CHAPTER VII

DEGRADATION OF 17ALPHA-METHYLTESTOSTERONE BY *NOCARDIOIDES AROMATICIVORANS* AND *NOCARDIOIDES NITROPHELOLICUS* ISOLATED FROM SEDIMENT AND WATER OF MASCULINIZING POND OF NILE TILAPIA FRY

7.1 Introduction

Treatment of 15-day old post-fertilized Nile tilapia fry (*Oreochromis niloticus*) for 21 days with 60 mg per one kilogram of fish food of 17alpha-methyltestosterone (MT), an anabolic androgenic steroid, can achieve sex reversal and produce all-male population (Fitzpatrick *et al.*, 1999). The all-male population is desirable to farmers as an all-male population has double the growth rate, larger body size and higher body weight than female tilapia (MacIntosh and Little, 1995; Green *et al.*, 1997).

In many of the masculinizing ponds, residual MT from uneaten and unmetabolized MT-impregnated food remained in the ponds and if released can contaminate the environment. Humans and animals may be impacted by MT as it can interfere with the normal functions of the reproductive system and serves as a possible carcinogenic agent. Soe *et al.* (1992) found that MT can induce nonmalignant tumors in the liver and cause prostatic cancer in human for a daily intake of 6-9 mg of MT for 30 years (Nakata *et al*, (1997). As an endocrine disruptor compound (Wason *et al*, 2003; Andersen *et al.*, 2006), Korsgaard (2006) found that the vitellogenin protein expressing female characteristic was decreased in female eelpout (*Zoarces viviparous*) when exposed to 10-500 ng/L of MT for ten days. Schulte-Oehlmann *et al.* (2004) found that male sex organs such as penis and vas deferens in female freshwater ramshorn snail (*Marisa cornuarietis*) were developed when exposed to 100-1,000 ng/L of MT for six months. MT in high dosage can be converted to 17α-methylestradiol (ME2) which has been found to produce estrogenic activity by aromatase enzyme (Hornung *et al.*, 2004)

Until now, studies on the occurrence of MT in environment have been limited. A study found that MT in the water inside the masculinization pond was about 3.6

μg/L at the end of MT treatment and then decreased to background concentrations (between non detectable and 0.02 μg/L) after one week. However, about 2.9 ng/g of MT was found to remain in the soil of masculinization pond for more than two months after the end of masculinization process (Fitzpatrick and Contreras-Sánchez, 2000).

Contaminated sediment and waters from masculizing ponds must be disposed of properly to prevent contamination of MT in nearby receiving waters and environment. An approach in eliminating MT without the androgen-like compounds as byproducts (see Chapter 4) is by biodegradation under aerobic condition. Thus far, responsible MT-degrading bacteria have not been identified and reported.

The objective of this study was to isolate, identify and characterize MT-degrading bacteria from sediment and water. In addition, this study investigated the effect of initial MT concentrations on biodegradation of MT and the androgenic activity of byproducts of MT.

7.2 Materials and methods

7.2.1 Chemicals

See section 4.2.5.

7.2.2 Collection of sediment and water samples

See section 5.2.2.

7.2.3 Inorganic salt medium

See section 5.2.3.

7.2.4 Enrichment of MT-degrading bacteria

Enrichment of MT-degrading bacteria was performed using the sediment and water sample with MT concentrations of 100 mg/L. Two mL of the stock solution of MT (5,000 mg/L) was added into a 250 ml Erlenmeyer flask. The methanol was dried by gently blowing the nitrogen gas. Ten mL of sediment and water (10% (vol./vol.)) were added into the 250 ml Erlenmeyer flask along with 90 mL of inorganic salt medium achieve an initial concentration of 100 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 a fresh 90 mL of inorganic salt medium containing MT at the same initial concentration. This process was repeated five times. The time needed for subculturing at initial MT concentration of 100 mg/L was about 2 weeks.

7.2.5 Isolation of MT-degrading bacteria

The serial 10-fold dilution of the enriched culture was prepared with fresh inorganic medium. One hundred μL of the diluted culture solution (10^1 to 10^9) was 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 in stock solution with concentrations of 5,000 mg/L were placed on the agar surface to achieve MT concentrations of 100 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 new plates. This procedure was repeated three times until a pure colony was obtained.

7.2.6 Analysis of 16S rRNA gene sequence

See section 6.2.6.

7.2.7 Physiological properties

Isolated MT-degrading bacteria were biochemically tested by Microbiological Resources Centre (Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand).

7.2.8 Cell morphology

See section 6.2.8.

7.2.9 Kinetics for MT degradation and growth

See section 6.2.9.

7.2.10 Kinetic model

See section 6.2.10.

7.2.11 Measurement of MT concentration

See section 4.2.5.

7.2.12 Analysis of androgenic activity

See section 4.2.6.

7.3 Results and discussions

7.3.1 Identification and characterization of isolated MT-degrading bacteria

According to the result from enrichment and isolation (see Chapter 5), MT-degrading bacteria stain SB100-05 and WB100-05 were selected to identify and characterize due to its ability to degrade MT at various initial MT concentrations. Using ARB program package, the sequences of strains SB100-05 and WB100-05 were aligned with the sequences in the SSU rRNA database. After that, sequences of strains SB100-05 and WB100-05 were added into the distant tree, which was constructed comparing>1400-bp sequences from the SSU rRNA database (Antwerm, Belgium). Then, the sequence of the strain SB100-05 and WB10005 together with some reference sequences selected from the SSU rRNA database were aligned and calculated and phylogenetic analysis conducted with three different methods (i.e. the distance matrix, maximum parsimony, and maximum likelihood) in the ARB program package to confirm the position of the bacteria strains sequence in the phylogenetic trees. The result from phylogenetic analysis with three methods showed that the position of strain SB100-05 on the phylogenetic tree was related closely to *Nocardioides aromaticivorans* with 100 % sequence identity (Figure 7.1). In case of strain WB100-05, the position of strain WB100-05 calculated by all method of phylogenetic analysis showed that strain WB100-05 was related closely to *Nocardioides nitrophelolicus* except with parsimony method. However, the phylogenetic result expressed that the position of strain WB100-05 was mostly identified as *Nocardioides nitrophelolicus* with 100 % sequence identity (Figure 7.1).

