4-aminophenol and 3-amino-5-methylisoxazole. Future studies are therefore essential on finding the enzymes, which are involved in SMX metabolism.

3.4. Chemotaxis of strain SA1, strain SC11 and strain SCD14 towards 3-amino-5-methylisoxazole and hydroquinone

We also studied the chemotactic behavior of pure bacterial cultures towards 3-amino-5-methylisoxazole and hydroquinone by drop plate assay, which were observed in culture filtrates of all

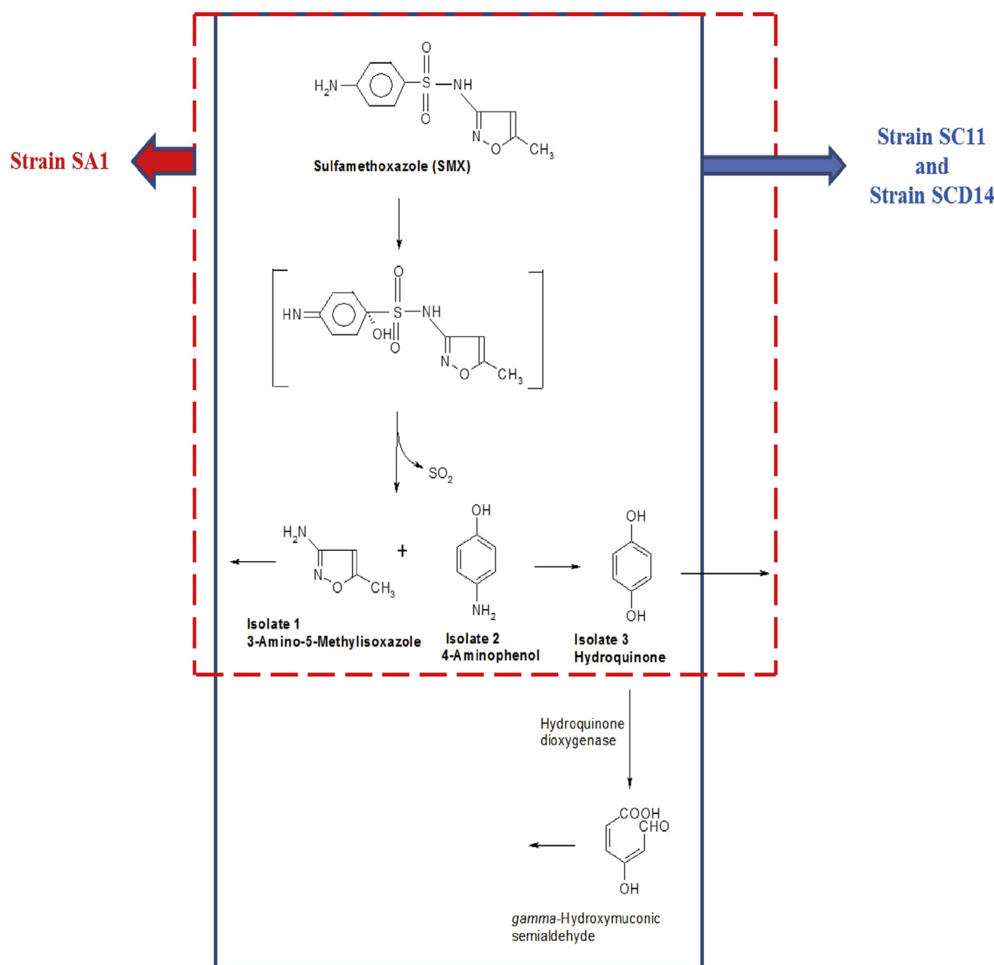

Fig. 6. A proposed pathway of SMX degradation in ***Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11** and ***Gordonia* sp. SMX-W2-SCD14**.

