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Degradation of 17α-methyltestosterone by *Rhodococcus* sp. and *Nocardioides* sp. isolated from a masculinizing pond of Nile tilapia fry

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ABSTRACT

17α-Methyltestosterone (MT), a synthetic anabolic androgenic steroid, is widely used in aquafarming for the production of an all male fish population such as Nile tilapia. This study isolated, identified and characterized MT-degrading bacteria in the sediment and water from a masculinizing pond of Nile tilapia fry. Based on the phylogeny, physiological properties and cell morphology, the three isolated MT-degrading bacteria were related closely to *Rhodococcus equi*, *Nocardioides aromaticivorans*, and *Nocardioides nitrophenolicus*. Growth of the three isolated strains was found to be inhibited for MT concentrations in the range of 1.0–10 mg/L. The inhibition of cell growth was found to be modeled using the Haldane's substrate inhibition model. The kinetic constants ranged from 0.13 to 0.19 h^{-1} for μ_{max}, 0.7–24.8 mg/L for K_s and 19.6–76.2 mg/L for K_i. Androgenic activity using β-galactosidase assay showed that all strains degraded MT to the products with no androgenic potency.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the most highly cultivated freshwater aquaculture fishes in many developing countries. In the production process of Nile tilapia, an all male population culture is desired, as male tilapia has double the growth rate and a larger body size and weight than the female tilapia [1,2]. Mono-sex production alleviates the problem of overpopulation and energy loss from stunting. An all male population can be achieved by feeding post-fertilized Nile tilapia fry for 21 days with food containing 17α-methyltestosterone (MT), a synthetic anabolic androgenic steroid, at a concentration of 60 mg per one kilogram of feed [3].

The residual MT from uneaten and unmetabolized MT-impregnated food remains in the ponds and if released, can contaminate the environment around the discharge points. MT has endocrine disrupting property at part per billion levels [4,5] and can interfere with the normal functions of the reproductive systems of humans and animals. Korsgaard [6] found that vitellogenin protein expressing female characteristic was decreased in female eelpout (*Zoarces viviparous*) after exposure to 10–500 ng/L of MT for ten days. Schulte-Oehlmann et al. [7] found that male sex organs such as penis and vas deferens in female freshwater ramshorn snail (*Marisa cornuarietis*) were developed when exposed to 100–1000 ng/L of MT for six months. Other studies include a decrease in the fecundity and fertility of medaka fish when exposed to about 46 ng/L of MT [8] and a decrease in the egg-laying rate of female Japanese quails (*Coturnix cotumix japonica*) and the fertility of male Japanese quails when exposed to 50–110 mg/L of MT for 3 weeks [9]. MT in high dosage can be converted to 17α-methylestradiol (ME2) which has been found to produce estrogenic activity by aromatase enzyme [10].

To date, reports on the occurrence of MT in masculinization process of Nile tilapia fry are extremely limited; but the potential risk of MT residue from masculinization process has been documented. Hulak et al. [11] showed that treated water from a masculinization pond continued to contain residual MT at the levels high enough to achieve 81–100% sex inversion of common carp (*Cyprinus carpio* L.) progeny. Our recent study [12] revealed that MT biodegraded in the sediments of a masculinizing pond of Nile tilapia fry. The biodegradation rates differed for different electron acceptor conditions. MT was transformed rapidly under aerobic, sulfate-reducing, and methanogenic conditions but was hardly degraded under nitrate and iron-reducing conditions. However, under methanogenic condition, the androgenic potency measured after 10 days of incubation was similar to the starting MT concentration even though the MT concentration was close to zero. The androgenic potency continued to persist even one month after the

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measurement on the tenth day. This persistent androgenic potency was not observed for aerobic condition. To avoid the distribution of residual MT and other androgenic-like compounds (MT metabolites) into the environment, contaminated sediment and water must be properly treated before they are discharged. In this study, MT-degrading bacteria were isolated from sediment and water of a masculinization pond of Nile Tilapia fry. The isolated MT-degrading bacteria were identified and characterized for their MT biodegradability.

2. Materials and methods

2.1. Sediment and water samples

Sediment and water samples were collected from a masculinizing pond of Nile tilapia fry in Thailand. The sediment sample was taken from the topmost layer at a depth of 0 and 5 cm using a coring device. The water sample was collected from the surface by a grab sampler. The sediment and water samples were kept on ice, transported to the laboratory (transportation time <1 h) and used immediately for enrichment.

2.2. Inorganic salt medium

Inorganic salt medium was prepared by dissolving 100 mg of NH_4Cl, 1 g of $NaNO_3$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.05 g of ethylenediaminetetraacetic acid-disodium ferric (EDTA-Fe), 0.05 g of $CaCl_2{\cdot}2H_2O$, 0.05 g of K_2HPO_4, 4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.6 mg of $MnCl_2{\cdot}4H_2O$, 0.5 mg of H_3BO_3, 0.1 mg of $ZnCl_2$, 0.1 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.6 mg of $CoCl_2{\cdot}6H_2O$, 0.12 mg of $NiCl_2{\cdot}6H_2O$, and 0.12 mg of $CuSO_4{\cdot}5H_2O$ in 1 L of Milli-Q water. The medium was autoclaved and the pH was adjusted to 7.5–8.0 by 1 N of NaOH [13].

2.3. Enrichment of MT-degrading bacteria

Enrichment of MT-degrading bacteria was performed with the sediment and water samples at initial MT concentrations of 10 and 100 mg/L. Two mL of MT stock solutions of 500 and 5000 mg/L in methanol was added to 250 mL Erlenmeyer flasks. The methanol was evaporated by gently blowing nitrogen gas into the flasks. 10 mL of sediment slurry was added into the flasks along with 90 mL of inorganic salt medium (10%, v/v). The flasks were incubated at 25 °C and rotated at a speed of 200 rpm. MT concentrations were monitored every 6–12 h and when the concentration was reduced by 70%, an aliquot of 10 mL was transferred and subcultured in fresh 90 mL of inorganic salt medium containing the corresponding initial MT concentration. This process was repeated five times. The time needed for each subculture enrichment was about 2 weeks.