The isolated MT-degrading bacteria strains SB100-05 and WB100-05 were biochemically tested (see details in Table 7.1). Both strains SB100-05 and WB100-05 were gram positive. The alkaline phosphatase, hydrolysis of esculin, leucine aminopeptidase, and catalase test were positive. This strain cans growth in group of

carbohydrate (as shown in Table 7.1) without the production of acidification. The physiological properties suggested that strain SB100-05 and WB100-05 were related closely with *Nocardioides*.

Scanning electron microscopy photo indicated that the strain SB100-05 had a rod shape with a length of 0.54-0.92 μm and a diameter of 0.35-0.42 μm (Figure 7.2 (a)) 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 (Figure 7.2 (b)). The result suggested that the strain SB100-05 and WB100-05 were similar to *Nocardioides*.

Based on the results from the phylogenetic analysis, physiological properties and cell morphology suggested that the strain SB100-05 and WB100-05 were closely related to *Nocardioides aromaticivorans* and *Nocardioides nitrophelolicus*, respectively which are a member of the genus *Nocardioidaceae* in family *Propionibacteria*. From the literatures, the strain in genus *Nocardioidaceae* can also degrade estrogen (estradiol (E2)). As study of Yu *et al.*, (2007), they isolated 17β-estradiol-degrading bacteria KC3 from activated sludge, which were also in genera *Nocardioides*. Moreover, some researches found that *Pimelobacter simplex* VKPM Ac-1632 (genus *Nocardioidaceae)* in the present of cyclodextrins (Druzhinina *et al.*, 2008) and bacteria in genus *Mycobacterium* (Voishvillo *et al.*, 2002) can also converse MT to methandrostenolone (ME2). However, many 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* (Voishvillo *et al.*, 2002). Moreover, *Nocardia*, *Arthorobacter*, *Mycobacterium* which were gram-positive bacteria and *Comamonas* and *Pseudomonas* which were gram-negative bacteria were able to use testosterone as carbon source (Horinouchi *et al.*, 2007).

7.3.2 Biodegradation of MT by strain SB100-05 and WB100-05

Experiments were conducted to examine the effect of various initial concentrations of MT (0.5-100 mg/L) on the biodegradation behavior of bacteria strain SB100-05 and WB100-05 (Figure 7.3 and 7.4). Strain SB100-05 can degrade MT without a lag time period for all initial MT concentration except for initial MT concentration of 100 mg/L, 12 hr of a lag time was observed (see Figure 7.3). The

strain WB100-05 can degrade MT without the acclimatized period for initial MT concentration of 0.5 and 1.0 mg/L, with 4 hr of acclimatized period for initial MT concentration of 5.0 and 10.0 mg/L and with 17 hr of acclimatized period for initial MT concentration of 50.0 and 100.0 mg/L. The initial degradation rate of MT for each initial MT concentration was evaluated using first order reaction kinetic. The initial first order degradation rate (±95% CI) were 0.36±0.09, 0.42±0.09, 0.30±0.05, 0.20±0.02, 0.11±0.05, and 0.06±0.01 hr^{-1} in MT degradation by strain SB100-05 and 0.16±0.02, 0.17±0.01, 0.13±0.03, 0.10±0.00, 0.08±0.02, and 0.04±0.01 hr^{-1} in MT degradation by strain WB100-05 at initial concentration of MT of 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 mg/L, respectively. In the degradation of MT by strain SB100-05 and WB100-05, the initial degradation rate increased when initial MT concentration increased to 1.0 mg/L but decreased for concentrations above 1.0 mg/L. The result from the biodegradation of MT indicated that bacteria strain SB100-05 and WB100-05 were able to use MT as a sole carbon source.

During the biodegradation of both strains, the metabolite was observed in the HPLC chromatogram at retention time of 16.2 min earlier than that of MT (17.6 min). and it has more polarity than MT. It implied that metabolite was probably more polarity and/or with a lower molecular weight than MT. However, both strains could degrade MT and metabolites throughout the end of incubation. The MT metabolite was not identified but possible metabolites may be methandrostenolone (ME2) which resulted from the clave of MT by *Pimelobacter simplex* VKPM Ac-1632 (genus of *Nocardioidaceae*) (Druzhimina *et al.*, 2008) and bacteria in genus Mycobacterium (Voishvillo *et al.*, 2002). Moreover, the other possible MT metabolites were 17α-methyl-5α-androstan-3α, 17β-diol and 17α-methyl-5 β -androstan-3α, 17β-diol and their isomer in minority which from the excretion of human and animal (Shinohara *et al* 2000; Rongone and Segaloff, 1962; Williams *et al*, 2000 and Mosbach *et al.*, 1968).

7.3.3 Effect of initial MT concentration on the growth of the strain SB100-05 and WB100-05

The number of cells of strain SB100-05 for initial MT concentration between 0.5 and 1.0 mg/L did slightly change of number (see Figure 7.3) which may suggest that the substrate concentration was not enough for bacteria growth but above the 1.0 mg/L of