three pure bacteria grown on SMX (5 mg L^{-1}). Drop plate experimental results revealed the development of bacterial ring near the crystals of 3-amino-5-methylisoxazole and hydroquinone after incubation between 4 and 6 h (Fig. 7). There are reports on various microorganisms having metabolism-dependent chemotactic response to various xenobiotics (Arora and Bae, 2014; Arora et al., 2014a; Hawkins and Harwood, 2002; Pandey et al., 2012). For example, *Burkholderia* sp. strain SJ98 showed chemotactic response towards substrates like 4-nitrophenol, 2-chloro-3-nitrophenol, 3-methyl-4-nitrophenol and 2-chloro-4-nitrophenol, which the strain can only mineralize or co-metabolize (Arora et al., 2014b). In another study, 4-nitrophenol degrading bacteria, *Pseudomonas* sp. WBC-3, showed metabolism-independent chemotaxis toward 4-nitrophenol, 3-nitrophenol, nitrocatechol, hydroquinone and other aromatic chemicals (Zhang et al., 2008). On the other hand, *Bacillus subtilis* PA-2 showed a negative chemotaxic response towards 4-chloro-2-nitrophenol, 4-nitrophenol, and 2,6-dichloro-4-nitrophenol (Arora et al., 2015). Our previous study (2016b) demonstrated three sulfadiazine degrading bacterial strains, *Paracoccus* sp. SDZ-PM2-BSH30, *Methylobacterium* sp. SDZ-W2-SJ40 and *Kribbella* sp. SDZ-3S-SCL47 also showed chemotactic response towards 2-aminopyrimidine. The microorganisms with positive chemotactic response have additional advantages for the removal of toxic pollutant(s) in the environment due to their strong attraction in nature towards such chemicals. Further studies should explore the molecular aspects related to the chemotactic response in our isolated microorganisms.

3.5. Characterization of heavy metal resistivity

Previous studies demonstrated that the antibiotic degrading and/or resistant microorganisms have also capability to resist different heavy metals (Singh et al., 2010; Holzel et al., 2012; Chen et al., 2015; Mulla et al., 2016b). Hence, in this study, sulfonamide antibiotic SMX degrading strain SA1, strain SC11 and strain SCD14 were exposed to heavy metals for the determination of the MIC values. All three pure bacteria showed resistance to different heavy metals like Copper (Cu), Cadmium (Cd), Chromium (Cr), Cobalt (Co), Lead (Pb), Nickel (Ni) and Zinc (Zn) (Table 1). The bacterial strain SA1 resist heavy metals like Pb, Cd and Zn up to 228 mg L^{-1}, 112 mg L^{-1} and 234 mg L^{-1}, respectively, whereas strain SC11 resist heavy metals like Cd as well as Zn up to 104 mg L^{-1} and 221 mg L^{-1}, respectively. The bacterial strain SCD14 resist heavy metals like Pb, Co, Ni and Zn up to 204 mg L^{-1}, 216 mg L^{-1}, 106 mg L^{-1} and 234 mg L^{-1}, respectively (Table 1). On the other hand, strain SC11 strain showed least MIC to Cr at 16 mg L^{-1} and Ni at 22 mg L^{-1} whereas strain SCD14 strain showed least MIC to Cu and Cd at 40 mg L^{-1} (Table 1). Antibiotics and heavy metals are widespread contaminants in the environment. Consequently, in multiple stressed conditions, bacterial cultures procure resistance by adjustments in genetic elements either by mutation or by transfer of resistant genes from the surrounded bacteria (Singh et al., 2010).

