CHAPTER VI

DEGRADATION OF 17ALPHA-METHYLTESTOSTERONE BY *RHODOCOCCUS EQUI* STRAIN SB010-03 ISOLATED FROM SEDIMENT OF MASCULINIZING POND OF NILE TILAPIA FRY

6.1 Introduction

In the production of some freshwater aquaculture products such as Nile tilapia, an all male population culture is achieved by the use of 17alpha-methyltestosterone (MT), an anabolic androgenic steroid. An all male population is more desirable because it provides about twice the growth rate, larger body size, and higher weight than female tilapia (MacIntosh and Little, 1995; Green *et al.,* 1997). In addition, it avoids the problems of overpopulation and energy loss from stunting for a bi-sexual population. In the case of Nile tilapia, the masculinization process can be achieved by feeding MT-impregnated food containing 60 mg of MT per kilogram of feed to Nile tilapia fry for 21 days after 15 days post-fertilization (Fitzpatrick *et al.*, 1999).

Uneaten and unmetabolized MT-impregnated food can be released into the environment and cause adverse effect to humans and animals. It has been reported that MT can induce the production of nonmalignant tumors in the liver of human (Soe *et al.*, 1992) and caused prostate cancer in humans taking medicine containing 6-9 mg of MT per day for 30 years (Nakata *et al.,* 1997). MT is also classified as an endocrine disruptor compound (Andersen *et al.*, 2006) as it interfers with the normal functions of animals when exposed to MT at low nanogram per liter level. Examples of effects include reduction of vitellogenin protein in female eelpout (*Zoarces viviparous*) (Korsgaard, 2006), development of male sex organ in female freshwater ramshorn snail (*Marisa cornuarietis*) (Schulte-Oehlmann *et al.*, 2004), and a decrease in fecundity and fertility of paired medaka (Kang *et al.,* 2008).

The low water solubility of MT at 3.39 mg/L at 25° C and the high log K_{ow} of 3.36 suggest that MT tends to be sorbed onto the solid phase rather than dissolved in the liquid phase. Therefore, in musculinizing ponds or in natural water bodies, MT

will most probably accumulate in the sediment. However, studies on the fate of MT in the environment are very limited. The only study on the fate of MT in masculinizing pond of Nile tilapia fry showed that MT was detected in the bottom soil of the pond with concentrations approximately 2.9 ng/g, two months after the end of treatment with MT-impregnated food (Fitzpatrick *et al.*, 2000), while in pond water, MT concentration was reduced from 3.6 µg/L to background level (between non detectable and 0.02 µg/L) after one week of the end of the treatment. A study done by Hulak *et al.* (2008) showed that MT-contaminated water from the musculinzing pond, treated with biological filters, continued to show androgenic potency as seen by 81-100% sex change of several common carp progeny (Hulak *et al*, 2008). A recent study revealed that MT was biodegradable by microorganisms in the sediment of masculinizing pond of Nile tilapia fry (see Chapter 4). However, 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 at the end of the experiment was similar to the starting MT concentration and continued to persist even one month after the experiment. This persistent androgenic potency was not observed for aerobic conditions which imply the presence of unknown metabolites of MT from the transformation of MT under methanogenic conditions. To avoid 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 simple and high efficiency treatment system without the persistence of androgenic compounds is biodegradation under aerobic condition. Thus far, the responsible microorganisms for the degradation of MT have not been identified and reported. In this study, MT degrading bacteria were isolated from the sediment of masculinization pond of Nile Tilapia fry, and identified and characterized for its MT biodegradability.

6.2 Materials and methods

6.2.1 Chemicals

See section 4.2.5.

6.2.2 Collection of sediment sample

See section 5.2.2.

6.2.3 Inorganic salt medium

See section 5.2.3.

6.2.4 Enrichment of MT-degrading bacteria

Enrichment of MT-degrading bacteria was performed using the sediment sample with MT concentration of 10 mg/L. Two mL of MT stock solution (500 mg/L) was added to a 250 mL Erlenmeyer flask. The methanol was evaporated by gently blowing nitrogen gas. Ten mL of sediment slurry (10% (vol/vol)) was added into the 250 ml Erlenmeyer flask along with 100 mL of inorganic salt medium to achieve a concentration of 10 mg/L. The flasks were incubated at 25 °C with a rotating speed of 200 rpm. MT concentrations were monitored and when the concentration was reduced by 70%, an aliquot of 10 mL was transferred and subcultured in fresh 100 mL of inorganic salt medium containing MT of the same initial concentration. This process was repeated five times. The time for subculturing at initial MT concentration of 10 mg/L was about 2 weeks.