MT, the number of cells increased significantly. In case of the strain WB100-05, the number of cells at MT concentration between 0.5 and 1.0 mg/L did not change (see Figure 7.4) which may imply as similar as in case of strain SB100-05. However, above 1.0 mg/L of MT concentration, the number of cells increased significantly. The specific growth rate (μ) calculated using $\ln (X/X_0) = \mu t$ equation where X_0 is the number of cells at initial time of incubation and X is the number of cells at present. The specific growth rate of both strains for various initial MT concentration tested was shown in Figure 7.5 (a) and (b). The specific growth rates of strain SB100-05 and WB100-05 were decreased as MT concentrations were increased for initial concentrations of MT above 5.0 and 10.0 mg/L, respectively, due to the possibility of substrate inhibitory on both strains. Using a nonlinear regression program in Microsoft Excel, the Haldane's substrate inhibition model was found to fit to the data with correlation coefficients (R^2) of 0.89 and 0.86 for strain SB100-05 and WB100-05, respectivly. The estimated kinetics constants μ_{max}, K_s, and K_i, in biodegradation of MT by the strain SB100-05 were 0.19 h^{-1}, 0.65 mg/L, and 19.63 mg/L, respectively. In case of biodegradation of MT by strain WB100-05, the estimated kinetic constants μ_{max}, K_s, and K_i were 0.16 h^{-1}, 1.50 mg/L, and 41.23 mg/L, respectively. Although, the two strains were in the same genus, they had different biodegradation activity on MT. The large value of μ_{max} and low value of K_s and K_i for strain SB100-05 rather than that for strain WB100-05 implied that strain SB100-05 was able to degraded MT faster but had lower resistance to high concentration of MT than strain WB100-05. It is possible that strain SB100-05 acclimated with MT due to the accumulation of MT in sediment rather than water.

7.3.4 Androgenic activity

Androgenic potency was used to measure the androgenic like compounds presenting during biotransformation of MT by using β-galactosidase assay and was also used as indirect measure of metabolite. Results from β-galactosidase assay expressed in term of testosterone equivalent (TEQ). TEQ was represented the overall androgen activity originated from all androgen like compounds (MT residue and MT metabolites). Relative TEQ was represented the androgen potency derived from MT only. Due to the high detection limit of β-galactosidase assay to MT concentration at 0.1 mg/L, this assay can not be detected the lower concentration of MT and its

metabolites as androgen-like compounds. Thus, the EC_{50} at initial MT concentration of 0.5 mg/L can not determine because of the lack of data series to draw the dose-response curve. With an exception for 0.5 mg/L as initial MT concentration, the tendency of TEQ was not significantly differed with relative TEQ during the incubation period of both strains except for an initial MT concentration of 5.0 mg/L in biodegradation test by strain SB100-05, TEQ was three times higher than relative TEQ at 5 hours (Figure 7.6 and 7.7). However, TEQ and relative TEQ reached undetectable levels at the end of the incubation for initial MT concentration of 5.0 mg/L and declined to lower than about 4.0 mg/L for initial MT concentration of 50 mg/L in both strains. The result implied that the strain SB100-05 and WB100-05 were able to degrade MT and its metabolites to non androgen-like compounds.

7.4 Conclusion

This is one of the first few studies that isolated MT-degrading bacteria from the sediment and water of musculinizing pond of Nile tilapia fry. Based on the 16s rRNA gene sequencing, physiological properties and cell morphology identification method, the strain SB100-05 and WB100-05 found to be closely related to *Nocardioides aromaticivorans* and *Nocardioides nitrophelolicus*, respectively. The biodegradation test with MT confirmed that these strains were capable to use MT as sole carbon source. Moreover, these strains could also degrade metabolite of MT occurring during the incubation period. Additionally, the androgenic activity also proved that MT was cleaved to non-androgenic compounds as byproducts by both strains. Biodegradation kinetic study showed that the strain SB100-05 was found to degrade MT more rapidly but lower resistance on MT than strain WB100-05. WB100-05.

(a)