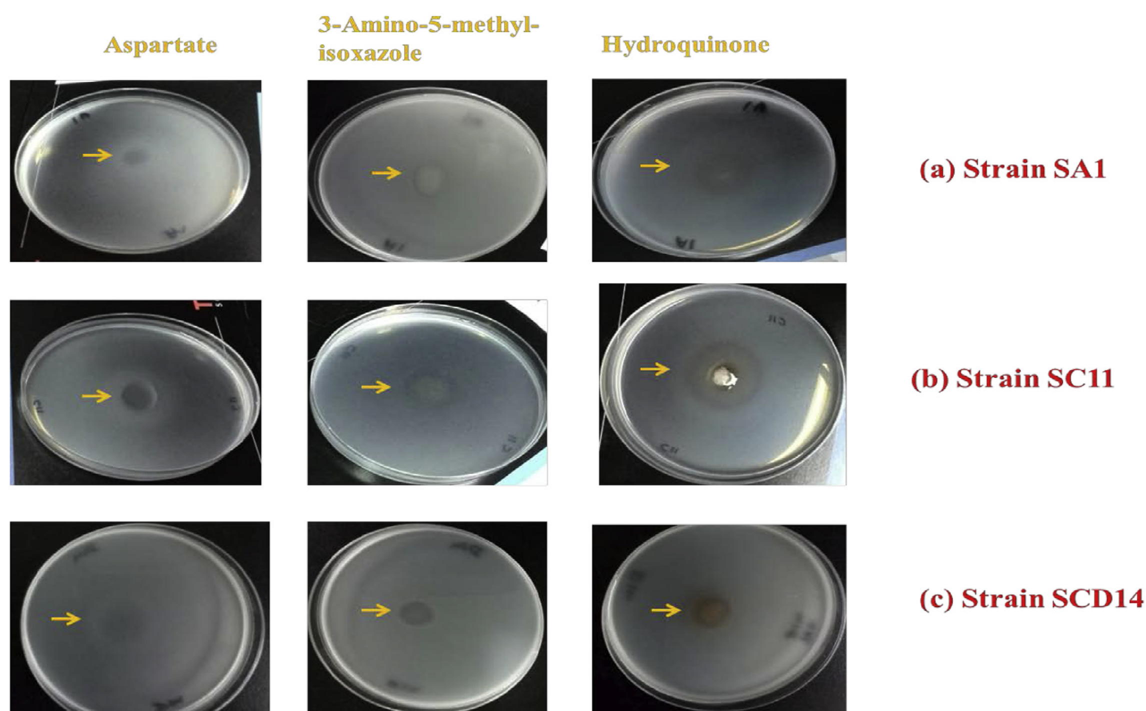

Fig. 7. Chemotaxis of ***Ochrobactrum*** **sp. SMX-PM1-SA1** (a), ***Labrys*** **sp. SMX-W1-SC11** (b), and ***Gordonia*** **sp. SMX-W2-SCD14** (c) towards 3-amino-5-methylisoxazole and hydroquinone. The bacterial cells were grown on SMX and tested on 3-amino-5-methylisoxazole and hydroquinone. Results were obtained by drop plate assays. The assays were performed in triplicate and the representative plates are shown here. Aspartate was used as the positive control.

Table 1
Minimum inhibitory concentration of heavy metal for bacterial isolates.

Bacteria	Minimum inhibitory concentration of heavy metals (mg L^{-1})						
	Pb	Cu	Co	Cr	Cd	Ni	Zn
Strain SA1	228	52	56	88	112	94	208
Strain SC11	38	46	54	16	104	22	221
Strain SCD14	204	40	216	52	40	106	234

Hence, these microorganisms might be useful to adsorb or transform heavy metals from contaminated sites.

4. Conclusions

SMX (5 mg L^{-1}) degradation was observed up to 45.2%, 62.2% and 51.4% in isolated individual pure bacteria, *Ochrobactrum* sp. SMX-PM1-SA1, *Labrys* sp. SMX-W1-SC11 and *Gordonia* sp. SMX-W2-SCD14, respectively. In all three bacterial cultures, SMX was sequentially converted into 3-amino-5-methylisoxazole, 4-aminophenol, and hydroquinone. All three bacteria completely degraded 3-amino-5-methylisoxazole at 4 mg L^{-1}, and further metabolites of 3-amino-5-methylisoxazole were not detected in the culture medium. These microorganisms also degraded 5 mg L^{-1} of 4-aminophenol. Moreover, cell free extracts of strain SC11 and strain SCD14 showed hydroquinone dioxygenase activity whereas strain SA1 did not contain the enzyme activity of hydroquinone dioxygenase. Hence, strain SC11 and strain SCD14 utilize the same degradation pathway of SMX which differs from strain SA1. Additionally, all bacterial strains were resistant to various heavy metals and exhibited positive chemotaxis toward 3-amino-5-methylisoxazole and hydroquinone. Hence these bacterial cultures could be useful for the biodegradation of SMX and its toxic by-products in the contaminated sites.

Competing financial interests

The authors declare that they have no competing interests.

Authors' contributions

SIM and CPY conceived and designed the study. SIM, AH, QS, JL, FS and MA performed experimental works. SIM, AH, QS and CPY analyzed the data and SIM and CPY wrote the paper. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jenvman.2017.10.029.

References

Akhtar, J., Amin, N.S., Aris, A., 2011. Combined adsorption and catalytic ozonation for removal of sulfamethoxazole using Fe_2O_3/CeO_2 loaded activated carbon. Chem. Eng. J. 170, 136–144.
Arora, P.K., 2012. Metabolism of *para*-nitrophenol in *Arthrobacter* sp. SPG. E3 J. Environ. Res. Manag. 3 (3), 52–57.

