Biodegradation of 17α-methyltestosterone and isolation of MT-degrading bacterium from sediment of Nile tilapia masculinization pond

Supreeda Homklin, Theerachit Wattanodorn, Say Kee Ong and Tawan Limpiyakorn

ABSTRACT

The fast growing and highly tolerant fish Nile tilapia is one of the most commonly raised fish in the aquaculture industry. To produce an all-male population, a common practice is to feed the Nile tilapia fry with 17α-methyltestosterone (MT)-impregnated food. Uneaten fish food with MT may accumulate in the masculinization ponds and be released into the receiving waters. Not much is known about the fate of MT in the fish farms and in the receiving streams. The objective of this study is to investigate the biodegradation of MT under aerobic condition and to isolate responsible microorganisms. Aerobic biodegradation tests were conducted with MT concentrations of 0.3, 1.0, 5.0, 7.0, and 10.0 mg/L using sediment from the masculinization pond as microbial seed. The results suggested that MT is biodegradable. Lag phase was not observed in all cases. With initial concentrations of 0.3, 1.0, 5.0, 7.0, and 10.0 mg/l, the first-order degradation rates were 0.52, 0.23, 0.17, 0.13 and 0.10 day^{-1}, respectively. Degradation rates were found to decrease with an increase in the initial MT concentration. Analysis of 16S rRNA gene sequences of a strain isolated from the sediment indicated that the strain was highly similar to *Pimelobacter simplex* strain S151 (100%) which is in the genus Nocardioidaceae. Using this strain, MT is degraded with a first-order degradation rate of 0.044 h^{-1} excluding the lag phase. This is the first work reporting biodegradation of MT and isolation of MT-degrading bacterium from environment.

Key words | aerobic biodegradation, 17α-methyltestosterone, isolation, *Norcardioides*, sediment

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INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is a very popular farmed-raised fish with aquaculture farmers as it is tolerant, easy to spawn, and easy to grow with various types of feed and has a high market value. However, growth of both male and female tilapia in the same pond can cause over-population and stunting. Culturing of only male Nile tilapia, which has double the growth rate of female tilapia and hence lead to larger body size, is therefore more desirable (MacIntosh & Little 1995). 17α-methyltestosterone (MT), an anabolic androgenic steroid, is commonly used to treat Nile tilapia fry for sex reversal. However, residual MT and MT metabolites in the food may accumulate in the masculinization ponds and be released into the receiving waters. MT is a questionable human carcinogen, producing nonmalignant tumors in the liver. It is a poison by the intraperitoneal route. It causes developmental abnormalities in the urogenital system (Lewis 1991). MT does not only affect humans but also the ecosystem, in particular, near the masculinization pond. Despite its widespread usage, not much is known about the fate of MT in fish farms and in receiving streams. The only study related to MT showed that between 2.8 and 2.9 ng/g of MT still remained in soils nearly three months

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after cessation of treatment (Fitzpatrick & Contreras-Sánchez 2000). The objectives of this study were to investigate the biodegradation of MT using sediment from masculinization pond of Nile tilapia fry and to isolate MT-degrading bacterium from the sediment.

MATERIALS AND METHODS

Chemicals

MT (>99% pure, HPLC grade) was purchased from Fluka (Buchs, Switzerland). Individual stock solutions of MT were prepared in methanol. Acetonitrite and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Medium for aerobic biodegradation

Medium used for aerobic biodegradation experiments with sediment was inorganic salt medium (IS medium) containg 100 mg of NH_4Cl, 1 g of $NaNO_3$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.05 g of EDTA-Fe, 0.05 g of $CaCl_2{\cdot}2H_2O$, 0.05 g of K_2HPO_4, 4 g of 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 $NiCl_2{\cdot}6H_2O$, 0.12 mg of $CuSO_4{\cdot}5H_2O$ dissolved in 1 liter of Milli-Q water and adjusted to pH 7–7.5 with 1 N NaOH solution (Chao *et al.* 2004).

Aerobic biodegradation of MT using sediment

To investigate the aerobic biodegradation of MT using sediment, three parallel batch tests comprising of biodegradation, recovery and control tests were carried out with five different initial MT concentrations of 0.3, 1.0, 5.0, 7.0 and 10.0 mg/l. Control tests were conducted for investigation of abiotic transformation which were generated by sterilizing IS medium and MT. Recovery tests were performed for investigation of physical and chemical transformation which were conducted using sterilized sediment, sterilized IS medium and MT. Biodegradation tests were conducted using sediment, sterilized IS medium and MT. Duplicate experiments were conducted for each concentration. The stock MT in methanol solution was added to the test tubes to achieve a final concentration as above. Methanol was allowed to evaporate. Five ml of the inorganic medium was added to the test tube. In the biodegradation test and recovery test, 10% (v/v) of sediment and sterilized sediment were added, respectively. Test tubes were incubated at 25°C and rotated at a speed of 200 rpm.