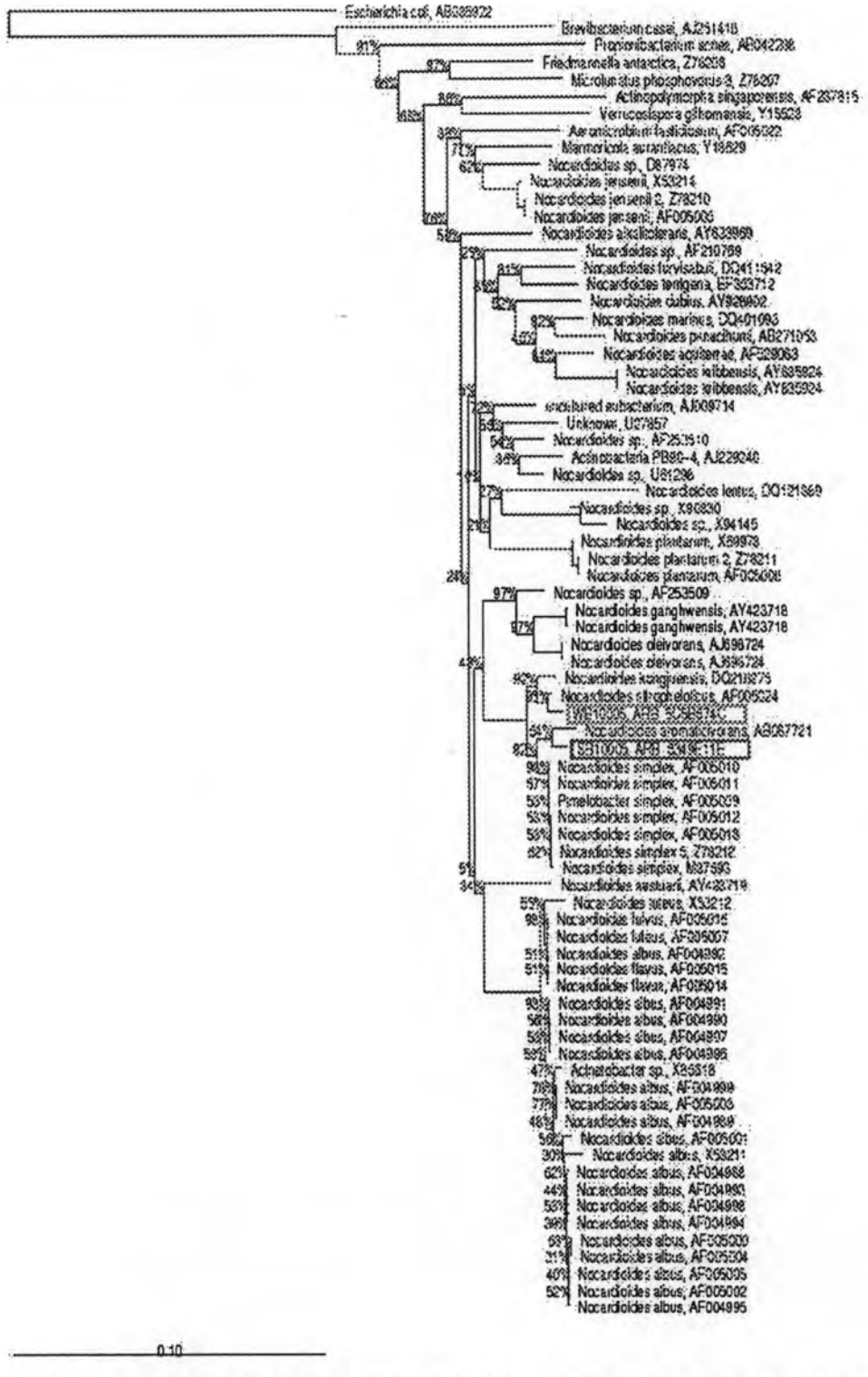


Figure 7.1 Phylogenetic tree showing 16S rRNA genes of the strain SB100-05 and WB100-05 constructed by adding full-length sequences of the strain SB100-05 and WB100-05 into the tree prior constructed by (a) neighbor joining (distance matrix) method, (b) parsimony method and (c) maximum likelihood method using > 1400-bp sequences of reference sequences.

(b)

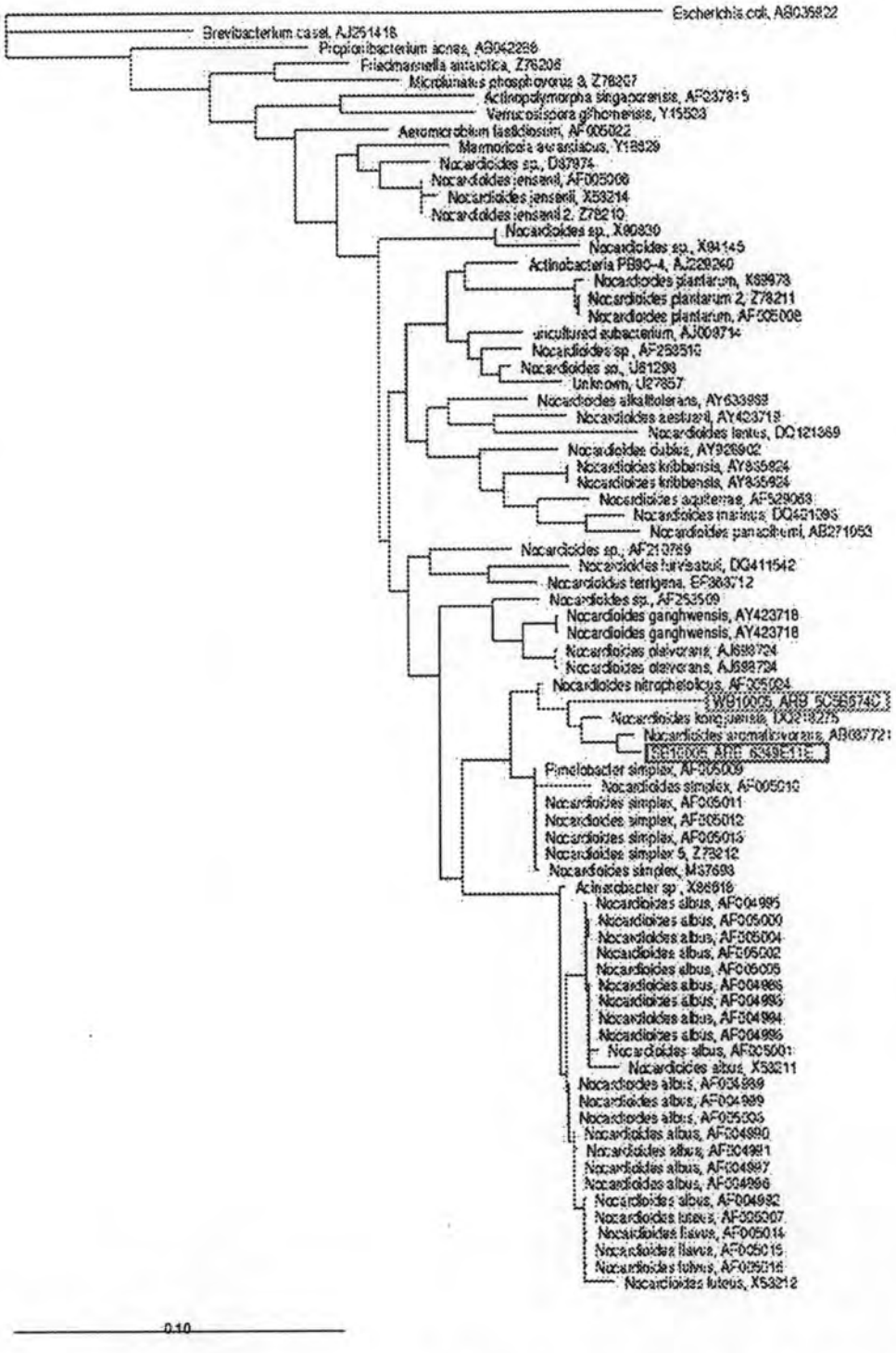


Figure 7.1 (Cont') Phylogenetic tree showing 16S rRNA genes of the strain SB100-05 and WB100-05 constructed by adding full-length sequences of the strain SB100-05 and WB100-05 into the tree prior constructed by (a) neighbor joining (distance matrix) method, (b) parsimony method and (c) maximum likelihood method using > 1400-bp sequences of reference sequences.