2.4. Isolation of MT-degrading bacteria

Serial 10-fold dilutions of the enriched cultures were prepared with fresh inorganic medium and 100 mL of each diluted culture solution (10^1 to 10^9) was spread onto agar plates and incubated at 25 °C. Agar plate was prepared by adding agar (Sigma, St. Louis, MO, USA) to the inorganic salt medium to obtain a final concentration of 17 g/L. Eighty μL of MT stock solutions of 500 and 5000 mg/L were placed on the agar surface. Methanol was allowed to evaporate. The plates were then incubated for two or three days. Single colony from the plates was picked and streaked onto the new plates. This procedure was repeated until a pure colony was obtained.

2.5. Identification of MT-degrading bacteria

DNA was extracted from the isolated MT-degrading bacteria using a DNA extraction kit (Qiagen, USA). The extracted product was verified by electrophoresis in 2% agarose (Bio-Rad, USA). Primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′) [14] were used to amplify almost-full length fragment of 16S rRNA gene. Extracted DNA was polymerase chain reaction (PCR)-amplified using the primer sets with *Taq* DNA polymerase (Fermentas, CA, USA) in a PTC-DNA engine cycler (Bio-Rad, USA). The PCR condition was 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by 10 min final extension at 72 °C. The PCR product was purified with QIAquick® PCR purified kit (Qiagen, USA) and analyzed for sequencing at Macrogen Inc., Korea with primers 27f, 341f (5′-CCTACGGGAGGCAGCAG-3′), 800r (5′-CATCGTTTACGGCGTGGAC-3′), 1100r (5′-GGGTTGCGCTCGTTG-3′) and 1492r. The analyzed sequences have been deposited in the GenBank database under the accession numbers GU735483, GU735484, and GU735485. The isolated MT-degrading bacteria SB010-03 was biochemically tested by the National Institute of Health (NIH) (Ministry of Public Health, Bangkok, Thailand) and the SB100-05 and WB100-05 were biochemically tested by Microbiological Resources Centre (Thailand Institute of Scientific and Technological Research, Bangkok, Thailand). Cell morphology was observed using a scanning electron microscopy (JEOL, JIM-5410LV, Spain).

2.6. Biodegradation of MT and kinetics of cell growth

MT degradation and cell growth were monitored under the condition where MT was the sole energy source. The tests were carried out with six different initial MT concentrations of 0.5, 1, 5, 10, 50 and 100 mg/L, each in triplicates. The tests were performed in 16 mL amber vials with 5 mL of inorganic salt medium containing 10^6 cells/mL of the isolated MT-degrading bacteria. Control tests were conducted without the isolated strains. The vials were incubated at 25 °C in dark with a rotating speed of 200 rpm. Cells were counted on a microscope-counting chamber (Hemacytometer) (Hausser Scientific, Horsham, PA, USA). Haldane's model, a substrate inhibitory model, was used to model the kinetics of cell growth [15]. The Haldane model is given as:

$$\mu = \frac{\mu_{max} S}{K_s + S + (S^2/K_i)} \quad (1)$$

where μ is the specific growth rate; μ_{max} is the maximum specific growth rate; S is the substrate concentration; K_s is the substrate half saturation coefficient; and K_i is the substrate inhibition coefficient.

2.7. Measurement of MT concentration

MT was measured by adding 5 mL of methanol (high-performance liquid chromatography (HPLC) grade, Merck, Germany) to 5 mL of the culture sample and the mixture was agitated for 1 min. The mixture was filtered with a syringe filter polytetrafluoroethylene (PTFE), 0.45 μm, Millipore Corp., Bedford, MA) and 50 μL of the filtered mixture was injected directly into a HPLC (1100 series, Agilent Technologies, USA) with a diode array detector (Agilent 1100 Series LC, Germany) to measure the MT concentration. Reverse phase C18 column (ODS Hypersil, 250 mm × 5 mm × 4.6 μm column, Hewlett Packard, Palo Alto, CA) was used and the column temperature was kept at 40.0 ± 0.5 °C. The mobile phase was acetonitrile (ACN) (HPLC grade, Merck, Darmstadt, Germany), and water at a flow rate of 0.5 mL/min with the following gradient: at time 0 min – 20% ACN, at time 19 min – 96% ACN, and at time 20 min – 20% ACN and post-run at 20% ACN for 10 min [16].

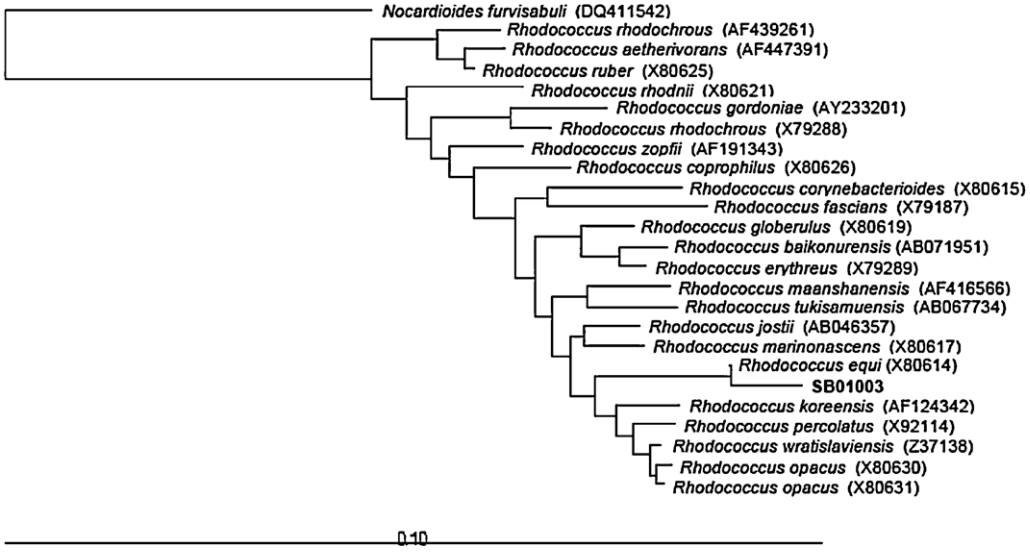

Fig. 1. Neighbor joining tree calculated using almost full-length sequences of 16S rRNA genes of MT-degrading bacterium SB010-03 and related bacteria.