Medium for enrichment and isolation of MT-degrading bacterium

Medium for enrichment and isolation of MT-degradation bacterium was prepared in the same manner as the medium for the aerobic biodegradation except that 1.7% of agar was added to the medium for isolation.

Enrichment and isolation of MT-degrading bacterium

MT in methanol stock solution was added to 250 ml Erlenmeyer flasks with a final concentration of 100 mg/l. Methanol was allowed to evaporate. Sediment, collected from the masculinization pond of Nile tilapia fry, was added (10% v/v) along with 100 ml of IS medium into the 250 ml of Erlenmeyer flasks containing MT. The cultures in the Erlenmeyer flasks after 3–4 days of incubation were then transferred to a similar Erlenmeyer flasks containing MT. Enriched cultures were incubated at 25°C and rotated at 200 rpm. Agar plates containing MT-inorganic salt medium were used for isolation.

Identification of MT-degrading bacterium

A colony of the isolated bacterium was incubated overnight (16–18 hours) in 5 ml LB broth. The culture was centrifuged to obtain a cell pellet. The DNA was extracted from pellet by phenol:chloroform extraction as described by Trochimchuk *et al.* (2003). The phenol:chloroform was adjusted to 1:1 (v/v). DNA was amplified by polymerase chain reaction (PCR) with universal primer 27f (5′AGA GTTTGATCC TGGCTCAG-3′) and 1492r (5′-GGCTACC TTGTTACGACTT-3′). The product from the PCR was purified by QIAquick® PCR purified kit (QIAGEN, Rockville, Maryland, USA) and sent to Macrogen Inc (Seoul, Korea) for sequencing. The sequencing result was analyzed by BLAST (NCBI, Washington, DC, USA).

Biodegradation experiment for isolated MT-degrading bacterium

To investigate the aerobic biodegradation of MT by the isolated MT-degrading bacterium, two parallel batch tests comprising of biodegradation and control tests were carried out with initial MT concentration of 100 mg/l. Each test was conducted in duplicate. Isolated MT-degrading bacterium was activated in the IS medium containing MT for three days. In the biodegradation test, 10% (v/v) of 10^4 cells/ml of inocula were added and incubated at 25°C at a rotational speed of 200 rpm.

Analysis of MT

To analyze for MT, equal volume of methanol was added to each test tube and vigorous mixed for 1 minute. Fifty μl of the methanol-water mixture was analyzed using a high-performance liquid chromatography (HPLC 1100 series, Agilent Technologies, Palo Alto, CA, USA) with RP C18 column (ODS Hypersil, 250 mm × 5 mm × 4.6 μm) at a flow rate of 0.5 ml/min and column temperature of 40.0 ± 0.5°C. MilliQ water and acetonitrile (ACN) were used as mobile phase with gradient program starting with 20% of ACN at time $t = 0$ min, 96% at $t = 19$ min, and 20% at $t = 20$ min. A 10-minute post run time was used to re-equilibrate the column. MT was detected at 245 nm using a diode array detector (DAD) (Marwah *et al.* 2005).

RESULTS AND DISCUSSION

Aerobic biodegradation of MT using sediment

Results of the aerobic biodegradation of MT as presented in Figure 1 showed that MT is biodegradable. The MT concentrations in the control tests were stable throughout the experiments while MT was found to decrease in all biodegradation experiments. No lag phase was found in all cases suggesting that time was not needed for the microorganisms in the sediment from the masculinization pond to acclimatize to MT. First-order degradation rates were determined and were found to be 0.52, 0.23, 0.17, 0.13 and 0.10 day^{-1} for initial concentrations of 0.3, 1.0, 5.0, 7.0 and 10.0 mg/l, respectively (R^2 values ranging from 0.88 to 0.98). Based on the estimated degradation rates, the degradation rates were found to decrease with an increase in the initial MT concentrations (Figure 2). It is interesting to note that there was a 50% reduction in the degradation rate for an initial concentration about 1 mg/l as compared to an initial concentration of 0.3 mg/L. Before the 50% reduction, the degradation rate was dramatically decreased whereas it was gradually decrease after 50% reduction. Several probable reasons include high activities of MT-degrading microorganisms at low initial MT concentrations as compared to high initial MT concentrations since the microorganisms in the sediment were exposed to very low level of MT. Another reason may be due to the substrate inhibition. MT may be toxic to the microorganisms and therefore inhibiting the biodegradation ability of some of the microorganism in sediment. Estimated half-lives were 2.4, 4.5, 7.0, 7.5 and 12.8 days with initial MT concentration of 0.3, 1.0, 5.0, 7.0 and 10.0 mg/l, respectively. Information on the fate of MT in the environment and biodegradation of MT is very limited and this is probably the first study providing information on the degradation rates.