(c)

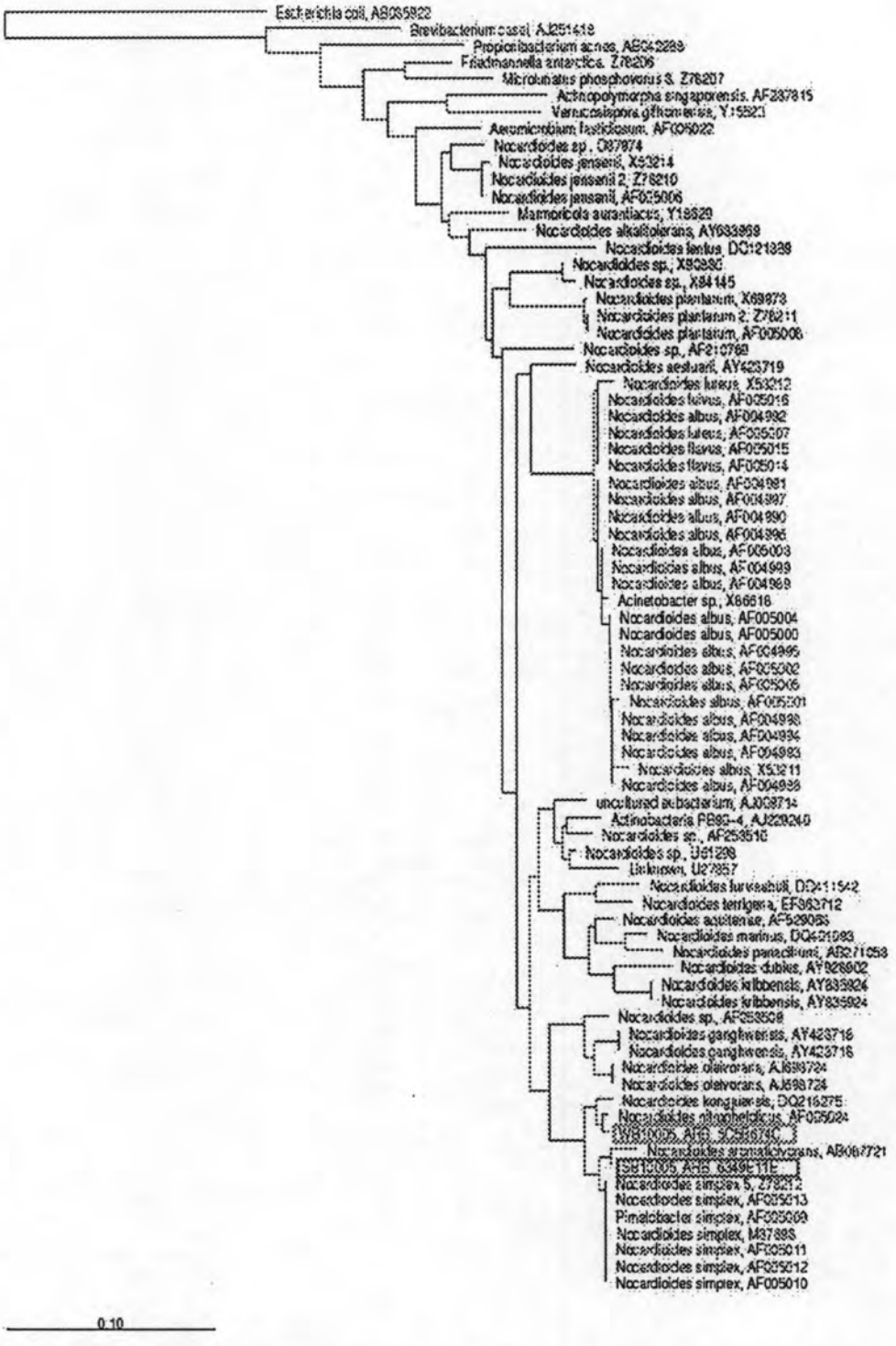


Figure 7.1 (Cont') Phylogenetic tree showing 16S rRNA genes of the strain SB100-05 and WB100-05 constructed by adding full-length sequences of the strain SB100-05 and WB100-05 into the tree prior constructed by (a) neighbor joining (distance matrix) method, (b) parsimony method and (c) maximum likelihood method using > 1400-bp sequences of reference sequences.

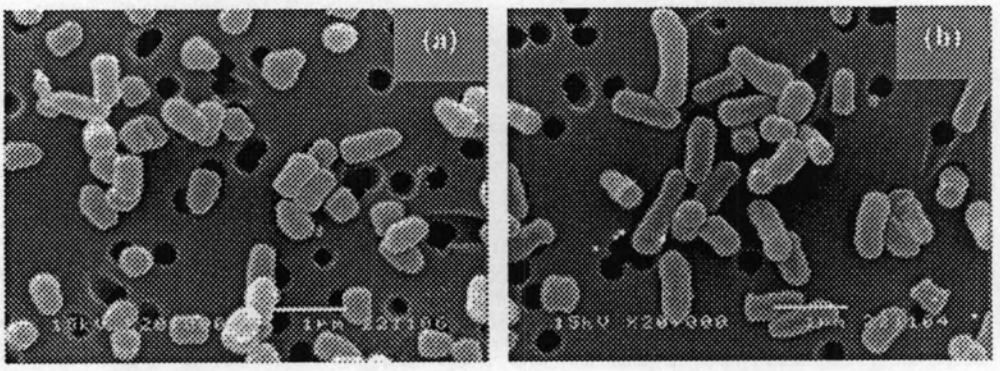


Figure 7.2 Scanning electron microscope photos of MT-degrading bacteria strains (a) SB100-05, (b) WB100-05 (20,000x)

Table 7.1: Physiochemical properties of MT-degrading bacteria strain SB100-05 and WB100-05

Characteristics	Reaction	
	SB100-05	WB100-05
Gram reaction	+ve	+ve
Nitrate reduction	-	-
Pyrrolidonyl arylmidase	-	-
Alkaline phosphatase	+	+
β – glucuronidase production	-	-
β – galactosidase production	-	-
α – galactosidase production	-	-
N-acetyl-β-glucosaminidase	-	-
Hydrolysis of esculin	+	+
Urease production	-	-
Acetoin production	-	-
Leucine aminopeptidase	+	+
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	+	+