2.8. Analysis of androgenic potency

Androgenic potency of MT and its metabolites was measured using β-galactosidase assay as described by Li et al. [17] with the yeast strain Y187. The strain Y187 was a recombinant yeast of *Saccharomyces cerevisiae* obtained from Zijian Wang (Research Center for Eco-Environment Sciences, Beijing, China). The androgen receptor (AR) plasmid was provided by Erik Jan Dubbink and J. Trapman (Department of Pathology, JNI, Erasmus University, Medical Centre, Rotterdam, Netherlands). Details on androgenic analysis are available in Ref. [12]. Briefly, the recombinant yeast was cultured in a selective medium (Supplementary material 1) for 16–18 h or until an optical density (OD_{600}) of approximately 0.8–1.0 was reached. Samples from the biodegradation tests were diluted into 7 series (1×, 0.5×, 0.1×, 0.05×, 0.01×, 0.005×, and 0.001×), in triplicate, with methanol. To start the yeast culture reaction, 10 μL of each dilution of the test sample and 2990 μL of the selective medium containing the yeast culture were mixed together and then incubated at 30 °C for 16–18 h with shaking at 300 rpm. To start the enzyme reaction, 50 μL from the yeast culture reaction was then transferred to a new vial containing 120 μL of Z-buffer and 20 μL of chloroform. After 5 min of incubation of the new solution at 30 °C, 40 μL of o-nitrophenyl-β-D-galactopyranoside (ONPG) (13.3 mM dissolve in Z-buffer) was added and mixed for 2 h at 30 °C with a rotating speed at 300 rpm. To stop enzyme reaction, 100 μL of 1 M Na_2CO_3 was added to the solution. The solution was centrifuged at 12,000 × *g* for 15 min and the color of the supernatant was measured by ultraviolet–visible (UV–vis) spectrometer at 420 nm. The β-galactosidase activity was calculated using the equation in Meng et al. [18]. The β-galactosidase activity against various volumes of the biodegradation samples was plotted as in a dose–response curve. In addition, the dose–response curves were plotted from the β-galactosidase activity against various concentrations of MT. The androgenic potency was reported in terms of testosterone equivalent (TEQ) [19].

3. Results and discussion

3.1. Identification of isolated MT-degrading bacteria

MT-degrading bacteria, identified as SB100-05 and SB010-03, and WB100-05 were isolated from the sediment sample (SB100-05 and SB010-03) and water sample (WB100-05). SB010-03 was retrieved from the culture enriched with an initial MT concentration of 10 mg/L, while SB100-05 and WB100-05 were recovered from 100 mg/L initial MT concentration cultures. Using the ARB program package (version 2.0; Department of Microbiology, Technische Universitat Munchen [http://www.arb-home.de]), the sequences of SB010-03, SB100-05 and WB100-05 were aligned with all sequences in the SSU rRNA database. Phylogenetic analysis was performed with three different methods (i.e. the distance matrix, maximum parsimony, and maximum likelihood). All methods exhibited the same positions of SB010-03, SB100-05 and WB100-05 in the three trees (data not shown). Figs. 1 and 2 show neighbor joining trees calculated with related sequences in the database. SB010-03 was closely related to *Rhodococcus equi* with 100% sequence identity when using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, USA). SB100-05 felled close to *Nocardioides aromaticivorans* with 100% identity. WB100-05 was related to *Nocardioides nitrophenolicus*. Blast analysis showed that 100% of the sequence identity of WB100-05 was related to *N. nitrophenolicus*.

Biochemical tests (Supplementary material 2) suggested that SB010-03 was Gram positive. The alkaline phosphatase, urease and catalase tests were positive. This strain can grow in various groups of carbohydrate without the production of acidification. The physiological properties suggested that SB010-03 was closely related with *Rhodococcus*. SB100-05 and WB100-05 were Gram positive. The tests for alkaline phosphatase, hydrolysis of esculin, leucine aminopeptidase, and catalase showed positive results. The strains can grow with a group of carbohydrate without production of acid. Physiological properties suggested that SB100-05 and WB100-05 were related to *Nocardioides*.

Images from scanning electron microscopy (Supplementary material 3) indicated that SB010-03 had a rod shape with a length of 0.78–1.67 μm and a diameter of 0.50–0.67 μm. The result suggested that this strain had similar cell morphology to *Rhodococccus*. SB100-05 had a rod shape with a length of 0.54–0.92 μm and a diameter of 0.35–0.42 μm and WB100-05 had a rod shape with a length of 0.71–1.19 μm and a diameter of 0.35–0.42 μm. The results indicated that SB100-05 and WB100-05 had similar cell morphology as *Nocardioides*.

The results from phylogenetic analysis, physiological properties and cell morphology confirmed that SB01003 was found to be closely related to *R. equi* which is a member of the genus *Nocardiaceae* in the family of *Corynebacterineae*. SB100-05 and WB100-05

Fig. 2. Neighbor joining tree calculated using almost full-length sequences of 16S rRNA genes of MT-degrading bacteria SB100-05 and WB100-05 and related bacteria.

were related closely to *N. aromaticivorans* and *N. nitrophenolicus*, respectively. They are members of genus *Nocardioidaceae* in family *Propionibacteria*.