Arora, P.K., Bae, H., 2014. Biotransformation and chemotaxis of 4-chloro-2-nitrophenol by *Pseudomonas* sp. JHN. Microb. Cell. Fact. 13, 110.
Arora, P.K., Jain, R.K., 2012. Metabolism of 2-chloro-4-nitrophenol in a gram negative bacterium, *Burkholderia* sp. RKJ 800. PLos One 7 (6), e38676.
Arora, P.K., Srivastava, A., Singh, V.P., 2014a. Degradation of 4-chloro-3-nitrophenol via a novel intermediate, 4-chlororesorcinol by *Pseudomonas* sp. JHN. Sci. Rep. 4, 4475.
Arora, P.K., Srivastava, A., Singh, V.P., 2014b. Bacterial degradation of nitrophenols and their derivatives. J. Hazard Mater. 266, 42–59.
Arora, P.K., Jeong, M.J., Bae, H., 2015. Chemotaxis away from 4-chloro-2-nitrophenol, 4-nitrophenol and 2,6-dichloro-4-nitrophenol by *Bacillus subtilis* PA-2. J. Chem., 296231
Boreen, A., Arnold, W., McNeill, K., 2004. Photochemical fate of sulfa drugs in then aquatic environment: Sulfa drugs containing five-membered heterocyclic groups. Environ. Sci. Technol. 38, 3933–3940.
Chen, S., Li, X., Sun, G., Zhang, Y., Su, J., Ye, J., 2015. Heavy metal induced antibiotic resistance in Bacterium Lsjc7. Int. J. Mol. Sci. 16, 23390–23404.
Dantas, R.F., Contreras, S., Sans, C., Esplugas, S., 2008. Sulfamethoxazole abatement by means of ozonation. J. Hazard Mater. 150, 790–794.
Dirany, A., Efremova Aaron, S., Oturan, N., Sires, I., Oturan, M.A., Aaron, J.J., 2011. Study of the toxicity of sulfamethoxazole and its degradation products in water by a bioluminescence method during application of the electro-Fenton treatment. Anal. Bioanal. Chem. 400, 353–360.
Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.
Hawkins, A.C., Harwood, C.S., 2002. Chemotaxis of *Ralstonia eutropha* JMP134 (pJP4) to the herbicide 2, 4-dichlorophenoxyacetate. Appl. Environ. Microb. 68, 968–972.
Holzel, C.S., Muller, C., Harms, K.S., Mikolajewski, S., Schafer, S., Schwaiger, K., Bauer, J., 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environ. Res. 113, 21–27.
Hoskeri, R.S., Mulla, S.I., Shouche, Y.S., Ninnekar, H.Z., 2011. Biodegradation of 4-chlorobenzoic acid by *Pseudomonas aeruginosa* PA01 NC. Biodegradation 22, 509–516.
Jia, Y., Khanal, S.K., Zhang, H., Chen, G.-H., Lu, H., 2017. Sulfamethoxazole degradation in anaerobic sulfate-reducing bacteria sludge system. Water Res. 119, 12–20.
Jiang, B., Li, A., Cui, D., Cai, R., Ma, F., Wang, Y., 2014. Biodegradation and metabolic pathway of sulfamethoxazole by *Pseudomonas psychrophila* HA-4, a newly isolated cold-adapted sulfamethoxazole-degrading bacterium. Appl. Microbiol. Biot. 98, 4671–4681.
Ju, K.S., Parales, R.E., 2010. Nitroaromatic compounds, from synthesis to biodegradation. Microbiol. Mol. Biol. Rev. 74, 250–272.
Kassotaki, E., Buttiglieri, G., Ferrando-Climent, L., Rodriguez-Roda, I., Pijuan, M., 2016. Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products. Water Res. 94, 111–119.
Khan, S.A., Hamayun, M., Ahmed, S., 2006. Degradation of 4-aminophenol by newly isolated *Pseudomonas* sp. strain ST-4. Enzyme Microb. Technol. 38, 10–13.
Kielhofner, G., 2005. Rethinking disability and what to do about it: Disability studies and its implications for occupational therapy. Am. J. Occup. Ther. 59, 487–496.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Larcher, S., Yargeau, V., 2011. Biodegradation of sulfamethoxazole by individual and mixed bacteria. Appl. Microbiol. Biot. 91, 211–218.
Lv, M., Sun, Q., Hu, A., Hou, L., Li, J., Cai, X., Yu, C.P., 2014. Pharmaceuticals and personal care products in a mesoscale subtropical watershed and their application as sewage markers. J. Hazard Mater. 280, 696–705.
Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995.
Mulla, S.I., Talwar, M.P., Hoskeri, R.S., Ninnekar, H.Z., 2012. Enhanced degradation of 3-nitrobenzoate by immobilized cells of *Bacillus flexus* strain XJU-4. Biotechnol. Bioproc. E. 17, 1294–1299.