+ = positive, - = negative

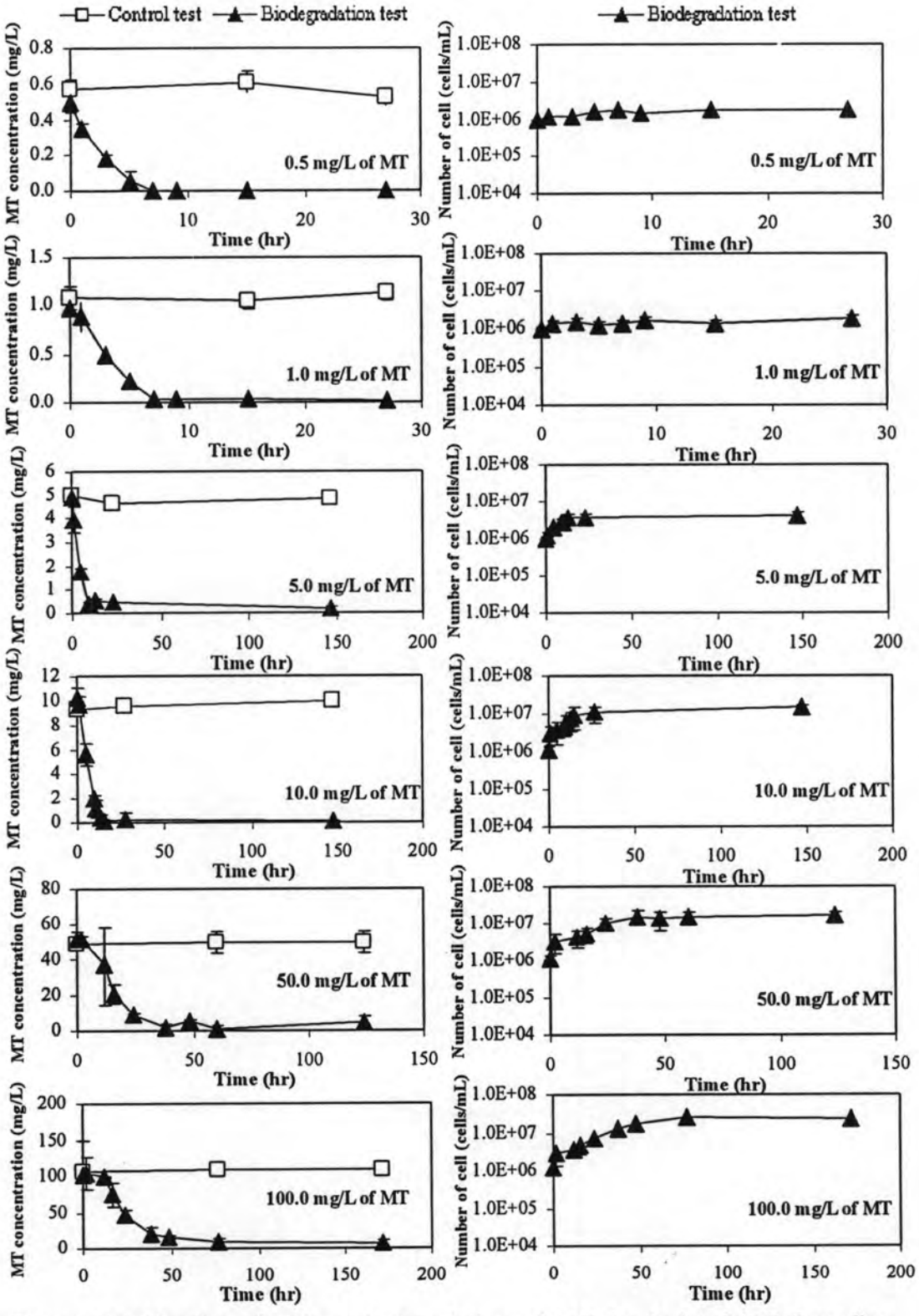


Figure 7.3 Number of cells and degradation of MT by isolated MT-degrading bacterium strain SB100-05 at different initial MT concentrations