Isolation of MT-degrading bacterium

The previous part showed the ability of microorganisms in sediment to degrade MT. To gain the responsible MT-degrading bacterium, the isolation of MT-degrading bacterium was conducted. Although, the degradation rates of biodegradation of MT by microorganism in sediment were decreased when increasing the initial MT concentrations, the high concentration as 100 mg/l was used to isolate MT-degrading bacterium to provide the environment plentifully with MT to select for only MT-degrading bacterium that use only MT as sole carbon source. A strain identified as Strain S-1 was isolated from the sediment at an MT concentration of 100 mg/l. The colony shape of Strain S-1 was white in color, circular in form, flat in elevation and erose in margin. The sequence of the 16S rRNA gene of Strain S-1 was analyzed and was found to be similar to *Pimelobacter simplex strain S151* (100%) (accession number of AY509240) which is in the genus Nocardioidaceae. Bacteria in this genus were also able to degrade other steroid hormone such as estradiol (Yu *et al.* 2007). Yu *et al.* (2007) isolated estradiol-degrading bacteria from activated

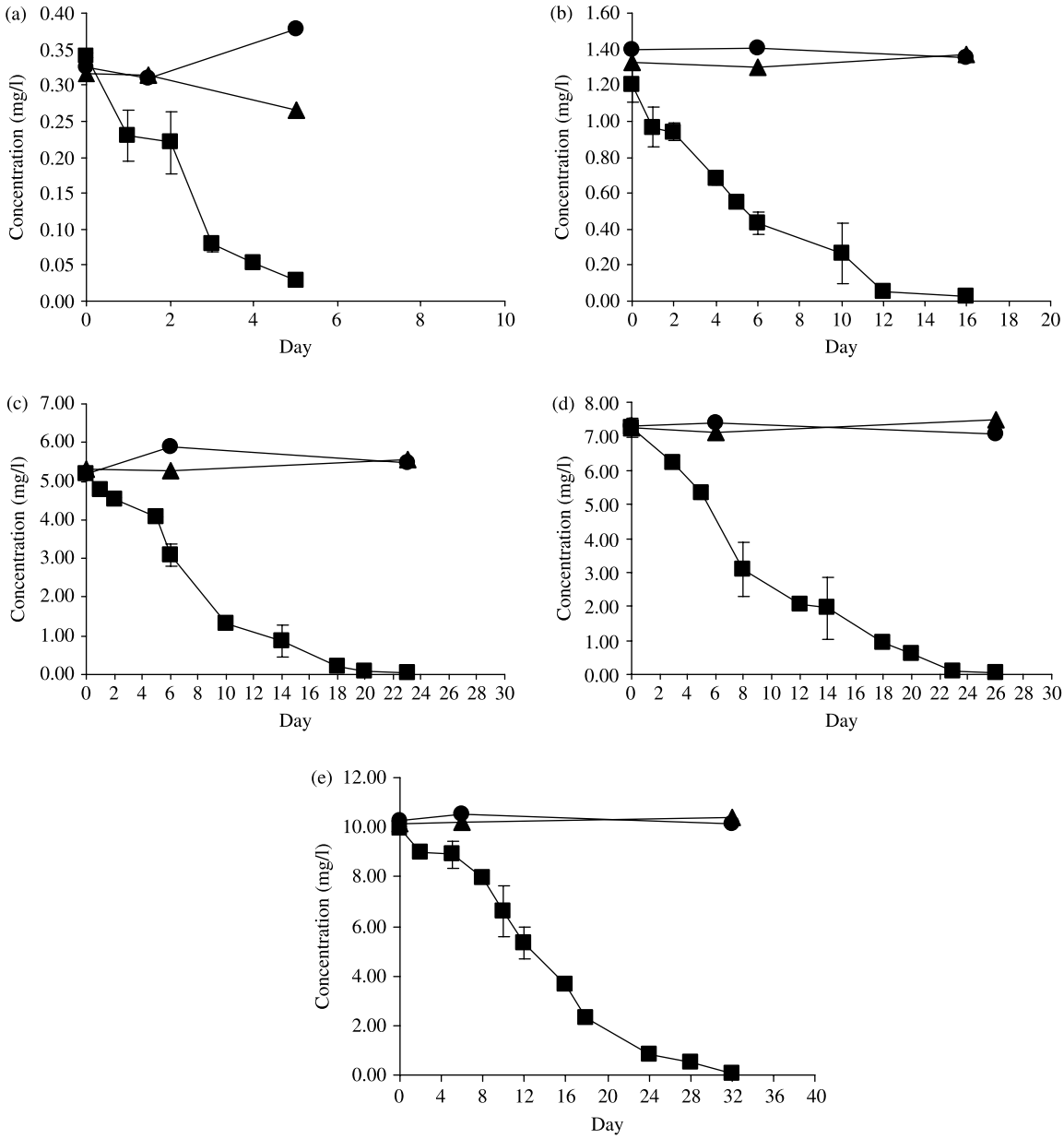

Figure 1 | Aerobic biodegradation of MT using sediment at initial MT concentrations of (a) 0.3 mg/l, (b) 1.0 mg/l, (c) 5.0 mg/l, (d) 7.0 mg/l, and (e) 10.0 mg/l in biodegradation test (■), recovery test (▲) and control test (●).

sludge. They found that estradiol-degrading bacteria Strain KC3 was in genus Nocardioidaceae.

Biodegradation of MT by MT-degradation bacterium

Strain S-1 was tested for the ability to degrade MT at the initial concentration of 100 mg/l and was found to degrade MT. A lag phase of 12 hours was observed. However, beyond the lag phase, MT was rapidly degraded with about 80% of MT degrading within the 48 h. From this point, MT degraded slowly to 0.9 mg/l over 216 h (data not shown). At the end of the experiment, MT remained in the sample at about 0.9 mg/l. The first-order degradation rate of MT for Strain S-1 excluding the lag phase was estimated to be $0.044\ h^{-1}$ ($R^2 = 0.96$). During the biodegradation of MT, an unknown metabolite at a retention time of 16.2 min was

