CHAPTER V

ISOLATION OF 17ALPHA-METHYLTESTOSTERONE-DEGRADING BACTERIA FROM SEDIMENT AND WATER OF MASCULINIZING POND OF NILE TILAPIA FRY

5.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is one of the more 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, larger body size, and are heavier than the female tilapia (MacIntosh and Little, 1995; Green *et al.*, 1997). Furthermore, mono-sex production alleviates the problem of overpopulation and energy loss from stunting. An all-male population can be achieved by post fertilizing Nile tilapia fry with food containing 17alpha-methyltestosterone (MT), a synthetic anabolic androgenic steroid (Fitzpatrick *et al.*, 1999).

The residual MT and unmetabolized MT-impregnated food from the masculinizing process can impact human, animals and ecosystem when released into the environment. MT is a suspect human carcinogen as it can induce production of nonmalignant tumors in the liver of human (Soe *et al.*, 1992). MT can cause prostate cancer in humans when exposed to 6-9 mg of MT per day for 30 years (Nakata *et al.*, 1997). MT is classified as an endocrine disrupting compound which can interfere with the normal function of endocrine and reproductive systems (Andersen *et al.*, 2006). Many researchers have found that MT can depress the vitellogenin protein of female eelpout (*Zoarces viviparous*), induce the imposex of female freshwater ramshorn snail (*Marisa cornuarietis*), and reduce the fecundity and fertility of paired medaka when exposed to nanograms per liter levels of MT. (Masanori *et al.*, 2004; Schulte-Oehlmann *et al.*, 2004; Selzsam *et al.*, 2005; Korsgaard, 2006). Lee et al. (2003) found that the highest female:male ratio was 1:10.2, when Korean rockfishs, *Sebastes schlegeli*, were fed with MT-impregnated food with 0.05 μg MT /g of diet for 29 days. They also observed the presence of ovarian cavity and sperm duct in the same

gonad of Korean rockfishs, *Sebastes schlegeli*, at MT concentrations greater than 0.5 μg/g diet.

The only study on the fate of MT in masculinizing process of Nile tilapia (Fitzpatrick and Contreras-Sánchez, 2000) indicated that MT concentration in the pond water reduced from 3.6 μg/L to the background level (between non detectable and 0.02 μg/L) within a week after the cessation of feeding of the MT-impregnated food whereas, three months later, MT continued to persist in the sediment at about 2.8 and 2.9 ng/g (Fitzpatrick and Contreras-Sánchez, 2000). The log K_{ow} (3.36) of MT suggests that this compound tends to be sorbed onto the solid phase rather than be dissolved in the liquid phase (Yalkowsky and He, 2003). Work done by Hulak *et al.* (2008) showed that residual MT in wastewater from masculinization ponds treated by biological filters was still high enough to reverse the sex of about 81-100% of a common crap progeny.

A study by our group showed that MT can be degraded by microorganisms in the sediment from masculinizing pond of Nile tilapia fry under various electron acceptor conditions (see chapter 4). MT was transformed rapidly under aerobic, sulfate-reducing, and methanogenic conditions, while it was hardly degraded under nitrate and iron-reducing conditions. Although MT was found to biodegrade under methanogenic condition, the androgenic potency of the final solution was found to be similar to that of the initial solution indicating that metabolites of MT with androgen-like activity were formed. To prevent the distribution of residual androgenic like compounds (MT and metabolite) into environment and receiving water body, contaminated sediment and water must be properly treated before discarded. The treatment by microorganism under aerobic condition was the proper system due to the simple and high MT removal efficiency without the persistence of androgenic compounds. However, the responsible MT-degrading bacteria have not been reported before. Therefore, in this study, MT-degrading bacteria were isolated from the sediment and water of a masculinization pond of Nile Tilapia fry. The enrichment and isolation of microorganisms were performed with three different MT concentrations of 1, 10, and 100 mg/L.

5.2 Materials and Methods

5.2.1 Chemicals

See section 4.2.1.