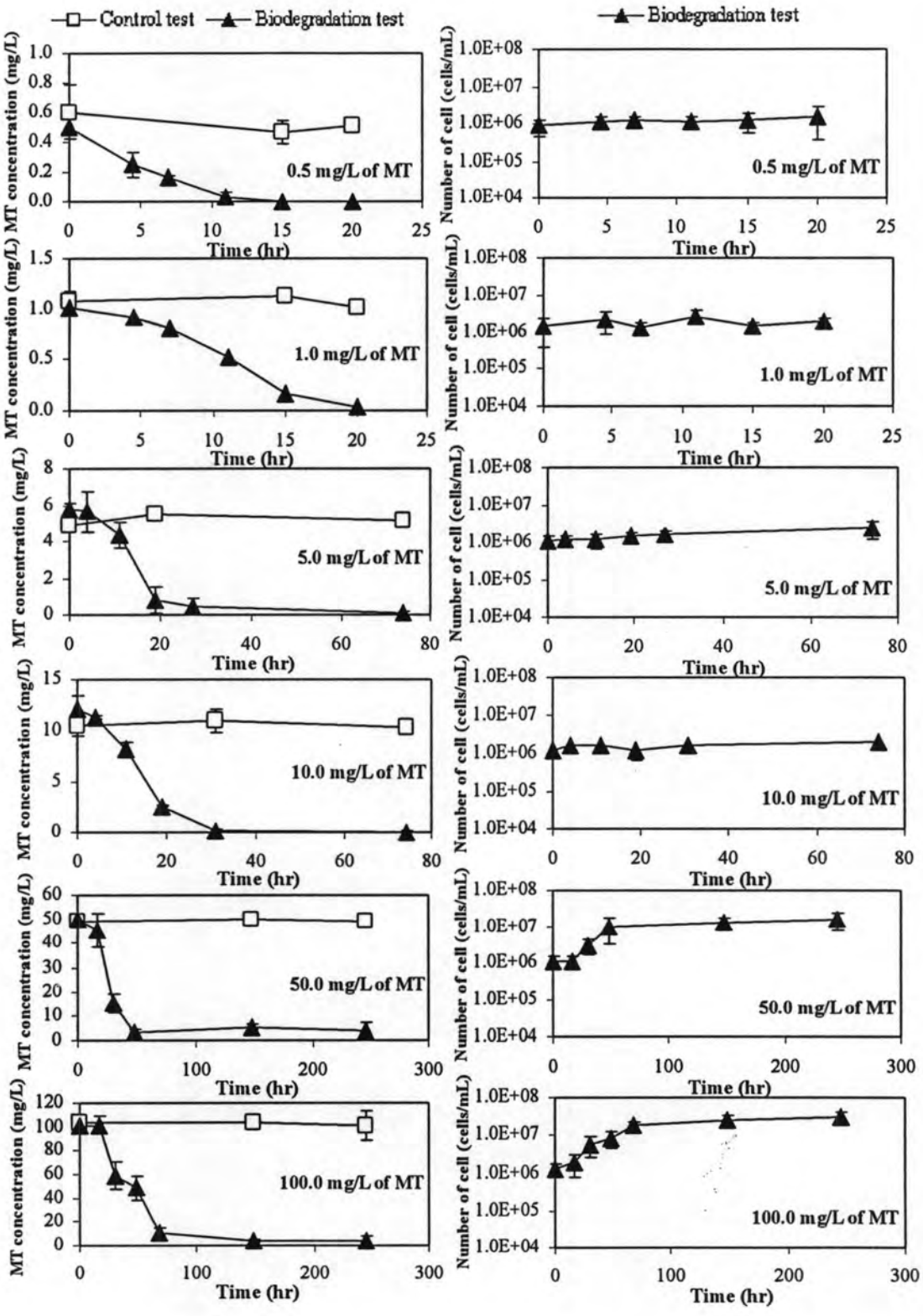
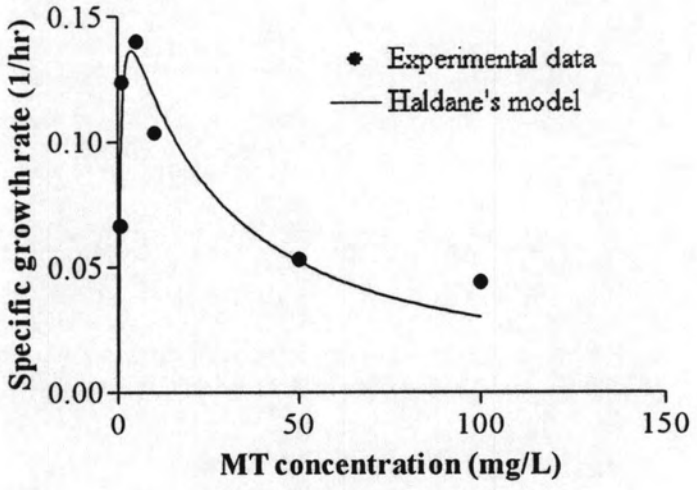
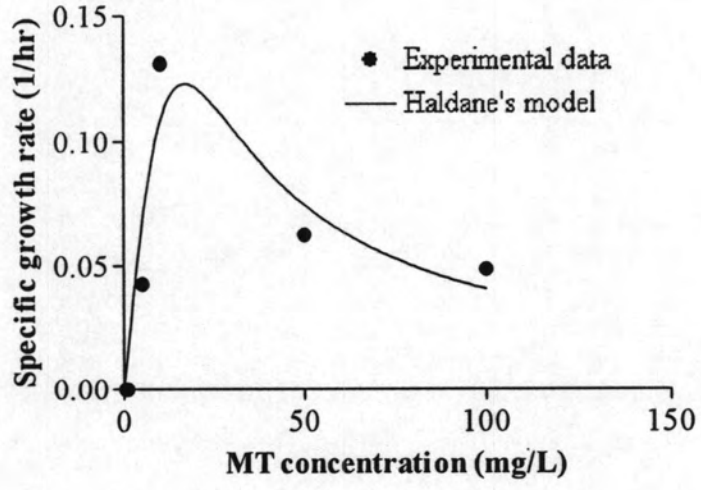


Figure 7.4 Number of cells and degradation of MT by isolated MT-degrading bacterium strain WB100-05 at different initial MT concentrations