In the literature, members of *Rhodococcus* have been found to utilize a number of substrates such as benzene [20], o-xylene [21], naphthalene [21], phenanthrene, anthracene, pyrene, fluoranthene and chrysene [22,23]. A possible reason for the ability of *Rhodococcus* to utilize these compounds may be due to the cell surface of *Rhodococcus* which contains aliphatic chain of mycolic acid, glycolipids, fatty acid, and polysaccharides that can easily sorb hydrophobic compounds [24,25]. In addition, *Rhodococcus* has the enzyme to cleave compounds with a steroidal skeleton structure. *Rhoddococcus* has been shown to be capable of degrading testosterone [26], 19-nortestosterone [27], and estrogens [28,29]. One strain of *Rhodococcus zopfii* (strain Y 50158) and three strains of *R. equi* (strains Y 50155,

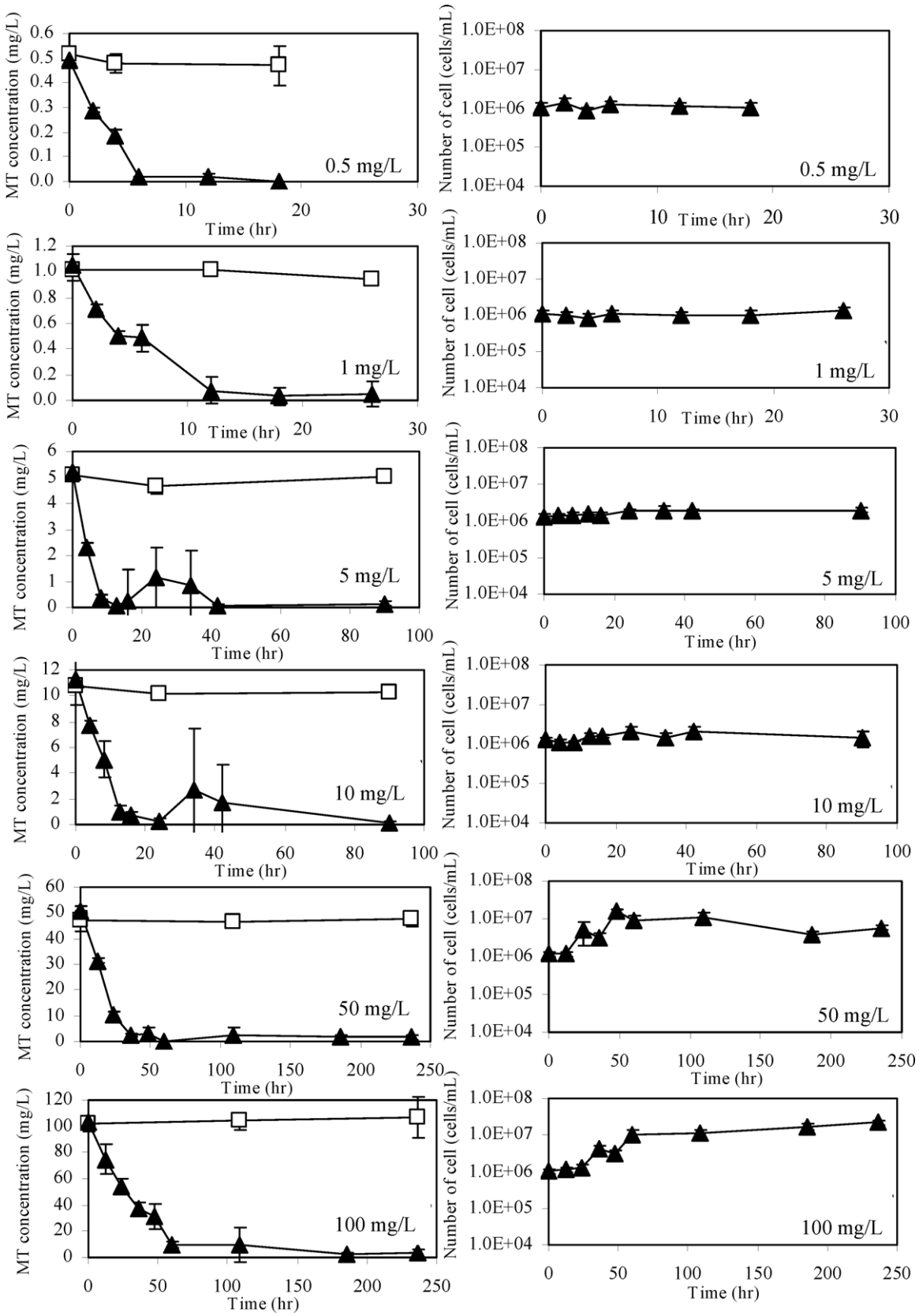

Fig. 3. Biodegradation of MT and change in cell numbers of MT-degrading bacterium SB010-03 under different initial MT concentrations: (▲) biodegradation test and (□) control test.