5.2.2 Collection of sediment and water sample

Sediment and water samples were collected from a masculinizing pond of Nile tilapia fry (see Figure 5.1) located in Pathumtani province, Thailand. The sediment sample was taken by a grab sampler at about 1.0 meter from the edge of the pond and at a sediment depth of 0 to 5 cm. The water sample was collected from the surface of the pond by a grab sampler. The sediment and water samples were placed in 1,000 mL polyethylene bottle containers and shipped to the laboratory in a cooler. The sediment and water were stored in a refrigerator at 4 ° C until use.

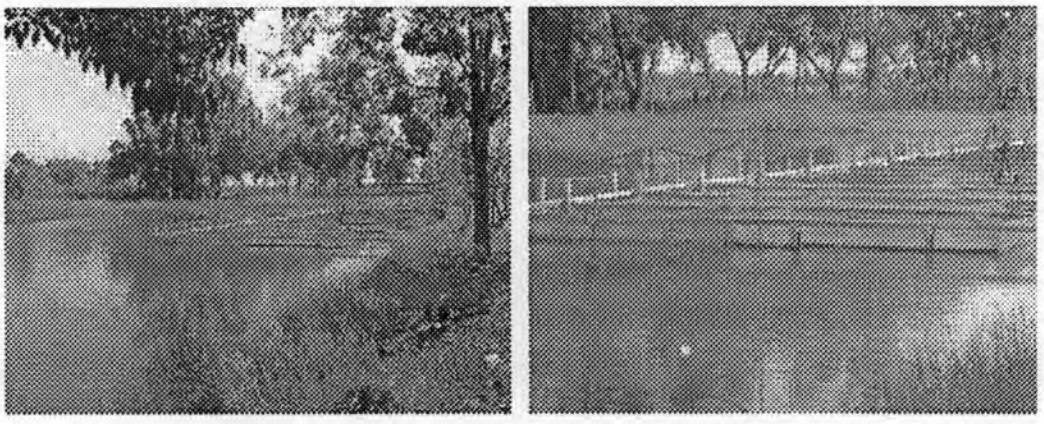

Figure 5.1: Masculinizing pond of Nile tilapia fry

5.2.3 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 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 $CuSO_4 \cdot 5H_2O$ in one liter of Milli-Q water. pH was adjusted to 7.5-8.0 by 1 N NaOH. The medium was sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C before use as the medium for enrichment and isolation.

5.2.4 Enrichment of MT-degrading bacteria

Enrichment of MT-degrading bacteria was performed using the sediment and water samples with three different MT concentrations of 1, 10, and 100 mg/L in 250 mL Erlenmeyer flasks. The final MT concentrations of 1, 10, and 100 mg/L were achieved by adding 0.2 and 2 mL of 500 mg/L MT stock solution and 2 mL of 5,000 mg/L MT stock solution into each flask, respectively. The methanol was evaporated by gently blowing nitrogen gas. Ten mL of sediment (10% (vol/vol)) or 10 mL of water sample was added to each 250 ml Erlenmeyer flask along with 100 mL of inorganic salt medium. The flasks were incubated at 25 °C and were rotated at a speed of 200 rpm. MT concentrations were monitored by removing aliquots of the aqueous phase for analysis and when the concentration was reduced by 70%, an aliquot of 10 mL was transferred and subcultured in fresh 100 mL inorganic salt medium containing MT of the same initial concentration. The enrichment process was repeated about twenty times for an initial MT concentration of 1 mg/L and five times for initial MT concentrations of 10 and 100 mg/L. The duration for the enrichment at initial MT concentration of 1 mg/L was about one month and about 2 weeks for the enrichment at initial MT concentrations of 10 and 100 mg/L.

5.2.5 Isolation of MT-degrading bacteria

The enriched culture was serially diluted with fresh inorganic medium. One hundred mL of the diluted culture solutions were spread onto agar plates and incubated at 25 °C. Agar plates were prepared by adding agar (Sigma, St. Louis, MO, USA) to the inorganic salt medium to obtain a final concentration of 17 g/L. Forty μL of MT with a concentration of 100 mg/L or 80 μL of MT with concentrations of 500 or 5,000 mg/L were placed on the agar surface to achieve final concentrations of 1, 10, or 100 mg/L of MT. Methanol was then allowed to evaporate. The plates were then incubated for two or three days. Single colony from the plates was picked and streaked onto new agar plates. This procedure was repeated three times until a pure colony was obtained.