6.2.5 Isolation of MT-degrading bacteria

The serial 10-fold dilution of enriched culture was prepared with fresh inorganic medium. One hundred mL of the diluted culture solution (10^1 to 10^9) 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. Eighty µL of MT stock solution (500 mg/L) were placed on the agar surface to achieve a MT concentrations of 10 mg/L. 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 the new plates. This procedure was repeated three times until a pure colony was obtained.

6.2.6 Analysis of 16S rRNA gene sequence

Colony was picked up with a loop and placed into 5 mL of LB broth. The 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, Maryland, 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 94^0C followed by 30 cycles of 1 min at 94^0C, 1 min at 55^0C, and 1 min at 72^0C, followed by 10 min final extension at 72^0C. 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 in full range with the primers 27f (5'- AGAGTTTGATCCTGGCTCAG-3'), 341f (5'- CCTACGGGAGGCAGCA G-3'), 800r (5'-CATCGTTTACGGCGTGGAC-3'), 1100r (5'-GGGTTGCGCTCG TTG-3'), and 1492r (5'-GGCTACCTTGTTACGACTT-3'). Sequence results were analyzed by Basic Local Alignment Search Tool (BLAST), (National Center for Biotechnology Information, Bethesda, MD, USA).

6.2.7 Physiological properties

Isolated MT-degrading bacterium was biochemically tested by the National Institute of Health (NIH), Ministry of Public Health, Bangkok, Thailand. The tests conducted included gram reaction, nitrate reduction, alkaline phosphatase, urease production, arginine dihydrolase, catalase, fermentative production of acid from D-glucose, D-ribose, D-xylose, D-mannital, D-lactose, sucrose, glycogen, D-fructose, D-mannose, xylital, D-melibiose, methyl- α-D-glucopyranoside, L-arabinose, D-sorbitol, D-trehalose, inulin, D-raffinose and starch.

6.2.8 Cell morphology

Cell morphology was observed using the images of a scanning electron microscopy (SEM) (JEOL, JIM-5410LV, Alcobendas, Spain).

6.2.9 Kinetics for MT degradation and growth

Kinetics for MT degradation and bacteria growth were studied with MT as the sole energy source for the isolated MT-degrading bacterium. The tests were carried out with six initial MT concentrations of 0.5, 1, 5, 10, 50 and 100 mg/L, and with each concentration conducted in triplicate. All experiments were performed in 16 mL amber vials with 5 mL of final volume of inorganic salt medium solution containing 10^6 cells/mL of isolated MT-degrading bacterium. The control tests were conducted without the isolated cells. The vials were incubated at 25 °C in the dark with a rotating speed of 200 rpm. MT concentration was measured by high performance liquid chromatography (HPLC; HPLC 1100 series, Agilent Technologies, Palo Alto, CA)) and cell count was analyzed by a microscope-counting chamber (Hemacytometer) (Hausser scientific, Horsham, PA, USA)

6.2.10 Kinetic model

Haldane's model, a substrate inhibitory model, was used to model the kinetics of MT degradation (Haldane, 1930). The Haldane model is given below:

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

where S is the substrate concentration (mg/L); μ is the specific growth rate (1/hr); μ_{max} is the specific growth rate (1/hr); K_s is the substrate half saturation coefficient (mg/L); and K_i is the substrate inhibition coefficient (mg/L). The kinetic parameters μ_{max}, K_s, and K_i were determined using the non-linear regression program of MS Excel.

6.2.11 Measurement of 17alpha-methyltestosterone concentration

See section 4.2.5.

6.2.12 Analysis of androgenic activity

See section 4.2.6.

6.3 Results and discussions

6.3.1 Identification and characterization of isolated MT-degrading bacterium

Of the different isolates, strain SB010-03 was isolated and selected due to it's the strong MT biodegradability (see Chapter 5). Using the ARB program package, the sequence of the strain SB010-03 was aligned with the sequences in the SSU rRNA database. The sequence of strain SB010-03 was added into the distant tree, which was constructed by comparing >1400-bp sequences in the SSU rRNA database (Antwerm, Belgium). After that, the sequence of the strain SB010-03 together with several reference sequences selected from the SSU rRNA database were calculated and a phylogenetic analysis conducted with three different methods (i.e., distance matrix, maximum parsimony, and maximum likelihood) in the ARB program package to confirm the position of the bacterium strain sequence in the phylogenetic trees. The position of strain SB010-03 on the phylogenic trees for all three methods showed that the MT-degrading bacterium strain SB010-03 was closely related to *Rhodococcus equi* (Figure 6.1) with 100 % sequence identity.