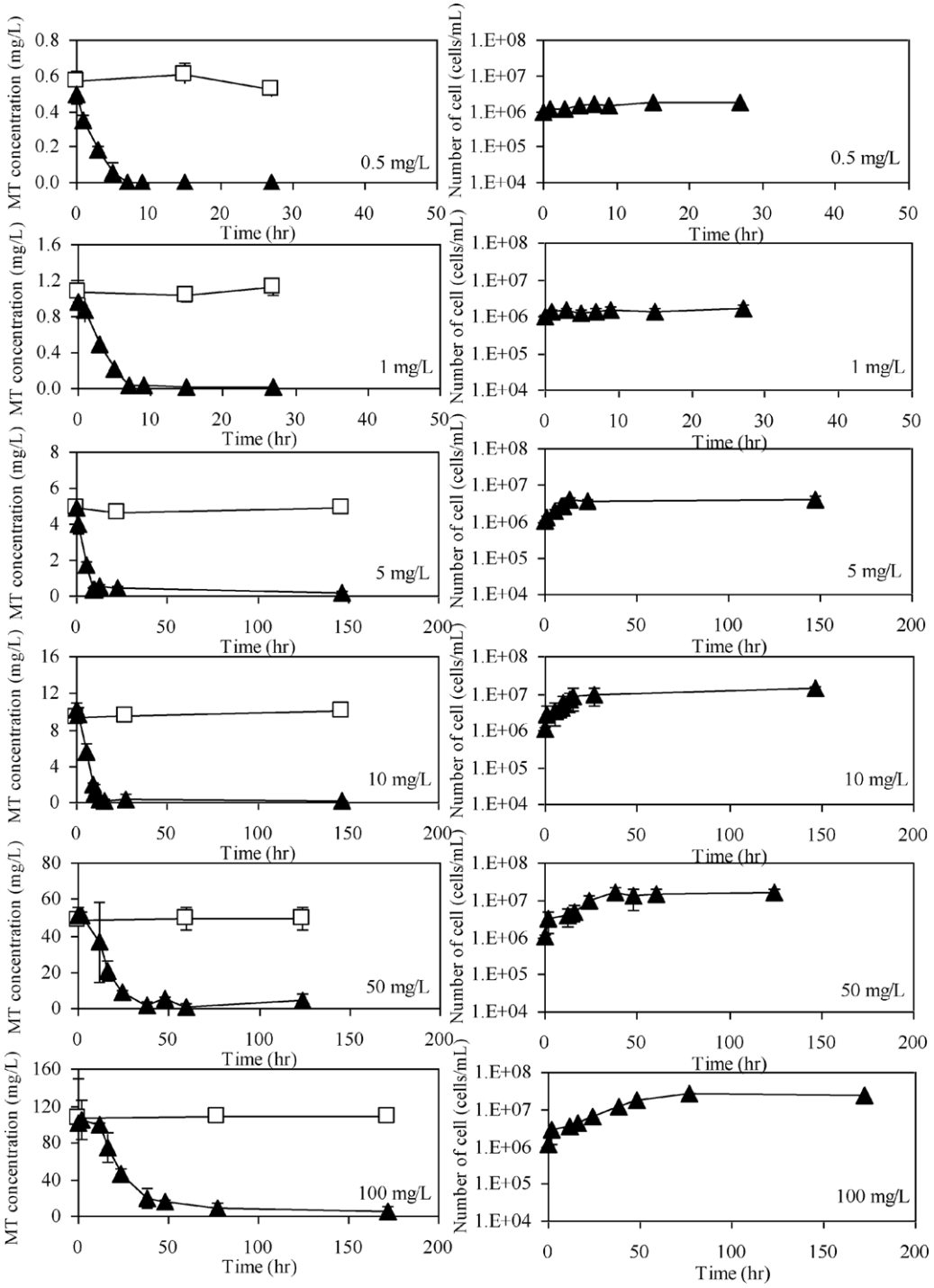

Fig. 4. Biodegradation of MT and change in cell numbers of MT-degrading bacterium SB100-05 under different initial MT concentrations: (▲) biodegradation test and (□) control test.

Y 50156, and Y50157) were isolated from activated sludge of wastewater treatment plants in Japan and were found to degrade estrone (E1), estriol (E3), and even ethynylestradiol (EE2) [28]. Additionally, one of the 14 isolated E2-degrading bacteria in the study of Yu et al. [29] (strain KC4) was found in the genera *Rhodococcus*. Another study by De las Heras et al. [30] found that *Rhodococcus rubber* strain Chol-4 used cholesterol, cholestenone, testosterone, 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD), pregnenolone, progesterone, androsterone, dihydroandrosterone, and β-estradiol as sole carbon sources.

Previously, strains in the genus *Nocardioidaceae* were reported to degrade compounds with similar chemical structure to MT. For example, 17β-estradiol-degrading bacterium strain KC3, a member of genus *Nocardioides*, was isolated from activated sludge [29]. Moreover, *Pimelobacter simplex* VKPM Ac-1632, a member of genus *Nocardioidaceae*, in the presence of cyclodextrins [31], and bacteria in genus *Mycobacterium* [32] can convert MT to methandrostenolone (ME2). Many bacteria in genus *Mycobacterium*, such as, *Mycobacterium album*, *Mycobacterium berolinense*, *Mycobacterium bovis*, *Mycobacterium chelonae*, *Mycobacterium cholesterolicum*, *Mycobacterium paraffinicum*, *Mycobacterium*