5.2.6 Criteria for selection of MT-degrading bacteria

5.2.6.1 Colony morphology

Colony morphology was used as the primary screening of the isolated MT-degrading bacteria. The basic morphology used included form, elevation, margin, diameter, and color.

5.2.6.2 MT biodegradation by colonies

Colonies of various types were randomly selected for their MT biodegradability under the concentrations used for enrichment and isolation. The tests were performed in 16 mL amber vials with 5 mL final volume of solution containing a culture directly picked from the agar plate. For each colony isolated, five biodegradation tests were conducted. The culture was incubated at 25 °C with a rotating speed of 200 rpm. Control test was carried out with no culture. The incubation period was 3 days.

5.2.6.3 Analysis of partial 16S rRNA gene sequences

Colony was picked and placed into 5 mL of LB broth. Culture was incubated at 25 °C with a rotating speed of 200 rpm for 16-18 hr or until the optical density (OD) at 600 nm reached 0.4-0.6.

DNA was extracted from the bacteria samples using a DNA extraction kit (QIAGEN, Germantown, MD, USA). The extraction steps followed the manufacturer's instructions. The product from DNA extraction was verified by electrophoresis in 2% agarose (Bio-Rad, Barcelona, Spain).

Universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3') were used to amplify the bacterial 16S rRNA gene fragments. Extracted DNA was PCR-amplified using the primer sets for 30 cycles in a 50 μL reaction volume. The PCR mixture was prepared using *Taq* DNA polymerase from Fermentas (Foster City, CA, USA) following the manufacturer's instructions. PCR amplification was performed in a PTC-DNA engine cycler (Bio-Rad, Hercules, CA, USA) under the condition of 3 min at 94 ^{0}C followed by 30 cycles of 1 min at 94 ^{0}C, 1 min at 55 ^{0}C, and 1 min at 72 ^{0}C, followed by 10 min final extension at 72 ^{0}C. The product from PCR was purified with QIAquick® PCR purified kit (Fermentas, Foster City, CA, USA). The purified DNA templates were

sent to Macrogen Inc. laboratory, Seoul, Korea for sequencing analysis. Sequence results were analyzed by Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, Bethesda, MD, USA).

5.2.7 Measurement of 17alpha-methyltestosterone concentration

See section 4.2.5.

5.3 Results and Discussion

5.3.1 Colony morphology

In total, five different colonies (identified as A-E) were isolated (Table 5.1 and 5.2). All five colonies (A-E) were obtained from sediment while four colonies (A-D) were obtained from the water sample with an initial concentration of 1 mg/L MT. For initial MT concentrations of 10 and 100 mg/L, only colonies A and B were found for sediment and water samples. As presented in the Figures 5.2 and 5.3, the first alphabet S or W stands for sediment and water, respectively and the second alphabet (A-E) stands for the colony. The first three numbers (001, 010, and 100) stand for the initial MT concentrations (1, 10, and 100 mg/L) and the last two numbers (01 – 05) stand for the strain selected from the agar plates.

5.3.2 MT biodegradation by colonies

Five strains (identified as 01, 02, 03, 04 and 05) representing each of the colony morphology for all conditions (see Tables 5.1 and 5.2) were selected for biodegradation tests at their corresponding initial MT concentrations.

For colonies isolated from the sediment, biodegradation results showed that all strains of colony B could degrade MT all initial MT concentrations (see Figure 5.2a, b and c). As shown in Figure 5a, nearly 100 % of MT was degraded for all five strains of Colony B while the percent of MT degraded for initial MT concentrations of 10 and 100 mg/L varied from 40 % to 95% depending on the strain (Figure 5.2b and c). For all the initial concentrations tested, only four strains of colony A showed some degradation of MT with the highest percentage about 20%. There were some degradation of MT at less than 5% for strains of Colony C, D and E but for only 1 mg/L of initial MT concentration (Figure 5.2a).