The isolated MT-degrading bacterium strain SB010-03 was biochemically tested (see details in Table 6.1). Strain SB010-03 was gram positive. The alkaline phosphatase, urease and catalase tests were positive. This strain can grow in various groups of carbohydrate (as shown in Table 6.1) without the production of acidification. The physiological properties suggested that strain SB010-03 was closely related with *Rhodococcus*.

Scanning electron microscope photos indicated that strain SB010-03 had a rod shape with a length of 0.78-1.67 μm and a diameter of 0.50-0.67 μm (Figure 6.2). The result suggested that this strain was closely related to *Rhodococccus*.

Based on the result from the phylogenetic analysis of full-length sequence of 16S rRNA gene physiological properties and cell morphology, strain SB01003 was found to be closely related to *Rhodococcus equi.* which is a member of the genus *Nocardiaceae* in the family of *Corynebacterineae.* In the literature, members of *Rhodococcus* have been found to utilize a number of substrates such as benzene (Paje

and Couper-White, 1996), o-xylene (Di Gennaro *et al.*, 2001), naphthalene (Di Gennaro *et al.*, 2001; Kulalov *et al.*, 2000), phenanthrene, anthracene, pyrene, fluoranthene and chrysene (Walter *et al.*, 1991; Dean-Ross *et al.*, 2001). A possible reason for the ability for *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 (Kim *et al.*, 1990; Peng *et al.*, 2007). In addition, *Rhodococcus* has the enzyme to cleave compounds with a steroidal skeleton structure. *Rhoddococcus* has been shown to be capable of degrading testosterone (Horinouchi *et al.*, 2007), 19-nortestosterone (Sallam *et al.*, 1995), and estrogens (Yoshimoto *et al.*, 2004; Yu *et al.*, 2007). One strain of *Rhodococcus zopfii* (strain Y 50158) and three strains of *Rhodococcus equi* (strains Y 50155, 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) (Yoshimoto *et al.*, 2004). Additionally, one of the 14 isolated E2-degrading bacteria in the study of Yu *et al*, (2007) (strain KC4) was found in the genera *Rhodococcus*. Another study by de las Heras *et al.* (2009) 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.

6.3.2 Biodegradation of MT by strain SB01003

The degradation results of strain SB010-03 for various initial concentrations of MT (0.5 – 100 mg/L) are presented in Figure 6.3. Results showed that strain SB010-03 can degrade MT without an acclimatization period for all initial MT concentration (see Figure 6.3). The initial degradation rate of MT for each initial MT concentration was estimated using first order reaction kinetics. The initial first order degradation rates (±95% CI) were 0.24±0.02, 0.26±0.10, 0.38±0.03, 0.19±0.03, 0.09±0.01, and 0.03±0.00 hr^{-1} at initial concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 mg/L, respectively. The initial degradation rate increased when the initial MT concentration increased to 5.0 mg/L but decreased for concentrations above 5.0 mg/L of MT. The results from the biodegradation of MT indicated that the bacteria strain SB010-03 was able to use MT as a sole carbon source.

During the biodegradation tests, a metabolite was identified by the HPLC with a retention time of 16.2 mins. Based on the retention time, the metabolite probably was more polar than MT or has a lower molecular weight. However, both MT and the metabolite can be degraded by strain SB010-03 throughout the incubation period. The metabolite was not identified but possible metabolites may be methandrostenolone (ME2) which may result from the cleavage of MT by *Pimelobacter simplex* VKPM Ac-1632 (genus of *Nocardioidaceae*) (Druzhimina *et al.*, 2008) and bacteria in genus Mycobacterium (Voishvillo *et al.*, 2002). Other possible MT metabolites include 17α-methyl-5α-androstan-3α, 17β-diol and 17α-methyl-5 β -androstan-3α, 17β-diol and their isomers which are found in the excretion of human and animal (Shinohara *et al* 2000; Rongone and Segaloff, 1962; Williams *et al*, 2000 and Mosbach *et al.*, 1968). It is possible that other MT metabolites may be present but not detected by the HPLC.