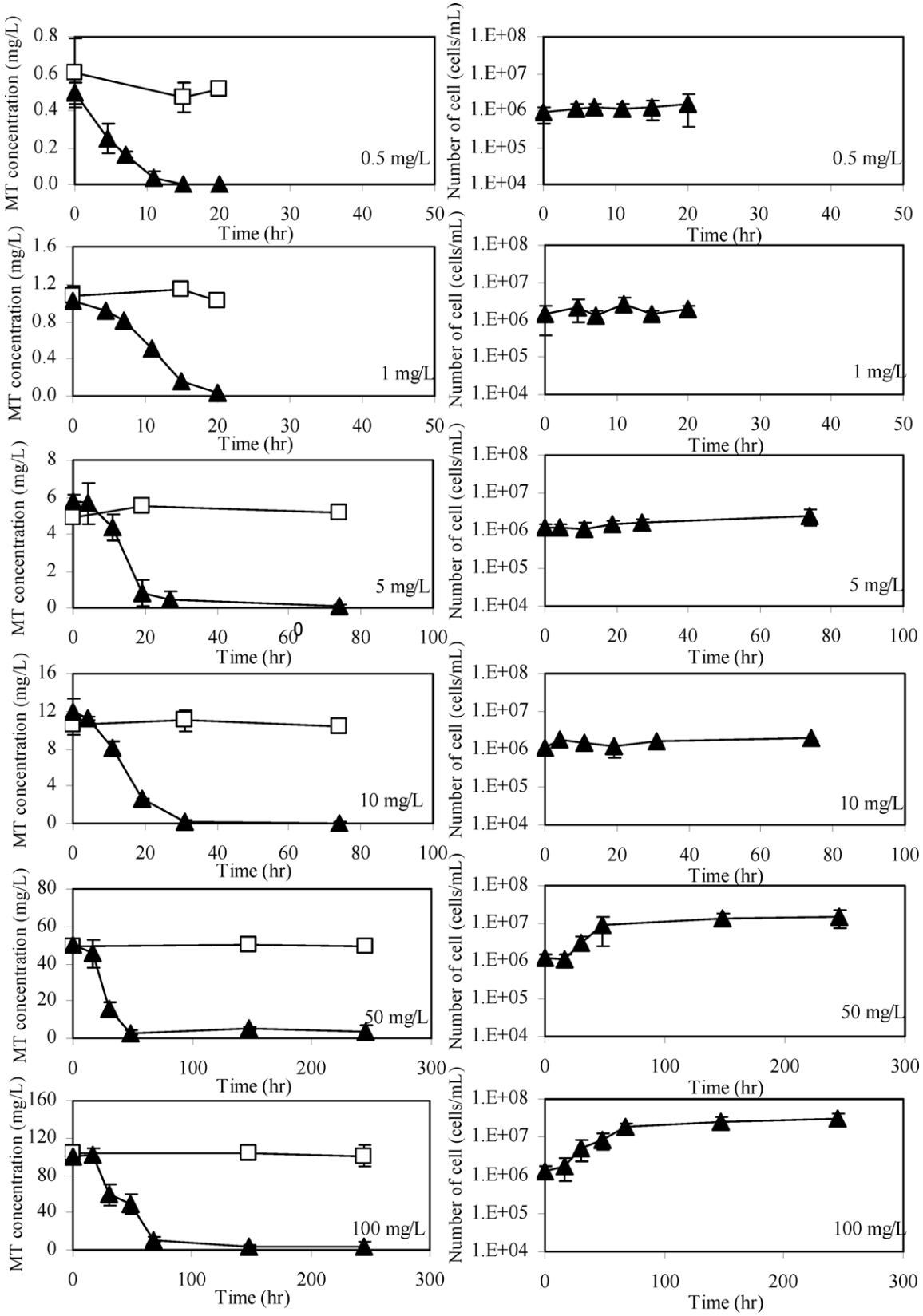

Fig. 5. Biodegradation of MT and change in cell numbers of MT-degrading bacterium WB100-05 under different initial MT concentrations: (▲) biodegradation test and (□) control test.

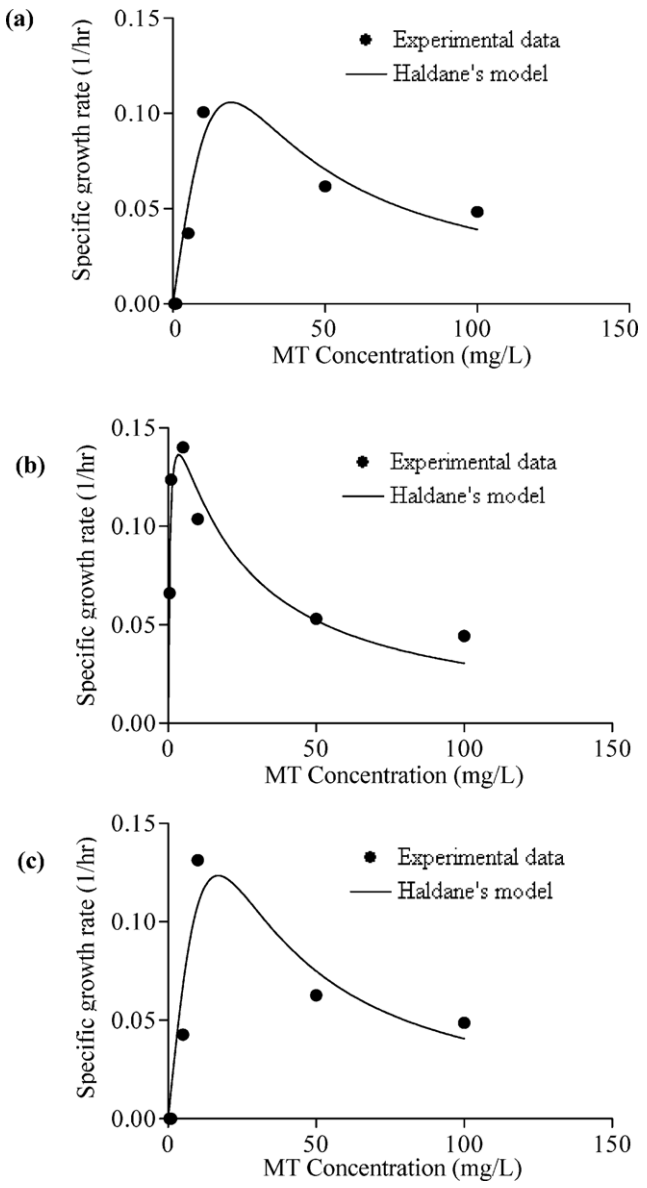

Fig. 6. Specific growth curves of MT-degrading bacteria SB010-03 (a), SB100-05 (b), and WB100-05 (c).

peregrinum, and mutant *Mycobacterium smegmatis* can cleave steroid and cholesterol compounds [32,33]. Horinouchi et al. [26] reported that *Nocardia*, *Arthorobacter*, and *Mycobacterium* which were Gram-positive bacteria and *Comamonas* and *Pseudomonas* which were Gram-negative bacteria were able to use testosterone as carbon and energy source.