For the colonies isolated from the water sample, biodegradation tests showed that all strains of colony B could degrade MT for all initial MT concentration (see Figure 5.3a, b and c). For an initial MT concentration of 1 mg/L, all five strains of colony B degraded nearly 100% of MT whereas for initial MT concentrations of 10 and 100 mg/L, the percent of MT degraded varied from 10% to nearly 100% depending on the strain (see Figure 5.3b and c). For the other colonies, three strains of colony A showed degradation of less than 20% of MT for initial MT concentrations of 10 and 100 mg/L (Figure 5.3b and c) while strains of colony C, D and E showed very little degradation of MT (< 5%) and only for an initial concentration of 1 mg/L (Figure 5.3a). As shown in Figures 5.2 and 5.3, the percent of MT degraded was lower for higher initial MT concentrations. A possible reason is that the strains did not have enough time to complete the degradation of MT when the starting concentrations were high.

5.3.3 Analysis of partial 16s rRNA gene sequences

All strains of colony B isolated were analyzed for partial 16s rRNA gene sequences (Table 5.3 and 5.4). The 30 analyzed sequences were found to closely related to *Pimelobacter simplex, Rhodococcus sp*, and *Nocardioides nitrophenolicus* with identification in the range of 99-100%. Twenty five sequences were in genus *Nocardioidaceae* whereas the other 5 sequences (SB010-01-SB010-05) were in genus *Nocardiaceae.*

The 16s rRNA gene sequences were classified into three groups closely related to *Pimelobacter simplex,* and *Nocardioides nitrophenolicus*, all of which were in genus *Nocardioidaceae* whereas *Rhodococcus sp.* was in genus *Nocardiaceae.* As all sequences came from the same group, a representative strain was selected from each group for further study. This included strain SB100-05 which was closely related to *Pimelobacter simplex*, SB010-03 which was closely related to *Rhodococcus sp*, and strain WB10005 which was closely related to *Nocardioides nitrophenolicus.*

The MT-degrading bacteria obtained from this study such as *Nocardioides* and *Rhodococcus* were reported previously by other researchers as estrogen degraders. Yu *et al.* (2007) isolated 17β-estradiol-degrading bacteria KC3 and KC4 from activated sludge, which were in the genera of *Nocardioides* and *Rhodococcus*, respectively. Yoshimoto *et al.* (2004) isolated four stains of estrogen-degrading bacteria which

were closely related to *Rhodococcus zopfii* and *Rhodococcus equi*. All four strains had strong efficiency in degrading both natural (estrone, estradiol, and estriol) and synthetic estrogens (ethylnylestradiol) at an initial estrogen concentration of 100 mg/L. Moreover, Druzhinina *et al.* (2008) found that MT can be cleaved to methandrostenolone by *Pimelobacter simplex* VKPM Ac-1632 in the presence of cyclodextrins. Additionally, MT can also be cleaved to methandrostenolone by bacteria in genus *Mycobacterium* (Voishvillo *et al.*, 2002). Voishvillo *et al.* (2002) reported that bacteria in genus *Mycobacterium* can cleave steroid and cholesterol compounds such as *Mycobacterium album*, *Mycobacterium berolinense*, *Mycobacterium bovis*, *Mycobacterium chelonei*, *Mycobacterium cholesterolicum*, *Mycobacterium paraffinicum*, and *Mycobacterium peregrinum*. Moreover, Horinouchi *et al.* (2007) found that gram positive bacteria in genus *Nocardia*, *Arthorobacter*, *Mycobacterium* and gram negative bacteria in genus *Comamonas* and *Pseudomonas* can use testosterone as carbon and energy sources.

5.4 Conclusion

MT-degrading bacteria have not been isolated and identified before. This is probably the first study isolating MT-degrading bacteria. Five different types of colony morphology (A-E) were obtained from the isolation from the sediment and water of masculinization pond under three initial MT concentrations. Only colony type B could degrade MT for all initial MT concentration. Using 16s rRNA gene analysis, 30 strains of colony type B were classified into three groups which closely related to *Pimelobacter simplex,* and *Nocardioides nitrophenolicus*, all of which were in genus *Nocardioidaceae* whereas *Rhodococcus sp.* was in genus *Nocardiaceae*.