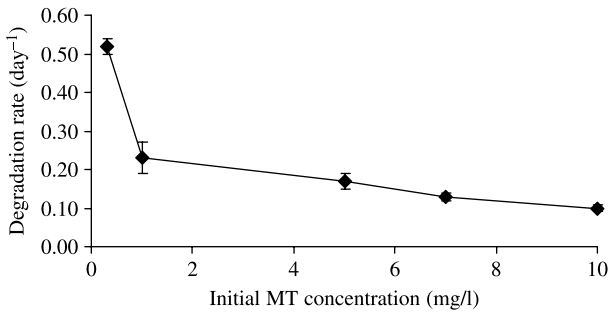

Figure 2 | Relationship between degradation rates (day^{-1}) and initial MT concentrations (mg/l).

found to appear after 12 h. The chromatogram peak area of the metabolite rapidly increased and then decreased within the next 48 h. After 48 h, the peak area of the metabolite gradually decreased but continued to be present until the end of experiment. From the reviews, the main metabolites of MT in urine and feces of human and animals by uptaking MT as clinical uses were 17α-methyl-5α-androstan-3α, 17β-diol and 17α-methyl-5β-androstan-3α, 17β-diol and their isomer in minority which were more polarity than MT (Rongone & Segaloff 1962; Shinohara *et al.* 2000; Williams *et al.* 2000). From the chromatogram of HPLC, peak of the unknown metabolite appeared on the chromatogram earlier than that of MT. Thus, the unknown metabolite has high polarity rather than that of MT as same as in the previous study. This is the first work for isolation of MT-degrading bacterium so this work needs the further study for identification the unknown metabolite of MT by biodegradation of MT-degrading bacterium.

CONCLUSION

MT was found to be biodegradable by microorganisms in sediment from the masculinization ponds of tilapia farms under aerobic condition. The first-order degradation rates were found to decrease with increasing initial MT concentrations and ranged from 0.52 to 0.10 day^{-1}. A MT-degrading strain was isolated from sediment and was found to be similar to *Pimelobacter simplex strain S151* which is in genus Nocardioidaceae using DNA sequencing.

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