Besides *Rhodococcus* sp. and *Nocardioides* sp., the MT-degrading bacteria found in the present study, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Serratia marcescens* were previously reported for their capability of degrading and utilizing MT as sources of carbon and energy. These MT-degrading bacteria were isolated from biofilm in biofilter units used to trap MT from effluent of a masculinization process [34].

3.2. Degradation of MT by isolated MT-degrading bacteria

Degradation of MT by SB010-03, SB100-05, and WB100-05 was observed under various initial MT concentrations (0.5–100 mg/L)

Table 1
First order biodegradation rate of MT-degrading bacteria SB010-03, SB100-05 and WB100-05.

Initial MT concentration (mg/L)	Biodegradation rate (h^{-1})		
	SB010-03	SB100-05	WB100-05
0.5	0.24 ± 0.02	0.36 ± 0.09	0.16 ± 0.02
1	0.26 ± 0.10	0.42 ± 0.09	0.17 ± 0.01
5	0.38 ± 0.03	0.30 ± 0.05	0.13 ± 0.03
10	0.19 ± 0.03	0.20 ± 0.02	0.10 ± 0.00
50	0.09 ± 0.01	0.11 ± 0.05	0.08 ± 0.02
100	0.03 ± 0.00	0.06 ± 0.01	0.04 ± 0.01

(Figs. 3–5). The results indicated that SB010-03, SB100-05, and WB100-05 were capable of using MT as a sole energy source. At all initial MT concentrations, SB010-03 degraded MT without a need of an acclimatization period. It is noted that the slight plateau at about 5 h for the initial MT concentration of 1 mg/L and the slight increases in MT concentrations between 20 and 40 h for the initial MT concentrations of 5 and 10 mg/L (Fig. 3) were probably due to variability in experimental procedures and analytical measurements and the concentrations between 20 and 40 h were statistically similar to the concentrations at 18 h and 90 h. SB100-05 degraded MT without a lag time period for all concentrations tested except for an initial MT concentration of 100 mg/L where a lag time of 12 h was required. WB100-05 degraded MT without an acclimatized time for initial MT concentrations of 0.5 and 1.0 mg/L. Acclimatization periods were required for this strain for initial MT concentrations higher than 5.0 mg/L. High octanol–water partition coefficient (K_{ow}) of 3.36 and low solubility of 3.39 mg/L (at 25 °C) of MT suggested that this compound tends to be sorbed on organic particles rather than getting dissolved in water. Sediments in and near the receiving water body may serve as a sink for MT where SB010-03 and SB 100-05 were acclimatized in the original sediment.

At each initial MT concentration, the initial degradation rate of MT was estimated using first order reaction rate kinetics (Table 1). For all strains, the initial degradation rates increased with increasing initial MT concentrations up to certain initial concentrations (5.0, 1.0, and 1.0 mg/L for SB010-03, SB100-05, and WB100-05, respectively). Above these initial concentrations, the initial degradation rates declined. The results indicated that high concentrations of MT can inhibit the activity of the isolated MT-degrading bacteria. Comparing the strains isolated, based on the degradation results, SB010-03 was found to be more tolerant to high concentrations of MT than the other strains isolated.

For all strains, a peak of a metabolite at a retention time of 16.2 min (17.6 min for MT) was shown in the HPLC chromatograms. Based on the retention time, it can be inferred that the metabolite probably was more polar and/or has a lower molecular weight than MT. However, disappearance of the metabolite with time indicated that all strains could completely degrade the metabolite. The metabolite was not identified but it may be methandrostenolone (ME2) which resulted from the cleavage of MT as reported for *P. simplex* VKPM Ac-1632 (genus *Nocardioidaceae*) [31] and bacteria in genus *Mycobacterium* [32]. Other possible metabolites can be 17α-methyl-5α-androstan-3α-17β-diol and 17α-methyl-5β-androstan-3α-17β-diol and their isomer, which are the main MT metabolites found in urine and feces of humans and animals [35–38]. Although the metabolite was not identified here, androgenic potency of MT and its metabolites was analyzed during degradation in Section 3.4.