Table 5.1 Colony morphology of MT-degrading bacteria isolated from sediment

Source	MT Initial concentration (mg/L)	Colony	Colony morphology			
			Color	Form	Elevation	Margin
Sediment (S)	1	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose
		C	Red	Circular	Convex	Entire
		D	Yellow	Circular	Convex	Entire
		E	Cream	Circular	Convex	Lobate
	10	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose
	100	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose

Table 5.2 Colony morphology of MT-degrading bacteria isolated from water

Source	MT Initial concentration (mg/L)	Colony	Colony morphology			
			Color	Form	Elevation	Margin
Water (W)	1	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose
		C	Red	Circular	Convex	Entire
		D	Yellow	Circular	Convex	Entire
	10	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose
	100	A	Cream	Circular	Convex	Entire
		B	White	Circular	Flat	Erose

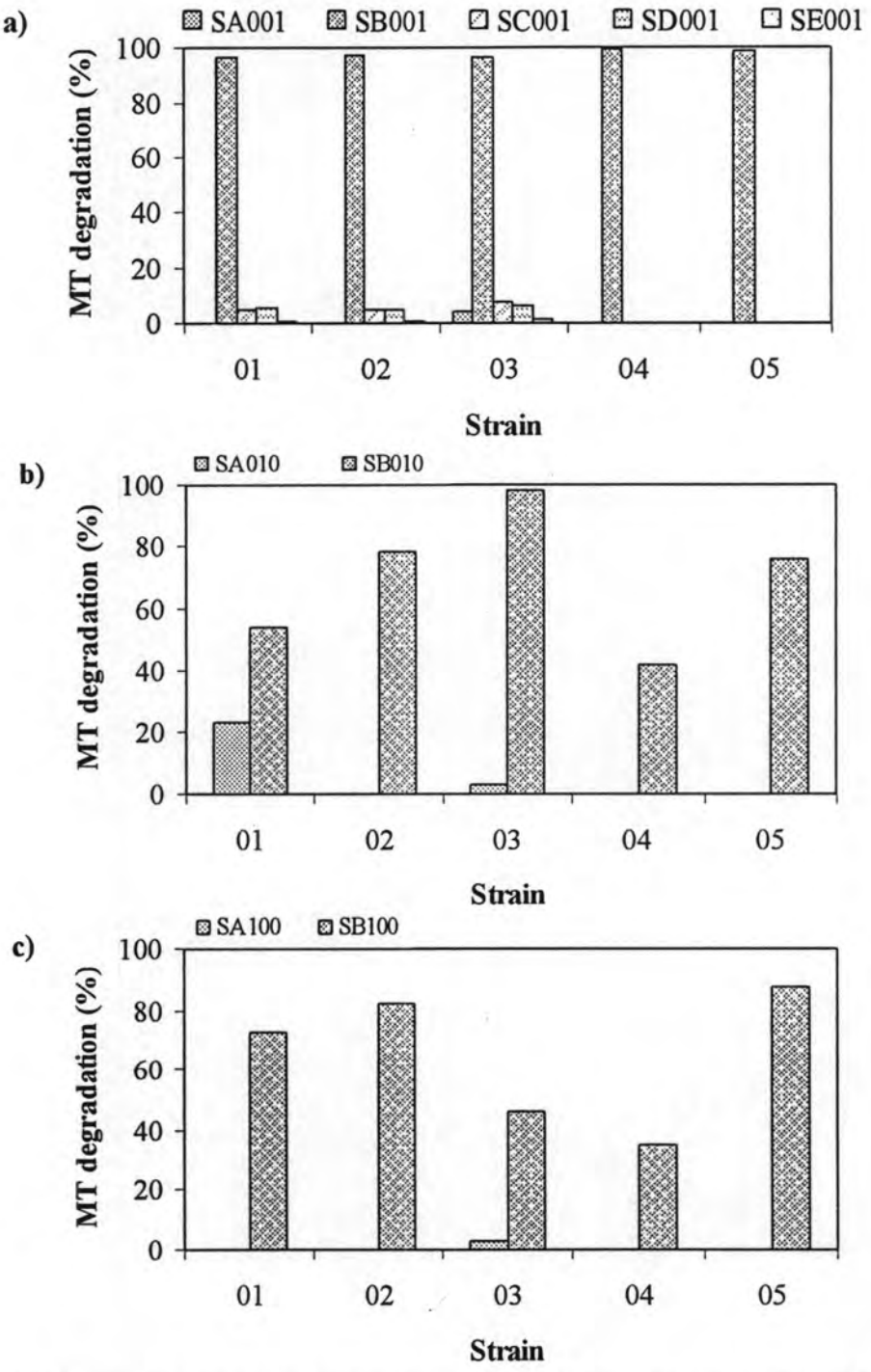

Figure 5.2 MT degradation in 3 day-incubation period by selected strains isolated from sediment of masculinization pond of Nile tilapia fry at a) 1 mg/L of initial MT concentration, b) 10 mg/L of initial MT concentration and c) 100 mg/L of initial MT concentration. The first alphabet (S) stands for medias (sediment). The second alphabet (A-E) stands for colony isolated. The first three numbers (001, 010, and 100) stand for initial MT concentrations (1, 10, and 100 mg/L). The last two numbers (01 – 05) stand for strain selected from the agar plate.