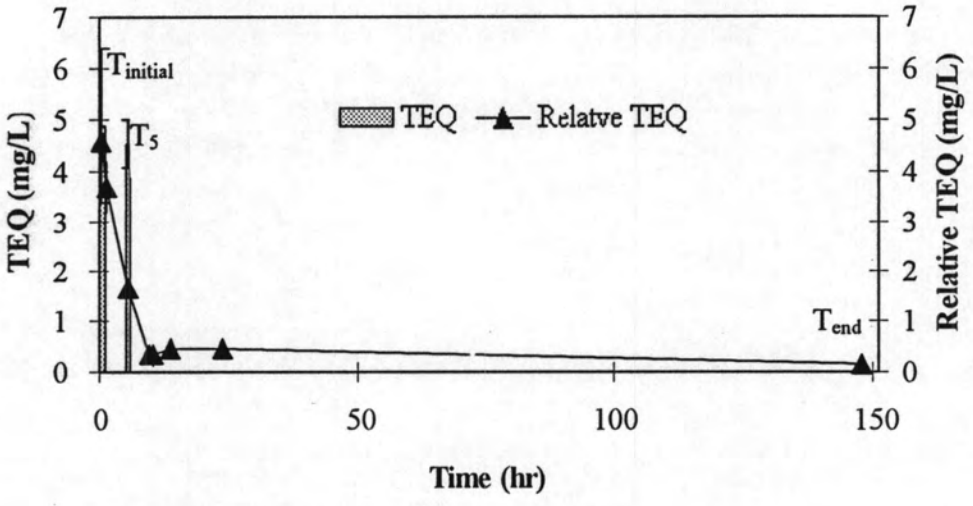


(a) Strain SB100-05

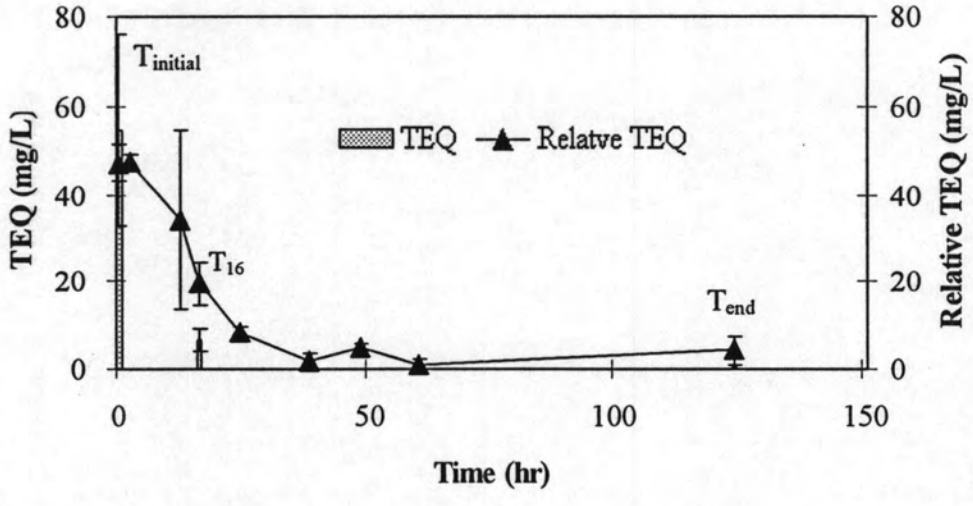


(b) Strain WB100-05

Figure 7.5 Specific growth rate as a function of initial MT concentration

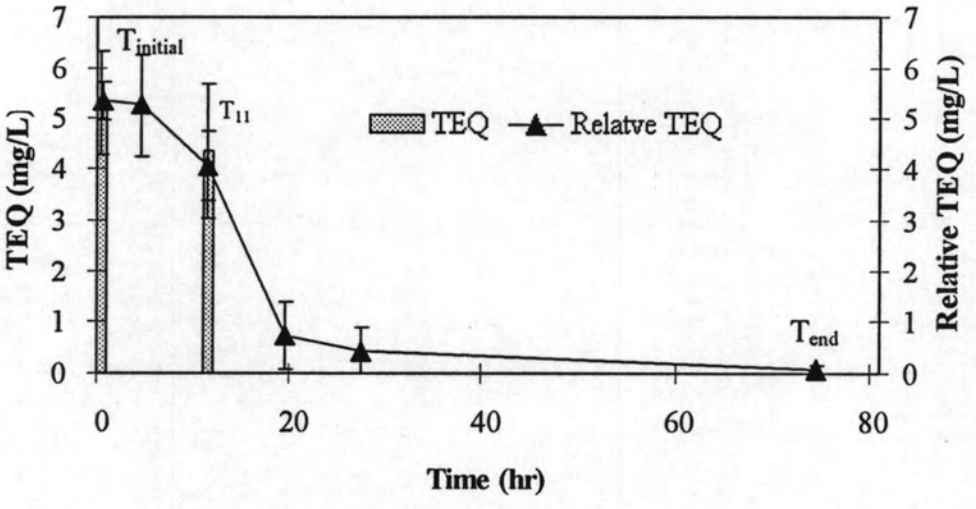


(a) Initial MT concentration of 5.0 mg/L

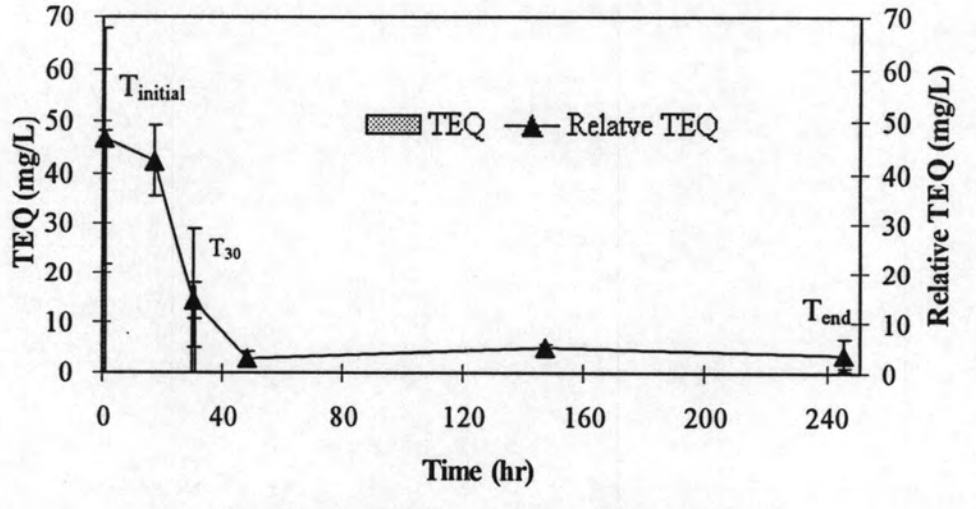


(b) Initial MT concentration of 50.0 mg/L

Figure 7.6 Biodegradation of MT by MT-degrading bacterium strain SB100-05 under different initial MT concentration at (a) 5.0 and (b) 50.0 mg/L



(a) Initial MT concentration of 5.0 mg/L



(b) Initial MT concentration of 50.0 mg/L

Figure 7.7 Biodegradation of MT by MT-degrading bacterium strain WB100-05 under different initial MT concentration at (a) 5.0 and (b)50.0 mg/L