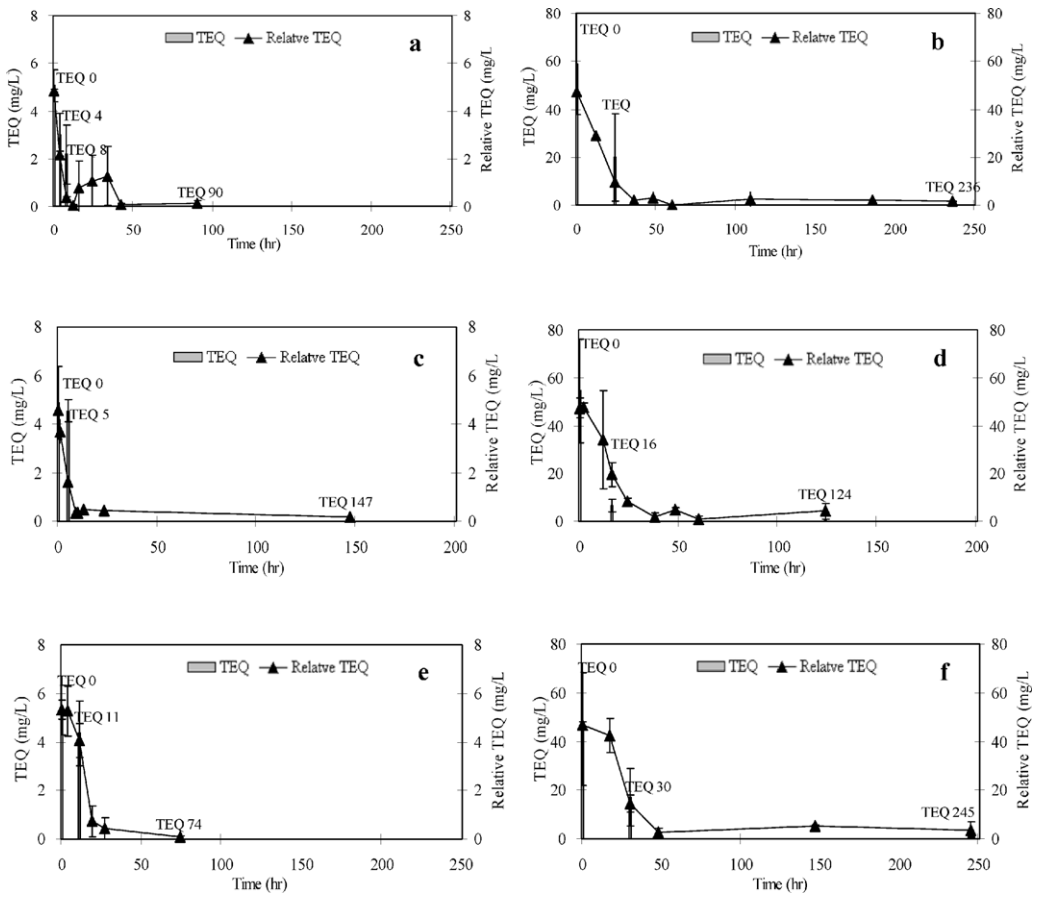

Fig. 7. Change in androgenic potency during biodegradation of MT by MT-degrading bacteria SB010-03 at the initial concentrations of 5.0 mg/L (a) and 50.0 mg/L (b); SB100-05 at the initial concentrations of 5.0 mg/L (c) and 50.0 mg/L (d); WB100-05 at the initial concentrations of 5.0 mg/L (e) and 50.0 mg/L (f).

3.3. Kinetics of cell growth

For all strains, cell growth cannot be observed for an initial MT concentration of 1.0 mg/L or less (Figs. 3–5). The results imply that these concentrations may not be enough to support cell growth or cell growth occurred, but was too small to be observed. Above 1.0 mg/L, the numbers of cells increased significantly. Fig. 6 shows specific growth curves of SB010-03, SB100-05, and WB100-05. Using nonlinear regression, the Haldane's substrate inhibition model was found to fit to the data with correlation coefficients (R^2) of 0.90, 0.89 and 0.86 for SB010-03, SB100-05, and WB100-05, respectively. The specific growth rates of SB010-03, SB100-05 and WB100-05 were found to decrease when the initial MT concentrations were above 10.0, 5.0, and 10.0 mg/L, respectively, indicating inhibition of the isolated MT-degrading bacteria. The estimated kinetic constants, μ_{max}, K_s, and K_i for SB010-03 were 0.13 h^{-1}, 24.8 mg/L, and 76.2 mg/L, respectively. For SB100-05, the estimated μ_{max}, K_s, and K_i were 0.19 h^{-1}, 0.7 mg/L, and 19.6 mg/L, respectively. In the case of WB100-05, the estimated μ_{max}, K_s, and K_i were 0.16 h^{-1}, 1.5 mg/L, and 41.2 mg/L, respectively. For the three strains, SB010-03 showed lower affinity and higher resistance to MT inhibition than SB100-05 and WB100-05. It should be noted that even though SB100-05 and WB100-05 were found to be closely related to *Nocardiaceae* sp., the growth rates and estimated rate constants for the two strains were different.

3.4. Androgenic potency

Androgenic potency monitored using β-galactosidase assay gave information on possible remaining androgencity after biodegradation. Results expressed in terms of testosterone equivalent (TEQ) represent the overall androgen activity originated from MT and all androgen-like compounds (MT metabolites) while the relative TEQ computed from the MT concentration represent the androgen potency derived from MT only (Fig. 7). The TEQs did not significantly differ from the relative TEQs during the incubation period of WB100-05 (Fig. 7e and f) but the TEQs were higher than relative TEQs about two to five times at the mid-incubation period for SB010-03 and SB100-05 (Fig. 7a–d). At the end of incubation of all strains, there were no difference between the TEQ and the relative TEQ. The higher TEQ compared to the relative TEQ at the mid-incubation period of SB010-03 and SB100-05 may be due to the occurrence of metabolites with androgen property. However, these metabolites were labile compounds which were degraded further by both strains. Overall, all strains were able to degrade MT and its metabolites to non-androgen-like compounds.

4. Conclusions

Phylogeny, physiological properties and cell morphology suggested that the isolated MT-degrading bacteria SB010-03, SB100-05 and WB100-05 were closely related to *R. equi*, *N. aromaticivorans* and *N. nitrophenolicus*, respectively. All isolated MT-degrading bacteria showed the similar patterns of MT degradation and cell growth. The initial degradation rates and cell growth increased with increasing initial MT concentrations but the isolated strains were inhibited above a certain MT concentration. Inhibition of the isolated strains was found to be modeled using the Haldane's substrate inhibition model. SB010-03 showed lower affinity and higher resistance to MT concentration than the other strains. All

isolated MT-degrading bacteria showed the capability of degrading MT to the products with no androgenic potency. Contaminated water and sediment from masculinizing ponds of Nile tilapia fry may need proper treatment before they are discharged to prevent contamination of MT to nearby receiving environmental media. Based on the degradation results, it is probable that in a contaminated area where MT levels are expected to be in the low ppm or ppb levels, SB100-05 and WB100-05 may thrive and be better suited to degrade MT than SB010-03.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.03.072.

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