6.3.3 Effect of initial MT concentration on the growth of the strain SB010-03

The number of cells for MT concentration between 0.5 and 1.0 mg/L did not change (see Figure 6.3) during the incubation which may imply that the substrate concentration was not enough for bacteria growth. However, above the 1.0 mg/L of MT, the number of cells increased significantly. The specific growth rate (μ) was estimated using $\ln (X/X_0) = \mu t$ where X_o is the initial number of cells and X is the number of cells at present. Figure 6.4 presents the specific growth rates of strain SB010-03 for the various initial MT concentrations tested. As seen in Figure 6.4, lower specific growth rates were observed for MT initial concentrations greater than 10 mg/L (Figure 6.4) which implied the possibility of substrate inhibition on the microbial strain by MT. Using a nonlinear regression program in Microsoft Excel, the Haldane substrate inhibition model was found to fit the data with a correlation coefficient (R^2) of 0.90. The estimated kinetics constants μ_{max}, K_s, K_i, were 0.13 h^{-1}, 24.75 mg/L and 76.18 mg/L, respectively. The K_s value indicates the affinity of microorganism for the substrate while the K_i value indicates the extent of inhibition with large values of K_i indicating the substrate has less inhibitory potential (Ong and Bowers, 1990). The initial concentrations used in this study were much higher than the reported concentrations found in the effluent water of the ponds. However, it

should be pointed out that when MT-impregnated food are fed to the fish, it is possible that localized high concentrations of MT may occur in the sediments due to accumulation of the food and in the water during the feeding process which can inhibit the microorganisms in the sediments.

6.3.4 Androgenic activity

The androgenic potency monitored using β-galactosidase assay gave information on possible remaining androgencity of MT after transformation and can also be used as an indirect measure of the presence of MT metabolites. Results expressed in term of testosterone equivalent (TEQ) which represent the overall androgen activity originated from all androgen like compounds (MT residue and MT metabolites) while the relative TEQ computed from the MT concentration which represent the androgen potency derived from MT only. Since the detection limit of β-galactosidase assay to MT was about 0.1 mg/L, the assay could not detect low concentrations of androgen-like compounds in the culture test for an initial MT concentration of 0.5 mg/L due to the serial dilutions. Therefore, the EC_{50} at initial MT concentration of 0.5 mg/L were not determined due to the lack of data to draw the dose-response curve. With the exception of 0.5 mg/L initial MT concentration, the TEQs were significantly higher that the relative TEQ during the incubation period (Figure 6.5 (a) and (b)). For example, the TEQ was six times higher than the relative TEQ at 8 hours for an initial MT concentration of 5 mg/L and two times higher at 24 hours for an initial MT concentration of 50.0 mg/L. However, the TEQ and relative TEQ reached undetectable levels at the end of the incubation. The androgenic activity results indicated that strain SB010-03 was able to degrade MT and its metabolites to non androgen-like compounds.

6.4 Conclusion

This is the first study isolating a MT-degrading bacterium from the sediment of musculinizing pond of Nile tilapia fry. Using 16s rRNA gene sequencing, physiological properties and cell morphology identification method, the strain SB010-03 was found to be closely related to *Rhodococcus equi*. This strain was tested in a biodegradation tests and was confirmed to be capable of degrading MT. This study also shows that MT was degraded as evident by the presence of a metabolite and the

disappearance of MT and the metabolite at the end of the incubation period. In addition, the absence of androgenic activity at the end of the incubation period indicated that the non-androgenic degradation products, if any, were produced. Biodegradation kinetics studies showed that high concentrations of MT were found to inhibit the growth of strain SB010-03.

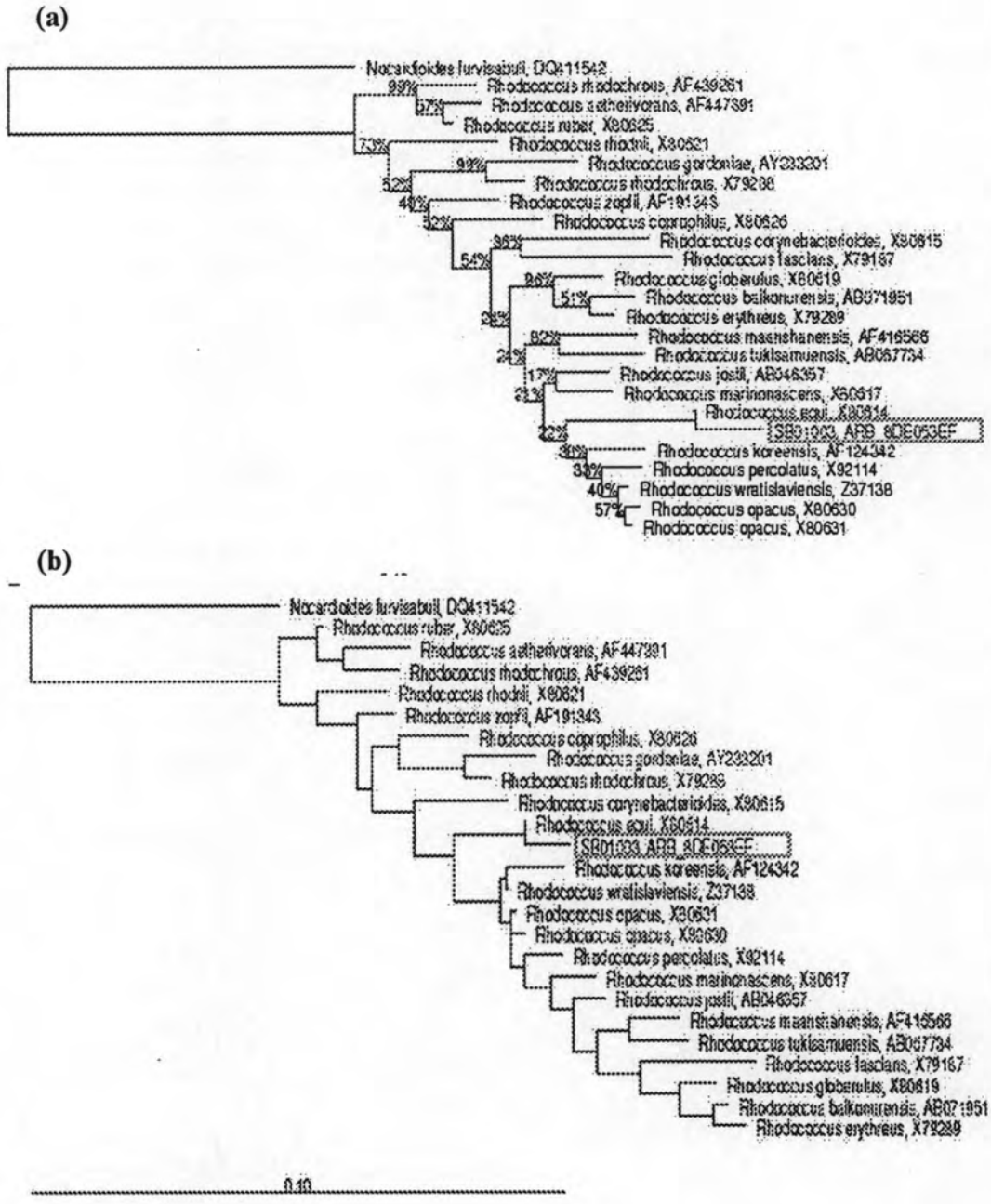


Figure 6.1 Phylogenetic tree showing 16S rRNA genes of the strain SB010-03 constructed by adding full-length sequences of the strain SB010-03 into the tree constructed by (a) neighbor joining (distance matrix) method, (b) parsimony method and (c) maximum likelihood using > 1400-bp sequences of reference sequences.

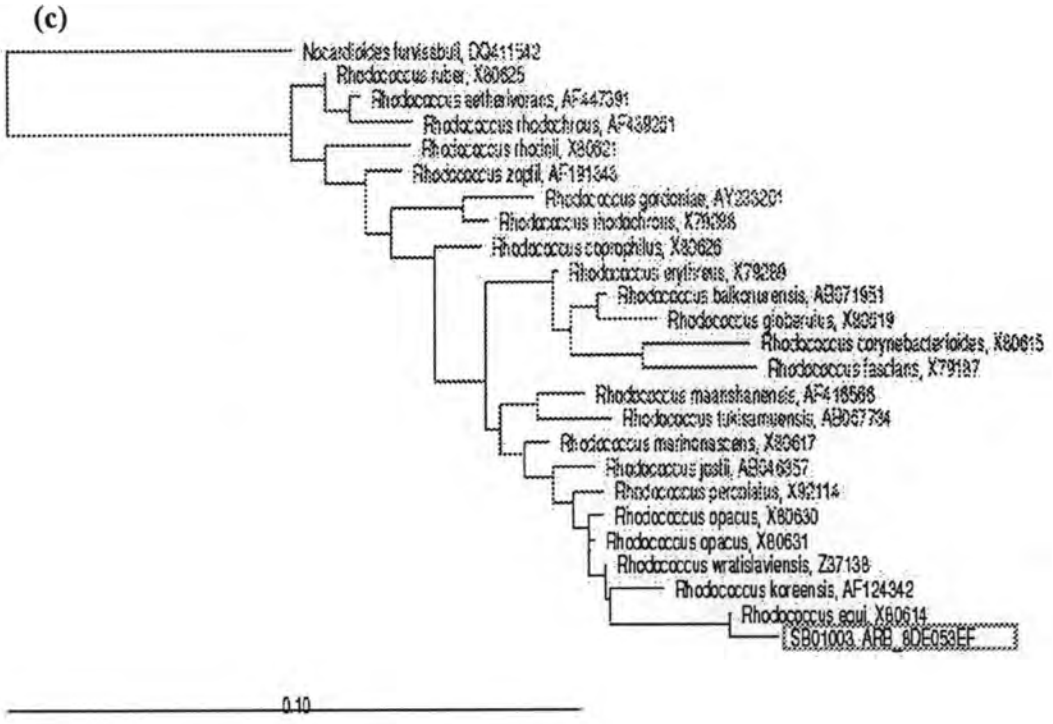


Figure 6.1 (cont') Phylogenetic tree showing 16S rRNA genes of the strain SB010-03 constructed by adding full-length sequences of the strain SB010-03 into the tree constructed by (a) neighbor joining (distance matrix) method, (b) parsimony method and (c) maximum likelihood using > 1400-bp sequences of reference sequences.

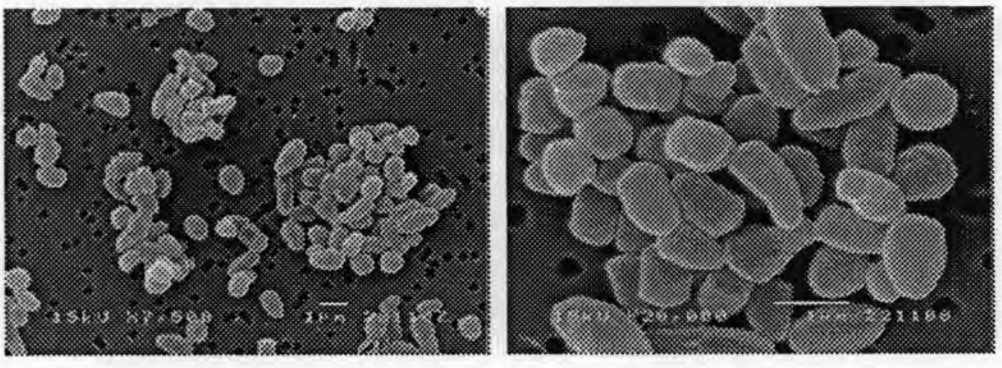


Figure 6.2 Scanning electron microscope photos of isolated MT-degrading bacterium strain SB010-03 (a=7,500x; b=20,000x)

Table 6.1 Physiological properties of isolated MT-degrading bacterium strain SB010-03

Characteristics	Result
Gram reaction	+
Nitrate reduction	-
Pyrrolidonyl arylmidase	n.a.
Alkaline phosphatase	+
β – glucuronidase production	n.a.
β – galactosidase production	n.a.
α – galactosidase production	n.a.
N-acetyl-β-glucosaminidase	n.a.
Hydrolysis of esculin	n.a.
Urease production	+
Acetoin production	n.a.
Leucine aminopeptidase	n.a.
Arginine dihydrolase	-
Fermentative production of acid from:	
- D-Glucose	-
- D-Ribose	-
- D-Xylose	-
- D-Mannital	-
- D-Maltose	-
- D-Lactose	-
- Sucrose	-
- Glycogen	-
- D-Fructose	-
- D-Mannose	-
- Xylitol	-
- D-Melibiose	-
- Methyl-αD-glucopyranoside	-
- L-Arabinose	-
- D-Sorbitol	-
- D-Trehalose	-
- Inulin	-
- D-Raffinose	-
- Starch	-
Catalase	+

n.a. = not analysis; + = positive, - = negative

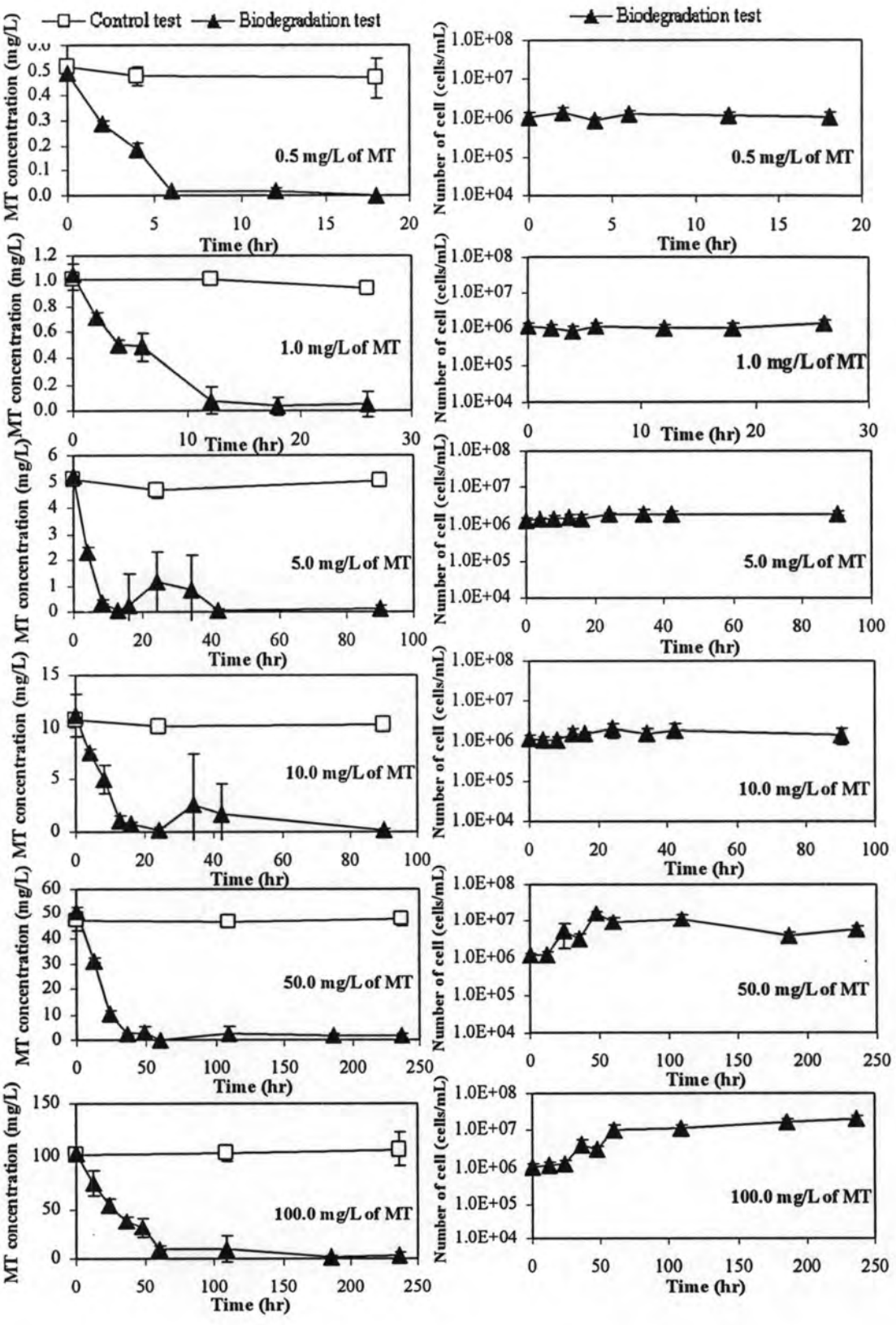


Figure 6.3 Number of cells and degradation of MT by isolated MT-degrading bacterium strain SB010-03 at different initial MT concentrations

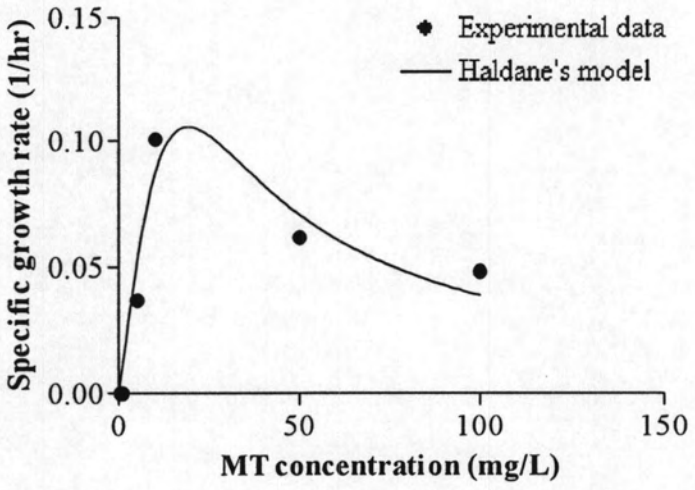
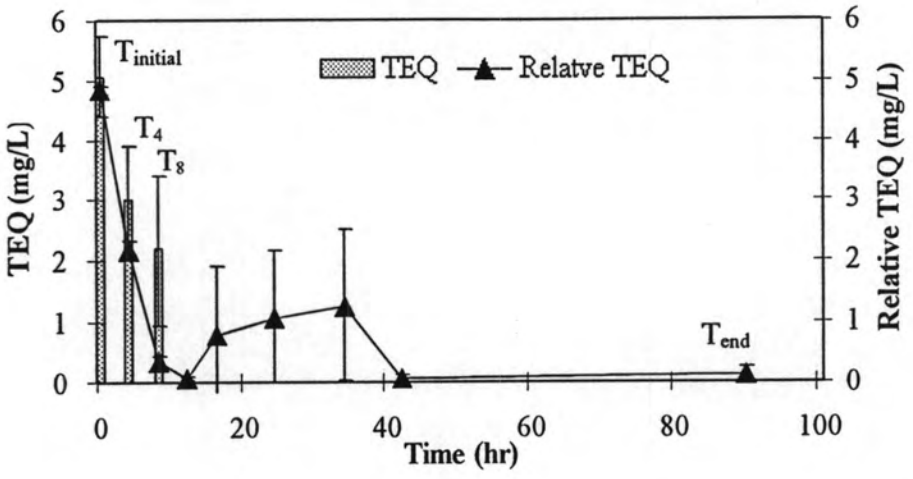
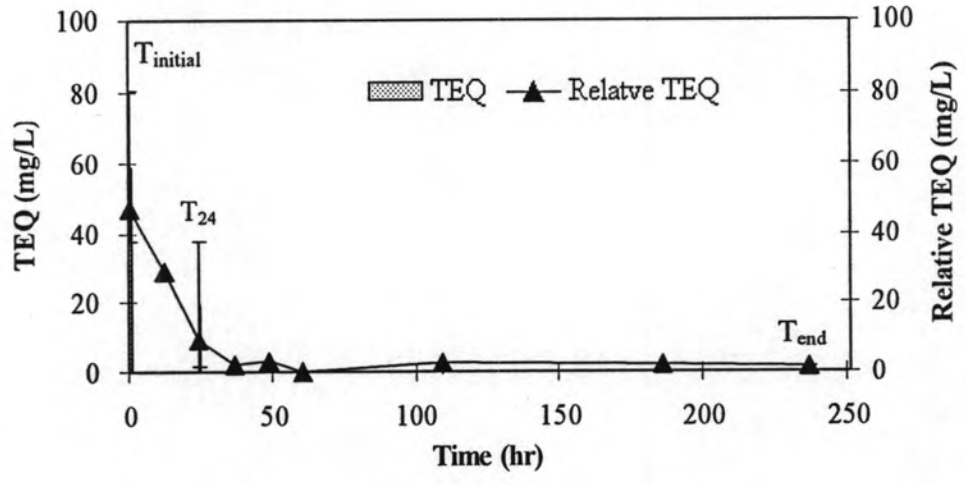


Figure 6.4 Specific growth rate as a function of initial MT concentration



(a) Initial MT concentration of 5.0 mg/L



(b) Initial MT concentration of 50.0 mg/L

Figure 6.5 Degradation of MT by isolated MT-degrading bacterium strain SB010-03 at initial MT concentration of (a) 5.0 and (b) 50.0 mg/L