Mulla, S.I., Hu, A., Wang, Y., Sun, Q., Huang, S.-L., Wang, H., Yu, C.-P., 2016a. Degradation of triclocarban by a triclosan-degrading *Sphingomonas* sp. strain YL-JM2C. Chemosphere 144, 292–296.
Mulla, S.I., Sun, Q., Hu, A., Wang, Y., Ashfaq, M., Eqani, S.A.M.A.S., Yu, C.-P., 2016b. Evaluation of sulfadiazine degradation in three newly isolated pure bacterial cultures. PLos One 11, e0165013.
Mulla, S.I., Wang, H., Sun, Q., Hu, A., Yu, C.-P., 2016c. Characterization of triclosan metabolism in *Sphingomonas* sp. strain YL-JM2C. Sci. Rep. 6, 21965.
Muller, E., Schussler, W., Horn, H., Lemmer, H., 2013. Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source. Chemosphere 92, 969–978.
Pandey, G., Jain, R.K., 2002. Bacterial Chemotaxis toward Environmental Pollutants: Role in Bioremediation. Appl. Environ. Microbiol. 68, 5789–5795.
Pandey, J.P., Sharma, N.K., Khan, F., Ghosh, A., Oakeshott, J.G., Jain, R.K., Pandey, G., 2012. Chemotaxis of *Burkholderia* sp. Strain SJ98 towards chloronitroaromatic compounds that it can metabolise. BMC Microbiol. 12, 19.
Reis, P.J., Reis, A.C., Ricken, B., Kolvenbach, B.A., Manaia, C.M., Corvini, P.F., Nunes, O.C., 2014. Biodegradation of sulfamethoxazole and other sulfonamides by *Achromobacter denitrificans* PR1. J. Hazard Mater. 280, 741–749.
Ricken, B., Corvini, P.F., Cichocka, D., Parisi, M., Lenz, M., Wyss, D., Martinez-Lavanchy, P.M., Muller, J.A., Shahgaldian, P., Tulli, L.G., Kohler, H.P., Kolvenbach, B.A., 2013. Ipso-hydroxylation and subsequent fragmentation: a novel microbial strategy to eliminate sulfonamide antibiotics. Appl. Environ. Microb. 79, 5550–5558.
Ricken, B., Fellmann, O., Kohler, H.P., Schaffer, A., Corvini, P.F., Kolvenbach, B.A., 2015. Degradation of sulfonamide antibiotics by *Microbacterium* sp. strain BR1-elucidating the downstream pathway. New. Biotechnol. 32, 710–715.
Singh, S.K., Tripathi, V.R., Jain, R.K., Vikram, S., Garg, S.K., 2010. An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease. Microb. Cell. Fact. 9, 59.
Spain, J.C., Gibson, D.T., 1991. Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. 57 (3), 812–819.
Sun, Q., Li, M., Ma, C., Chen, X., Xie, X., Yu, C.P., 2016. Seasonal and spatial variations of PPCP occurrence, removal and mass loading in three wastewater treatment plants located in different urbanization areas in Xiamen, China. Environ. Pollut. 208, 371–381.
Trovo, A.G., Nogueira, R.F., Aguera, A., Fernandez-Alba, A.R., Sirtori, C., Malato, S., 2009. Degradation of sulfamethoxazole in water by solar photo-Fenton. Chemical and toxicological evaluation. Water Res. 43, 3922–3931.
Wang, L., Wu, Y., Zheng, Y., Liu, L., Zhao, F., 2015. Efficient degradation of sulfamethoxazole and the response of microbial communities in microbial fuel cells. RSC. Adv. 5, 56430–56437.
Xu, B., Mao, D., Luo, Y., Xu, L., 2011. Sulfamethoxazole biodegradation and biotransformation in the water-sediment system of a natural river. Bioresource. Technol. 102, 7069–7076.
Yargeau, V., Huot, J.C., Rodayan, A., Rouleau, L., Roy, R., Leask, R.L., 2008. Impact of degradation products of sulfamethoxazole on mammalian cultured cells. Environ. Toxicol. 23, 492–498.
Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J., 2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 1613–1617.
Zhang, J., Xin, Y., Liu, H., Wang, S., Zhou, N., 2008. Metabolism-independent chemotaxis of *Pseudomonas* sp. strain WBC-3 toward aromatic compounds. J. Environ. Sci. 20, 1238–1242.
Zhang, J.J., Liu, H., Xiao, Y., Zhang, X.E., Zhou, N.Y., 2009. Identification and characterization of catabolic para-Nitrophenol 4-Monooxygenase and para-Benzoquinone reductase from *Pseudomonas* sp. Strain WBC-3. J. Bacteriol. 191, 2703–2710.
Zhang, D., Pan, B., Zhang, H., Ning, P., Xing, B., 2010. Contribution of different sulfamethoxazole species to their overall adsorption on functionalized carbon nanotubes. Environ. Sci. Technol. 44, 3806–3811.