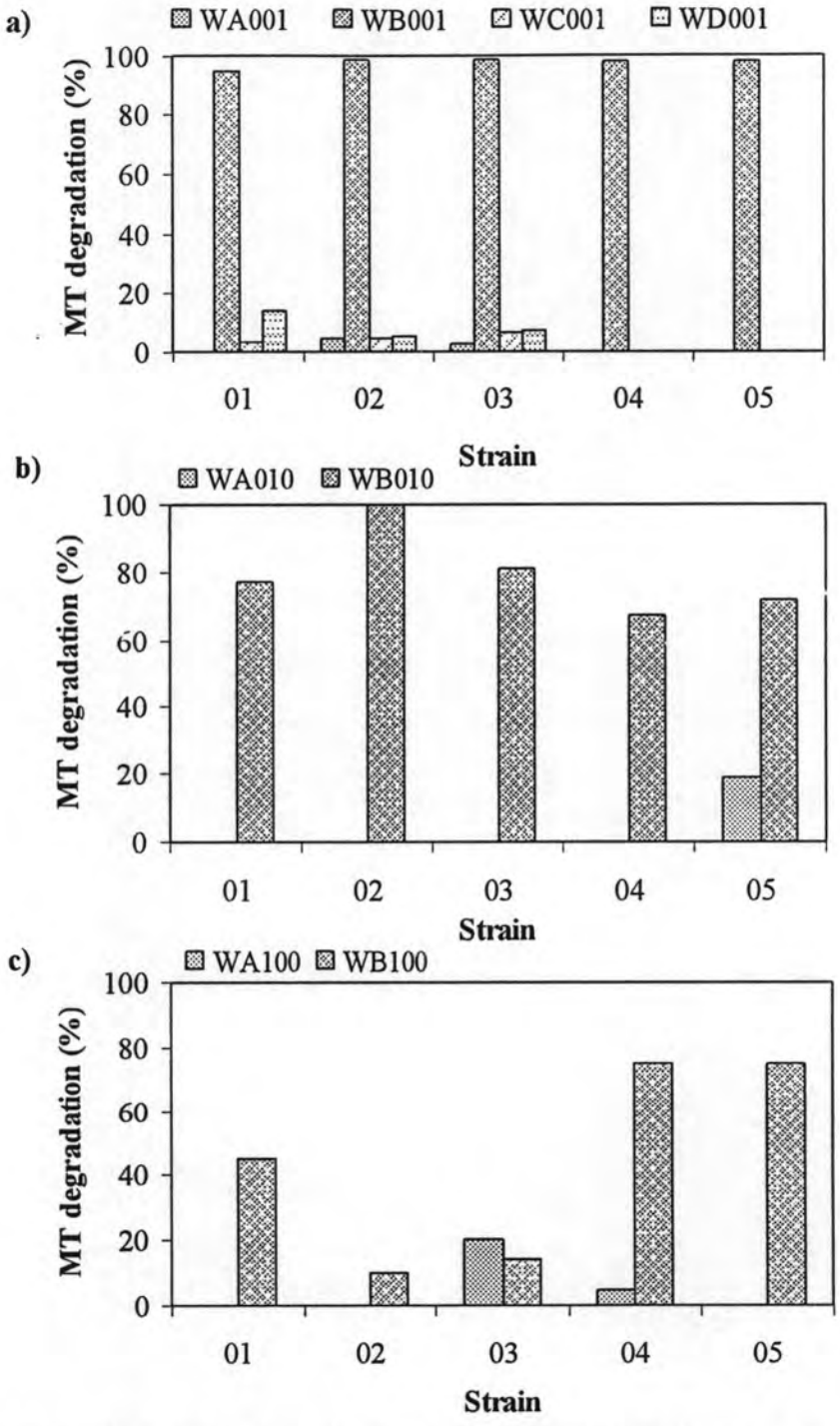

Figure 5.3 MT degradation in 3 day-incubation period by selected strains isolated from water of masculinization pond of Nile tilapia fry at a) 1 mg/L of initial MT concentration, b) 10 mg/L of initial MT concentration and c) 100 mg/L of initial MT concentration. The first alphabet (W) stands for medias (water). The second alphabet (A-E) stands for colony isolated. The first three numbers (001, 010, and 100) stand for initial MT concentrations (1, 10, and 100 mg/L). The last two numbers (01 – 05) stand for strain selected from the agar plate.

Table 5.3 Closely related neighbor of partial 16S rRNA gene sequences of selected colonies isolated from sediment of masculinizing pond of Nile tilapia fry

Name	Score	Ass. No.	Clone	Identity	Gap	Organism
SB001-01	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB001-02	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB001-03	913	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	499/501 (99%)	1/501 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB001-04	918	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/501 (99%)	1/501 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB001-05	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB010-01	907	FJ905295	*Rhodococcus sp. 4-8* 16S ribosomal RNA	495/497 (99%)	0/497 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus.
SB010-02	896	FJ905295	*Rhodococcus sp. 4-8* 16S ribosomal RNA	495/500 (99%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus.
SB010-03	904	FJ905295	*Rhodococcus sp. 4-8* 16S ribosomal RNA	492/493 (99%)	1/493 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus.
SB010-04	900	FJ905295	*Rhodococcus sp. 4-8* 16S ribosomal RNA	491/493 (99%)	0/493 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus.
SB010-05	924	FJ905295	*Rhodococcus sp. 4-8* 16S ribosomal RNA	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus.
SB100-01	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB100-02	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB100-03	739	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB100-04	739	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
SB100-05	555	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	300/300 (100%)	0/300 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.

Table 5.4 Closely related neighbor of partial 16S rRNA gene sequences of selected colonies isolated from water of masculinizing pond of Nile tilapia fry

Name	Score	Ass. No.	Clone	Identity	Gap	Organism
WB001-01	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
WB001-02	905	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	498/501 (99%)	3/501 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
WB001-03	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
WB001-04	918	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/501 (99%)	1/501 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
WB001-05	924	AY509240	*Pimelobacter simplex strain S151* 16S ribosomal RNA gene	500/500 (100%)	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Pimelobacter.
WB010-01	739	AF005024	*Nocardioides nitrophenolicus* strain NSP 41 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB010-02	739	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB010-03	739	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB010-04	739	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene,	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB010-05	739	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene,	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB100-01	924	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene,	500/500 (100%	0/500 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB100-02	917	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	498/499 (99%)	0/498 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB100-03	739	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	400/400 (100%)	0/400 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB100-04	920	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	498/498 (100%)	0/498 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.
WB100-05	721	AF005024	*Nocardioides nitrophenolicus strain NSP 41* 16S ribosomal RNA gene	398/401 (99%)	3/401 (0%)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides.