DECIPHERING ACTIVE ESTROGEN-DEGRADING MICROORGANISMS IN BIOREACTORS

A Dissertation

by

HYUNG KEUN ROH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Civil Engineering

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Approved by:

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ABSTRACT

Deciphering Active Estrogen-Degrading Microorganisms in Bioreactors. (August 2009)

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Estrogens are a group of endocrine disrupting compounds capable of causing abnormalities in the reproductive systems of the wildlife. Wastewater is a major source of environmental estrogens, in part due to incomplete removal of estrogens in biological wastewater treatment processes. This dissertation investigated factors affecting estrogen biodegradation in bioreactors. Specifically, research efforts were placed on characterization of several bacterial estrogen degraders (model strains: *Aminobacter* strains KC6 and KC7, and a *Sphingomonas* strain KC8) and examination of the effects of operating parameters on estrogen removal and estrogen-degrading microbial community structure.

Sphingomonas strain KC8 can use 17β-estradiol as a sole carbon source, suggesting that estrogen degradation by KC8 is a growth-linked, metabolic reaction; however, estrogen degradation by strains KC6 and KC7 might be a non-growth linked, cometabolic reaction. One important finding was that strain KC8 can also degrade and further utilize testosterone as a growth substrate. Strain KC8 was characterized in terms of its utilization kinetics toward estrogens and testosterone with the results that showed

relatively smaller kinetic parameters than the typical values for heterotrophs in activated sludge. Strain KC8 can also grow on other organic constituents (glucose, succinate, and acetate). Strain KC8 retained its ability to degrade both 17β-estradiol and estrone (after 15 d of growth on a complex nutrient medium without 17β-estradiol).

Effective removals (>98.7 %) of 17β-estradiol with no significant differences were observed in sequencing batch reactors (SBRs) under three solid retention times (SRTs of 5, 10, 20 d). The population ratios of known estrogen degraders (strains KC8 and ammonia-oxidizing bacteria (AOB)) and *amo*A gene (associated with ammonia oxidation) to total bacteria decreased as SRT increased in SBRs. These observations correspond to the decreasing percentages of 17β-estradiol biodegraded in SBR when SRT increased from 5 to 20 d, when the sorption of 17β-estradiol onto biomass was considered. Real-time terminal restriction fragment length polymorphism showed that more ribotypes were observed in SBR-20d than SBR-5d. The species evenness (*E*) in microbial community structures in SBRs was not affected by SRT. However, diversity indices (Shannon-Weaver diversity index (*H*) and the reciprocal of Simpson's index (*1/D*)) suggest that longer SRTs might lead to a more diverse microbial community structure.

Dedicated

to

my beloved parents,

wife, Sunsook,

and son, Hyunchul

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I wish to express my sincere gratitude to my advisor, Dr. Kung-Hui Chu, for her academic guidance and unconditional support throughout the course of this research. This dissertation could not have been possible without her endless patience and encouragement. My special thanks are extended to Dr. Robin L. Autenrieth, Dr. Thomas K. Wood, and Dr. Kirby C. Donnelly for being my dissertation committee members.

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Finally, I thank God for the grace and blessings during my studies and in my life.

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1. INTRODUCTION

1.1 Introduction

Endocrine disrupting compounds (EDCs) in water environments have raised a great public concern in recent years. The exposure to EDCs like estrogens is known to cause reproductive and sexual disruption in aquatic wildlife (1-4). For example, feminization of male fish was observed after exposure to estrogenic compounds in treated wastewater (5-8). Baronti et al. (9) and Purdom et al. (10) reported that even a few ng/L of estrogens can provoke reproductive disruption in riverine fish. Despite the controversial viewpoints of human health effects, previous studies reported that exposure to estrogens is linked to the development of breast and testicular cancers (11, 12). The US Environmental Protection Agency (EPA) began an EDC screening program in 1996 (13, 14). Pesticides and other substances are currently screened for their potential effects on the endocrine system (13). Estrogens, produced by humans and animals or used for personal care like hormone therapy, are excreted in urine and feces that enter into sewage for wastewater treatment (15, 16). Estrogens remaining in the effluent are subsequently released into the environment via effluent discharge (17). Estrogens have been detected in the effluent of wastewater treatment plants (WWTPs), in rivers, and even in drinking water (18, 19). Estrogens can be removed via the activated sludge process in wastewater treatment plants (19-21). As such, the runoff from sewage/ biosolids applications for agriculture is another source of environmental estrogens (22).

This dissertation follows the style of *Environmental Science & Technology.*

While many studies have been focused on advanced chemical and physical processes to remove estrogens from wastewater, studies on the biodegradation of estrogens and the microorganisms responsible for their degradation are few. Layton et al. (21) reported that biosolids from municipal WWTPs mineralized 70-80 % of amended 17β-estradiol into carbon dioxide within 24 h, suggesting that biodegradation would be an important removal mechanism for estrogen during wastewater treatment. Later, several estrogen-degrading bacteria, including *Nitrosomonas europaea*, *Novosphingobium tardaugens* (ARI-1), *Rhodococcus* species, *Achromobacter* species, and *Ralstonia* species have been isolated from activated sludge (23-26). More recently, Yu et al. (27) isolated fourteen estrogen-degrading isolates (strains KC1 through KC14) from activated sludge. Among the fourteen isolates, only three strains (*Aminobacter* strains KC6 and KC7, and *Sphingomonas* strain KC8 (refered as "KC6", "KC7", and "KC8", respectively, hereafter)) could degrade both 17β-estradiol and estrone. Strain KC8 could degrade 17β-estradiol to non-estrogenic metabolites and/or end products. However, these three strains have not been fully characterized, and the potential of their application for enhanced estrogen removal in engineered bioreactors has not been addressed. In this study, a series of experiments were conducted to characterize estrogen-degrading microorganisms and to examine their potential for improving estrogen removal in bioreactors.

1.2 Hypothesis, Goal, and Objectives

The overall **hypothesis** of this research is that *enhanced estrogen degradation can be achieved by promoting the growth of estrogen degraders in engineered bioreactors.* The **goal** of this research is to *better understand factors affecting biodegradation of estrogens in engineered systems, as the first step in developing advanced biological treatment strategies for enhanced estrogen removal in wastewater treatment plants*. The hypothesis was tested through two specific objectives:

1. **Characterization of a *Sphingomonas* strain KC8** (27) focusing on (i) growth; (ii) degradation kinetics; (iii) its ability to utilize macro- and micro-pollutants that co-exist in wastewater; and (iv) investigation of other factors, such as complex nutrients, on its estrogen degradability.
2. **Investigation of factors affecting estrogen removal in bioreactors**. Particularly the effects of solids retention time (an important operating parameter for engineered bioreactors) on estrogen-degraders and their performance in estrogen removal were studied.

Strains KC6, KC7, and KC8, *Novosphingobium tardaugens* (ARI-1), and ammonia-oxidizing bacteria were used as model microorganisms in this study. These strains were chosen because of their degradation ability toward estrogens.

1.3 Dissertation Overview

There are seven sections in this dissertation. The literature review for this research is in Section 2. Materials and methods used in this study are described in Section 3. The ability of strains KC6, KC7, and KC8 to use estrogens as a growth substrate and the presence of nonspecific oxygenase enzymes are addressed in Section 4; the results of this section have been published in a peer-reviewed journal, *Environmental Science & Technology*. Section 5 describes characteristics of strain KC8, including its degradation kinetics of estrogen-testosterone and its ability to use common pollutants in wastewater. The effects of complex nutrients on estrogen degradability of strain KC8 were also examined. Section 6 describes the effects of solids retention time on estrogen degraders and their associated microbial community in lab- and full-scale bioreactors. Finally, Section 7 summarizes significant findings of this research and recommendations for future study.

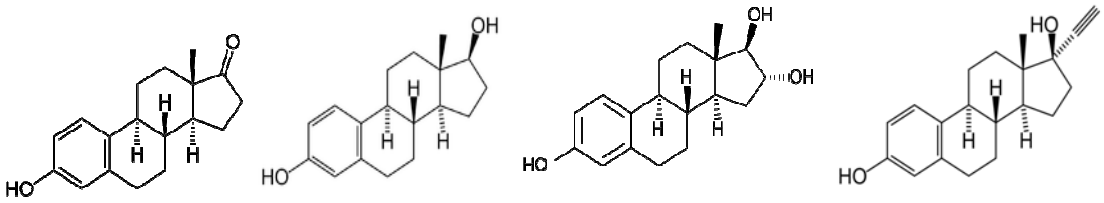
2. LITERATURE REVIEW

2.1 Sources and Properties of Estrogens and Their Roles in Endocrine System

Estrogens are considered as endocrine disrupting compounds (EDCs) that have shown adverse health impacts on aquatic wildlife (1-10). Synthetic estrogens, such as DES (diethylstilbestrol), are known to disrupt the human reproductive system (11, 28). Estrogens are reproductive hormones and can be produced naturally and synthetically (18). Natural estrogens include estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-estradiol (an isomer of E2), and synthetic estrogen includes 17α-ethinyl estradiol (EE2) commonly used for oral contraceptives.

Estradiol is produced mainly from the ovaries, the corpus luteum, and the placenta to develop follicles in premenopausal women. In postmenopausal women and men, estradiol is produced from many extragonadal sites including breast, osteoblasts and chondrocytes of bone, vascular endothelium and aortic smooth muscle cells, and brains (29). More than 50% of the estrone is produced the peripheral tissue conversion of androstenedione in ovaries (30). Estriol is derived from estrone, estradiol, and androstenedione in the body (30).

Structurally, natural estrogens contains 18-carbon steroids with a phenolic-A ring, which has a high-affinity binding to the estrogen receptors, while the structure of 17α-ethinyl estradiol is similar to natural estrogen with the ethinyl substitution at carbon 17 (Figure 2.1).



Estrone (E1, natural) | 17β-estradiol (E2, natural) | Estriol (E3, natural) | 17α-ethinyl estradiol (EE2, synthetic)

Figure 2.1. Chemical structures of estrogens (18, 31).

The solubility of estrogens in water ranges from 1.3 to 13 mg/L (Table 2.1); however, the solubilities in nitrate mineral salts medium are 2.6-3.0 mg/L for 17β-estradiol and 1.0-1.3 mg/L for estrone in our laboratory. The vapor pressure of estrogens is low (6×10^{-15}~2×10^{-10} mm Hg) and the melting temperature is high for all estrogens (Table 2.1) (32-35). Estrogens have been used as oral contraceptives, in hormone replacement therapy for human, and veterinary medicine (16). Estrogenic products are also used to enhance the growth of livestocks (18). After being metabolized in the human body, estrogens are excreted with their metabolites such as estriol, 2-methoxy-estrone, 2-hydroxy-ethinyl estradiol, and 2-methoxy-ethinyl estradiol in urine and feces (16, 36). A normal female usually releases 5-100 μg/d, and a pregnant woman can excrete estrogens up to 30 mg/d. Even men release 2-25 μg/d of estrogens (16). Similarly, estrogens are released from livestock (37). The excreted estrogens from humans may be introduced into wastewater treatment plants. After wastewater treatment processes, the effluent of wastewater treatment plants containing untreated estrogens and

the sludge treatment for agricultural use are an important contaminant source of estrogens in environment (5, 18, 19, 38).

Table 2.1 Properties of estrogens

Name	17α-estradiol	Estrone (E1)	17β-Estradiol (E2)	Estriol (E3)	17α-ethinyl estradiol (EE2)
Chemical formula	$C_{18}H_{24}O_2$ (natural)	$C_{18}H_{22}O_2$ (natural)	$C_{18}H_{24}O_2$ (natural)	$C_{18}H_{24}O_3$ (natural)	$C_{20}H_{24}O_2$ (synthetic)
Molecular weight	272.4	270.4	272.4	288.4	296.4
Melting Point (°C)[1,2]	223-226	255-262	178-179	280-282	-
Water Solubility (mg/L) [1,2,]	1.3[3]-3.9	12.4-13	13	13	4.8
$LogK_{ow}$ [1,2]	3.5[4]-4.0	3.43	3.94	2.81	4.15
Vapor pressure (mmHg) [1]	-	$2.3x10^{-10}$	$2.3x10^{-10}$	$6.7x10^{-15}$	$4.5x10^{-11}$

[1] Ingerslev and Halling-Sorenson, 2003 (32)
[2] Beck et al., 2008 (33)
[3] Rytting et al., 2005 (35)
[4] Xia et al., 2005 (34)

2.2 Current Technologies for Estrogen Removal

2.2.1 Physical Process

Powdered activated carbon (PAC) has been reported to remove estrogens successfully (> 95%) from raw drinking water (39). PAC removed endocrine disrupting compounds in the order: 17β-estradiol > 17α-ethinyl estradiol > bisphenol A (40). Longer contact time and higher dose of PAC are required for better removal efficiencies of estrogens, and natural organic matters (NOMs) may inhibit the estrogen removal by PAC (39, 40). Granular activated carbon (GAC) adsorbs 17β-estradiol from deionized water quickly (41). However, the coagulation-filtration process is not effective for estrogen removal (42).

2.2.2 Chemical Process

Estrogens have low solubility in water. However, estrogens are lipophilic and can be removed by chemical processes such as ozonation, UV radiation, advanced oxidation, and chlorination. Chemical oxidation has been shown as an effective process for removing estrogen compounds. Huber et al. (2003) reported that conventional ozonation and advanced oxidation processes could oxidize 17α-ethinyl estradiol in a drinking water treatment plant (43). Titanium dioxide (TiO_2) suspension under UV radiation can degrade natural and synthetic estrogens (44). Rosenfeldt and Linden (2004) applied ultraviolet (UV) radiation photolysis and the UV/hydrogen peroxide (H_2O_2) advanced oxidation process (AOP) to degrade endocrine disrupting compounds including 17α-ethinyl estradiol and 17β-estradiol. Both processes removed all the tested EDCs effectively and the UV/H_2O_2 AOP was better than UV photolysis (45). Synthetic

manganese oxides (MnO_2), a chemical oxidizing agent, were able to oxidize natural and synthetic estrogens in water effectively at pH 4.0 (46). Chlorination is a common disinfection process for water and treated wastewater. During the chlorination process, 17β-estradiol was rapidly degraded, and chlorinated derivatives of estrone were produced. Low or no estrogenic activities were found in the chlorinated derivatives (47-49).

2.2.3 Biological Process

Numerous studies have investigated the fate of estrogens in wastewater treatment plants (WWTPs) and observed the degradation of natural and synthetic estrogens by biological process. (9, 17, 21, 50-56). The removals of estrogens by WWTPs varied from 19 to 98% for estrone, from 76 to 98% for 17β-estradiol, and 83-90% for 17α-ethinyl estradiol, depending on the operation status, location of plants, and influent estrogen concentrations (9, 17, 21, 50-56). Several studies isolated estrogen-degrading microorganisms from activated sludge (23-26, 57, 58). *Novosphingobium tardaugens* ARI-1 was isolated from activated sludge as a 17β-estradiol degrader (24). Nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea* degraded natural (estrone, 17β-estradiol, estriol) and synthetic estrogens (17α-ethinyl estradiol) significantly. The first-order degradation rate constants are 0.056 h^{-1} for estrone, 1.3 h^{-1} for 17β-estradiol, 0.03 h^{-1} for estriol, and 0.035 h^{-1} for 17α-ethinyl estradiol (23). Shi et al. also isolated ethinyl estradiol degrading bacterium *Fusarium proliferratum* strain HNS-1 from a cowshed sample (57). Two *Rhodococcus* strains were isolated from activated sludge as estrogen degraders (25). *Rhodococcus zopfii* and *Rhodococcus equi*

degraded natural and synthetic estrogens rapidly and completely, and no estrogenic activities were observed after degradation. Weber et al. found that *Achromobacter xylosoxidans* and *Ralstonia picketii* transformed 17β-estradiol and estrone into estriol and 16α-hydroxyestrone (26). Three estrogen degraders were also isolated from a sandy aquifer (58). *Acinetobacter* strains (LHJ1 and LHJ3) and a *Sphingomonas* strain CYH degraded 17β-estradiol and estrone both in aerobic and anoxic conditions. Li et al. (59) showed the estrogen degradation by activated sludge with a first-order degradation rate constants of rate 0.2-4.8 h^{-1}. Fourteen phylogenetically diverse 17β-estradiol-degrading cultures (strains KC1 through KC14) were isolated from activated sludge in our laboratory (27). These strains showed three different degradation patterns of estrogens. Details of the degradation patterns toward estrogens were available on pages 42-45.

A microbial degradation pathway for 17β-estradiol has not been identified. On the other hand, a 17α-ethinyl estradiol degradation pathway by *Sphingobacterium* sp. JCR5 (Figure 2.2) and a testosterone degradation pathway by *C. testosteroni* TA441 (Figure 2.3) have been proposed by Haiyan et al.(60) and Horinouchi et al. (61).

As shown in Figure 2.2, the A-ring of 17α-ethinyl estradiol was cleaved by a dioxygenase (R4 in Figure 2.2), while the A ring of testosterone was cleaved by meta-cleavage enzyme (reaction 5 in Figure 2.3). Since 17β-estradiol is structurally similar to 17α-ethinyl estradiol and testosterone, the degradation pathway of 17β-estradiol might be similar to those two proposed pathways. Hence, these two proposed pathways for 17α-ethinyl estradiol and testosterone can be used as a starting point for elucidating the degradation pathway of 17β-estradiol.

In this study, the activity of nonspecific oxygenase enzymes in three estrogen-degraders (strains KC6, KC7, and KC8) was examined using nonspecific monooxygenase and dioxygenase enzyme assays. The activity of nonspecific monooxygenase enzyme was detected using a naphthalene oxidation assay (62). The activity of a nonspecific dioxygenase was detected using an indol oxidation assay (63).

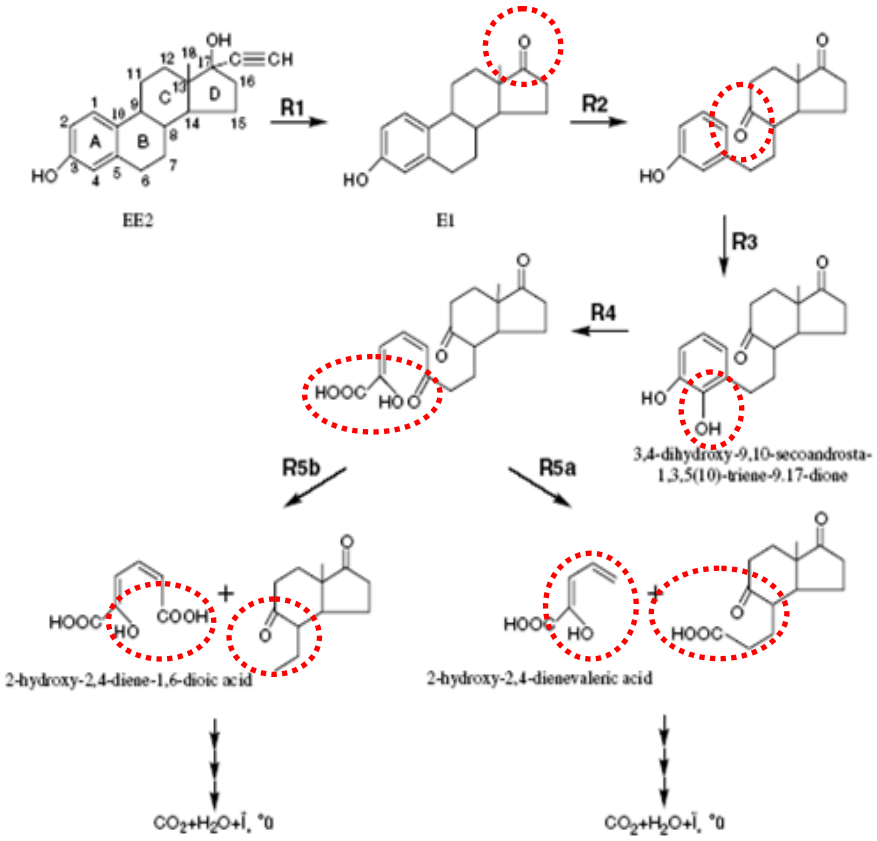


Figure 2.2 Proposed pathway of 17α-ethinyl estradiol degradation in *Sphingobacterium*. JCR5 (60). Dioxygenase enzyme might catalyze the reaction R4 for cleavage between C4 and C5.

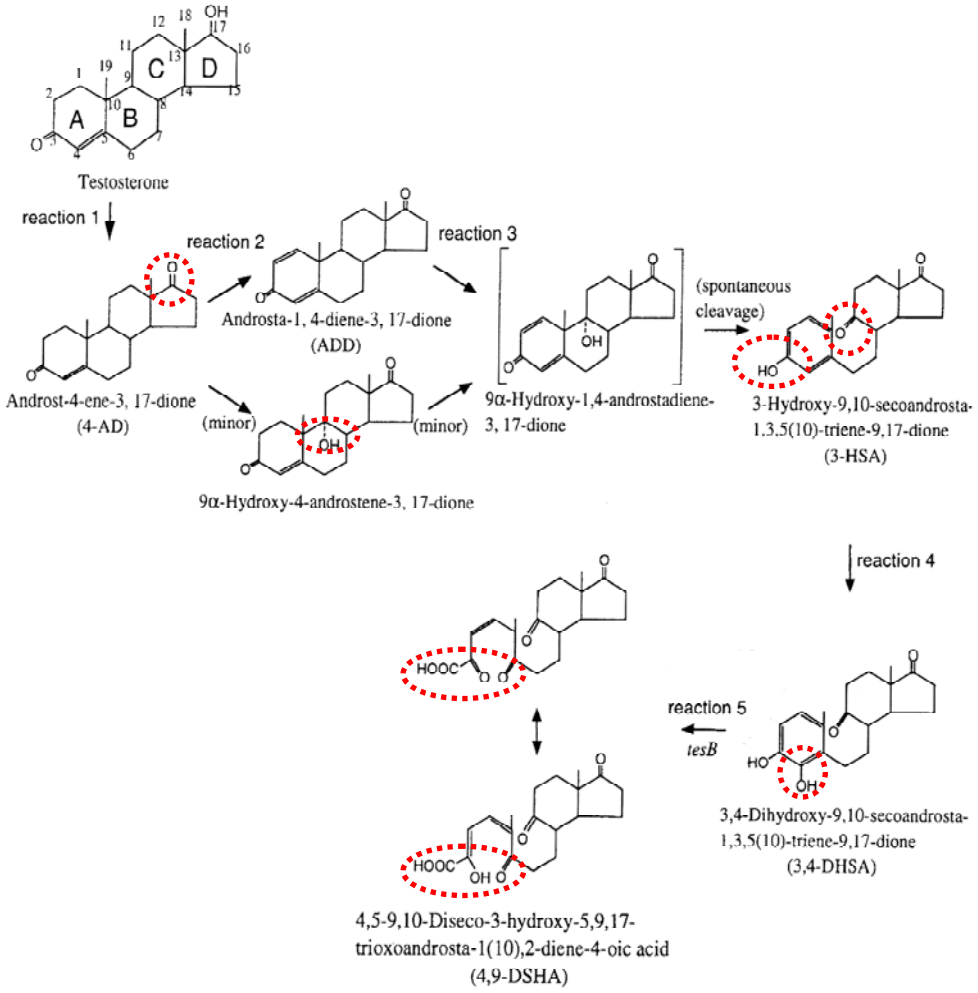


Figure 2.3 Proposed pathway of testosterone degradation by *Comamonas testosteroni* (61). *Meta*-cleavage enzyme gene, *tesB* might catalyze the conversion of 3,4-DHSA to 4,9-DHSA in reaction 5.

2.3 Growth and Degradation Kinetics

2.3.1 Yield and Doubling Time

Cellular yield (Y) was estimated by dividing the amount of cell biomass produced over the amount of substrate utilized. The Y is expressed as:

$$Y = -\frac{\Delta X}{\Delta S} = -\frac{(X_1 - X_2)}{(S_1 - S_2)} \tag{2-1}$$

in which

Y= cellular yield (biomass produced/mass of substrate utilized);
ΔX= amount of biomass produced (mass/volume);
X_1 = initial biomass concentration (mass/volume);
X_2 = final biomass concentration (mass/volume);
ΔS= amount of substrate concentration utilized (mass/volume);
S_1 = initial substrate concentration (mass/volume);
S_2 = final substrate concentration (mass/volume).

Cellular yields of aerobic heterotrophs were reported to be 0.49 gVSS(volatile suspended solids)/gBOD_L with carbohydrate BOD and 0.42 gVSS/gBOD_L with other electron donors (64).

Doubling time (τ_d) is defined as the time needed for a microbial population to double in number. From a growth curve, τ_d can be estimated from the exponential growth phase using the following equation:

$$\tau_d = \frac{Ln2}{\mu} = \frac{0.693}{\mu} \tag{2-2}$$

in which

τ_d = doubling time (time);
μ = specific growth rate (time^{-1}).

2.3.2 Monod Kinetics

The widely applied relationship to address bacterial growth kinetics is the Monod equation which shows the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (64):

$$\mu_{syn} = (\frac{1}{X_a}\frac{dX_a}{dt})_{syn} = \mu_m \frac{S}{K_m + S} \tag{2-3}$$

in which

μ_{sny} = specific growth rate due to cell syntheses (time^{-1});
X_a = active biomass concentration (mass/volume);
t = time;
S = substrate concentration (mass/volume);
μ_m = maximum specific growth rate (time^{-1});
K_m = half velocity constant or saturation constant (mass/volume). Substrate concentration corresponding to one-half of maximum specific growth rate.

Then μ and μ_m in equation (2-3) can be replaced with equation (2-4) into equation (2-5) for the nonlinear regression analysis.

$$\mu = q \cdot Y \quad \text{and} \quad \mu_m = q_m \cdot Y \tag{2-4}$$

in which

q = substrate utilization rate (time^{-1}).

q_m= maximum substrate utilization rate (time^{-1})

$$q = \frac{q_m S}{K_m + S} \tag{2-5}$$

The kinetic parameter, maximum specific growth rate (μ_m), describes the rate of cell growth and half velocity constant (K) indicates the affinity to substrates. For aerobic heterotrophs in activated sludge, typical maximum substrate utilization rate (q_m) and maximum specific growth rate (μ_m) are 20-27 $gBOD_L/gVSS\text{-}d$ and 8.4-13.2 $gVSS/gBOD_L\text{-}d$, respectively (64). Nonlinear regression using Sigmaplot 2002 (SPSS Inc.) was conducted to estimate the kinetic parameters, the maximum substrate utilization rate (q_m) and half velocity constant (K). Then the maximum specific growth rate (μ_m) was calculated by equation (2-4).

2.4 Molecular Tools Used in This Study

2.4.1 Real-Time PCR

While the traditional PCR amplifies specific DNA sequences from a DNA mixture of DNAs, the real-time PCR, a fluorescence-based kinetic PCR assay, allows rapid quantification of the initial number of copies of the target gene (65, 66). The real-time PCR assay amplifies a target gene with a fluorescence-labeled primer and a probe and quantifies a fluorescent signal which increases proportionally with the amount of PCR product. The amount of fluorescence emitted at each cycle is recorded and the PCR reaction for the first significant increase of PCR product correlating to the initial amount of target template is monitored. The significant increase of the fluorescence occurs when the starting copy number of the target gene is higher. The significant increase of fluorescence signal is compared to the standard curve and quantifies the target gene (67). Real-time PCR has been used to quantify various targets such as pathogens(68, 69), viruses(70, 71), and specific species (72-77)).

2.4.2 Real-Time-t-RFLP

Real-time terminal restriction fragment polymorphism (real-time-t-RFLP) is a quantitative fingerprinting method capable of determining the diversity and the abundance in a microbial community structure simultaneously (78). Real-time-t-RFLP assay was developed by combining two molecular technologies, real-time PCR (65, 66, 79) and t-RFLP (80-82). During the first step, the 16S rRNA genes of genomic DNA sample are amplified and quantified by a fluorescence-labeled primer and a fluorescence-labeled probe. While the fluorescent-labeled primer is incorporated into

the PCR products, the fluorescent-labeled probe emits a signal for rapid quantification after being cleaved from a complementary region of the target gene. Then the harvested labeled-PCR products are digested with a restriction enzyme, followed by analysis with an automatic sequencer to produce a series of T-RF profiles. Finally the relative abundance of each species in a microbial community is determined (Figure 2.4) (78).

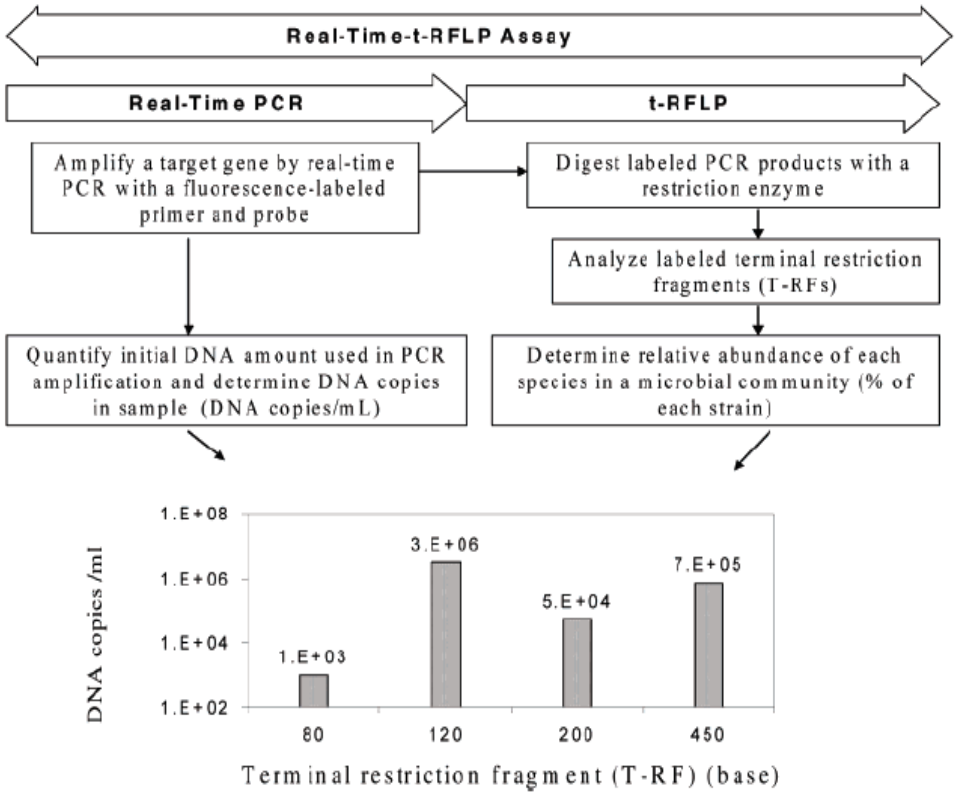


Figure 2.4 Real-time-t-RFLP assay for quantitative fingerprinting of microbial communities (78).

2.5 Estrogenic Screen Assays

Due to concern for the adverse effects of EDCs on wildlife and human health, it is necessary to screen and detect the present of EDCs and their estrogenic activities in environments. The estrogenic potential can be determined via two different types of estrogenic screen assays, *in vivo* and *in vitro* assays. The *in vivo* estrogenic assay measures the *in vivo* responses to estrogen exposure. Mouse uterotrophic assay and vitellogenin (VTG) assay are two commonly used *in vivo* assays. The *in vitro* estrogenic assays are used to evaluate estrogenic potencies by using transgenic human breast cancer cell lines (MVLN or MCF cells), or a recombinant yeast cell. Compared to the *in vitro* assays, the *in vivo* assay are more costly and time consuming. Relative estrogenic potencies measured by *in vivo* and *in vitro* assays are shown in Table 2.2.

2.5.1 *In Vivo* Estrogenic Screen Assay

Numerous studies investigated estrogenic activities by using *in vivo* assays. The classic and sensitive *in vivo* estrogenic assay is the mouse uterotrophic assay. The mouse uterotrophic assay is able to detect estrogenic activity for multiple estrogen compounds (83). Another commonly used *in vivo* estrogenic assay is the vitellogenin (VTG) assay. Vitellogenin is a specific protein and yolk protein precursor in females of fish, amphibians, reptiles (including birds), insects and the platypus. Exposure to estrogenic compounds can induce vitellogenin and vitellogenin can be detected at very low levels in males. Therefore, the vitellogenin become an effective biomarker for estrogenic activity (5, 10, 84, 85). However, it is still not clear how the vitellogenin levels are closely related to the adverse impacts on gonads and reproductive success or development of

progeny (86). Folmar et al. investigated the estrogenic activity of the effluent of wastewater treatment plants (5). The plasma vitellogenin level was elevated in male carp in the downstream of the effluent of sewage treatment plants; on the contrary, testosterone level in male carp decreased (5, 18). Segner et al. (87) and Hemmer et al. (88) investigated the induction of vitellogenin in zebra fish and sheepshead minnow with the *in vivo* VTG estrogenic screen assay.

2.5.2 *In Vitro* Estrogenic Screen Assay

In vitro assays are relatively more rapid and cost effective than *in vivo* assays. The yeast estrogen screen (YES) with a recombinant yeast strain (*Saccharomyces cerevisiae*) was developed by expressing human estrogen receptor (hER) and two estrogen response elements (ERE) linked to the *lacZ* gene (84, 87, 89, 90). Since the MCF-7 human breast cancer cells proliferate in the presence of chemicals that directly or indirectly activate the estrogen receptor, the E-screen assay was developed by comparing the cell number achieved by similar inocula of MCF-7 cells in the absence and presence of 17ß-estradiol and a range of concentrations of concern (84, 91-93). The MVLN-assay was developed by using the MVLN-cell, the human breast cancer cell (94). These assays were applied to detect estrogenic activity in sewage effluent, surface, and other water environments (19, 92, 95). Relative estrogenic potencies are shown in Table 2.2.

Table 2.2 Relative estrogenic potencies

Name	*In vitro* assay			*In vivo* assay
	YES[a]	MVLN[b]	E-screen[c]	VTG[d]
17β-Estradiol	1	1	1	1
Estrone	0.4 [(1)]	0.01 [(2)]-0.2 [(1)]	0.01 [(4)]	0.5 [(5)]-0.8 [(1)]
Estriol	-	0.083 [(2)]	-	0.001 [(6)]
17α-ethinyl estradiol	0.7 [(4)]-0.96 [(3)]	1.25 [(2)]-1.6 [(1)]	1.25 [(2)]-1.9 [(4)]	30.6 [(1)]

[(1)] Van den Belt et al. (86)
[(2)] Gutendorf and Westendorf (92)
[(3)] Segner et al. (87)
[(4)] Folmar et al. (84)
[(5)] Routledge et al. (38)
[(6)] Metcalfe et al. (96)

a YES: Yeast estrogen screen assay

b MVLN: Assay using MVLN, the human breast cancer cell lines

c E-screen: Assay using MCF-7, the human breast cancer cell lines

d VTG: Vitellogenin assay

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Three estrogens (17β-estradiol (> 99% pure), 17α-estradiol (> 98% pure), and estrone (> 98% pure)) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Testosterone was purchased from Pfaltz and Bauer, Inc. (Waterbury, CT). Due to low solubility of estrogens and testosterone, stock solutions were prepared in acetone and stored at 4 °C. Bicinchoninic acid (BCA) protein assay reagent kit, pyridine, dimethylformamide (DMF) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Sodium formate (ACS reagent grade) and glycerol (ultra pure) were purchased from MP Biomedicals, Inc. (Solon, OH). Tetrazotized *o*-dianisidine was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Naphthalene (99.6%) was obtained from Alfa Aesar (Ward Hill, MA). NADH was produced from Research Organics (Cleveland, OH). Dithiothreitol (DTT) was produced from Promega Corp. (Milwaukee. WI). Ferrous sulfate, triclosan (97%) and indol (>99%), phenol were purchased from Mallinckrodt Baker, Inc. (Philipsburg, NJ) and Sigma-Aldrich Inc. (St. Louis, MO), respectively. Naphthalene (99.6%) and sodium succinate were purchased from Alfar Aesar (Ward Hill, MA). Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) and naphthol were obtained from TCI America (Portland, OR). Glucose was obtained from EM Science (Gibbstown, NJ). GeneScan 500 Rox size standard was purchased from Applied Biosystems (Warrington, United Kingdom). QuantiTect probe PCR supermix and SYBR Green I (as a double

stranded DNA binding fluorescent dye) were purchased from Qiagen (Valencia, CA). For synthetic wastewater, sodium acetate, ammonium chloride, ammonium sulfate, sodium bicarbonate, magnesium sulfate, potassium biphthalate, sodium hydroxide, sodium nitrite, sodium nitrate, zinc sulfate, and cobalt chloride were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Calcium chloride, ferrous sulfate, manganese chloride, potassium phosphate, sodium carbonate, sodium phosphate, and EDTA were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Copper sulfate and ammonium molybdate were obtained from VWR International (West Chester, PA).

3.1.2 Bacterial Strains and Growth Conditions

Three estrogen-degrading bacterial strains, KC6, KC7, and KC8 (all gram negatives, Genbank accession numbers DQ066436-DQ0664368, respectively) were isolated from activated sludge of a wastewater treatment plant in Knoxville, Tennessee (27). *Novosphingobium tardaugens* ARI-1 (gram negative, Genbank accession number AB070237) was purchased from the American Type Culture Collection (ATCC #BAA-531). *Nitrosomonas europaea* was a gift from Dr. Michael Hyman at the Department of Microbiology in North Carolina State University. Strains KC6, KC7, and KC8, and ARI-1 were grown in tryptic soy broth (TSB) containing 3mg/L of 17β-estradiol at 30 °C at 150rpm for 2 d before harvesting for experimental use. The growth medium for *Nitrosomonas europaea* contains 3.3g $(NH_4)_2SO_4$, 0.41g KH_2PO_4, 0.75mL of 1M $MgSO_4$, 0.2mL of 1M $CaCl_2$, 0.33mL of 30mM $FeSO_4$/50mM EDTA, 0.01mL of 50mM $CuSO_4$, 5.444g KH_2PO_4, 0.48g NaH_2PO_4, 0.04% Na_2CO_3 in 1.0 L. NaOH(10N) was used to adjust a pH 8.0 (97). The cells were grown in dark room at 30 °C at 150rpm

for 3-5 d before harvesting for experimental use. The nitrate mineral salts (NMS) used in this study contains 11.76 mM $NaNO_3$, 0.98 mM K_2SO_4, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.07 mM $CaSO_4 \cdot 2H_2O$, 0.08 mM $FeSO_4 \cdot 7H_2O$, 3.9 mM KH_2PO_4, 6.1 mM Na_2HPO_4, 0.1 mM H_2SO_4, 0.001 mM KI, 0.002 mM $ZnSO_4 \cdot 7H_2O$, 0.002 mM $MnSO_4 \cdot H_2O$, 0.002 mM H_3BO_3, 0.004 mM $CoMoO_4 \cdot H_2O$ (98). The acetone-free nitrate mineral salts (NMS) medium containing estrogens or other targets (testosterone, bisphenol A, or triclosan) was prepared as follows: Following the additions of the known amounts of target stock solutions in acetone, acetone was evaporated by purging with nitrogen gas for 5-10 minutes. Then NMS medium was added in the reactor, and the targets were resuspended at 150rpm at room temperature for 2-3 d.

3.1.3 Sequencing Batch Reactors

Three laboratory-scale sequencing batch reactors (SBR) were constructed with Erlenmeyer flasks (2.0 L). Water-vapor-saturated air was continuously bubbled into the bioreactors to maintain dissolved oxygen concentrations in the range between 4 and 6 mg/L. The water-vapor-saturated air was generated by passing ambient air though a flask containing DI water. The SBR reactors were inoculated with activated sludge in synthetic wastewater (1.5 L), resulting in an initial MLVSS concentration of 1,320 mg/L. The activated sludge was collected from a local municipal WWTP in College Station, TX. The synthetic wastewater contained acetate (measured as total organic carbon (TOC) =175 mg/L), ammonia (as total nitrogen =25 mg/L) and inorganic nutrients. The synthetic wastewater contained 512 mg $NaCH_3COO$, 166 mg $NaHCO_3$, 107 mg NH_4Cl, 75.5 mg $NaH_2PO_4 \cdot 2H_2O$, 90 mg $MgSO_4 \cdot 7H_2O$, 36 mg KCl, 14 mg $CaCl_2 \cdot 2H_2O$, 18 mg

EDTA, 1 mg yeast extract, 1.5 mg $FeSO_4 \cdot 7H_2O$, 0.0015 mg H_3BO_3, 0.48 mg $CuSO_4 \cdot 5H_2O$, 0.003 mg KI, 1.5 mg $MnCl_2 \cdot 4H_2O$, 0.33 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.66 mg $ZnSO_4 \cdot 7H_2O$, and 0.015 mg $CoCl_2 \cdot 6H_2O$ in 1.0L (99). A cell suspension containing strain KC8 was spiked into the SBR to result in an initial concentration of $2.2x10^8$ 16rRNA gene copies of strain KC8/mL. All reactors were operated at a hydraulic retention time of 36 h and a cycle of 24 h. The 24-h cycle consisted of a 22-h fill-mix-reaction phase and a 2-h settle-and-draw phase. In the beginning of each cycle (i.e., every 24 h), 17β-estradiol was added into the SBRs to bring the initial concentration of 1mg/L. The three SBRs (referred as SBR-5d, SBR-10d, and SBR-20d hereafter) were operated under three different SRTs of 5, 10, and 20 d. Each solids retention time of 5, 10, and 20 d was controlled by removing 300, 150, and 75 mL of MLSS including the sample volume from the reactor at each cycle. Also, hydraulic retention time (36 h) was controlled by removing 700, 850, and 925 mL of supernatant after settle phase from the reactor operating under 5, 10, 20 d of SRT, respectively. Liquid samples were collected from the bioreactors after mixing phase and after settling phase of each cycle. Collected samples were analyzed for estrogen, MLVSS, TOC, total nitrogen (TN), and estrogen-degrading cultures. Synthetic wastewater (1.0 L) was added to keep the reactor volume (1.5 L) at the fill phase into each reactor with 17β-estradiol.

3.1.4 Sampling from Wastewater Treatment Plants

Three WWTPs in College Station, Austin, and Houston, TX, were chosen for this study because of their different configurations in biological treatment processes and operating conditions. These WWTPs are designed for BOD removal and nitrification to

treat 10 to 200 million gallons of wastewater daily. The SRTs of the three WWTPs ranged from 7 to 30 d. Other information of each WWTP is summarized in Table 3.1. Activated sludge samples were collected from the aeration tanks (MLVSS = 1.1~2.2 g/L) of the WWTPs. The genomic DNA of the activated sludge samples were extracted and used for molecular quantification of 16S rRNA genes of strains ARI-1, KC8, AOB, and *amo*A genes.

Table 3.1. Operation parameters of WWTPs for activated sludge sampling

WWTPs		WWTP #1	WWTP #2	WWTP #3	
				1st stage	2nd stage
Operating parameters	Capacity (MGD)	9.5	75	200	
	SRT (d)	7-8	10-12	2	20-30
	MLVSS (g/L)	2.15	1.52	1.35	1.10
	BOD_5 removal (%)	97-98	97-99	91-99	
	Ammonia removal (%)	95	99	84-99	

3.2 Experimental Methods

3.2.1 Estrogen Degradation Tests

Experiments were designed to examine whether strains KC6, KC7, and KC8 were capable of degrading 17β-estradiol and estrone. 17β-Estradiol and estrone were used as two model compounds. The degradation tests were conducted in a series of 300-mL vials containing 100 mL of resting estrogen-degrading cells, and an initial concentration of 3 mg/L of 17β-estradiol or estrone. Strains KC6, KC7, and KC8 were pre-grown overnight in tryptic soy broth (TSB) medium containing 3 mg/L of 17β-estradiol before harvesting by centrifugation for experimental use. The harvested cells were washed with 10 mM phosphate buffered saline (pH 7.0, containing 10 mM NaCl and 2.5 mM KCl) before re-suspending in the acetone-free, NMS-estrogen growth medium to an optical density (OD_{600}) of 0.5. The vials were incubated on a rotary shaker at 150 rpm at 30ºC. Liquid samples collected over time were analyzed for 17β-estradiol and estrone concentrations by GC/MS analysis. All degradation tests were performed in duplicate. Autoclave-killed controls were used.

3.2.2 Cell Growth Tests

Strains KC6, KC7, and KC8 capable of degrading both17β-estradiol and estrone were further examined for their abilities to use 17β-estradiol as a sole carbon source. Sufficient 17β-estradiol in acetone-free NMS growth medium during the tests was prepared as described below. A known amount of stock solution (containing 20 mg of 17β-estradiol) was first added into a 300-mL flask followed by gentle purging with nitrogen to evaporate the acetone in the flask. After complete evaporation of acetone,

100 ml NMS medium was added into the flask to dissolve estrogen. The flask was shaken at 150 rpm for 2 d before inoculated with isolates. The flasks were incubated at 30 °C at 150 rpm for 25 d. Negative controls containing only NMS medium and cells (no 17β-estradiol) were used. Samples were collected over time for 17β-estradiol analysis and protein content measurements. The protein content was determined using a BCA protein assay kit.

3.2.3 Ability to Utilize and/or Degrade Macro- and Micro-pollutants in Wastewater

Experiments were conducted to examine whether strain KC8 can utilize and/or degrade common organic constituents (both macro- and micro-pollutants) in wastewater. Four different organics (phenol, glucose, sodium succinate, and sodium acetate) and three micropollutants (testosterone, bisphenol A and triclosan) were used. The substrate utilization tests were conducted similarly in flasks as described by Yu et al. (27). The flasks (300 mL) were inoculated with strain KC8 (an initial optical density OD_{600} of 0.02) and one of the organic substrates: 20 mg phenol (to avoid toxicity (100)), 300 mg glucose, 300 mg sodium succinate, 175 mg sodium acetate in 100 mL, or 20 mg testosterone in nitrate minimum salts (NMS) medium (150 mL). The flasks were incubated at room temperature at 180 rpm on a shaker. Negative controls containing autoclave-killed cells in NMS medium were used. Cell growth was monitored over time. The biomass concentrations were expressed as turbidity at A_{600} (OD_{600}) or as protein content. The absorbance of liquid samples at A_{600} was measured using a Hewlett Packard G1130A UV-visible spectrophotometer. The protein content was determined using a BCA protein assay kit.

The substrate degradation tests for micro-pollutants (triclosan, bisphenol A, and testosterone) were conducted in 300 or 500 mL Erlenmeyer flasks containing NMS medium with resting cells of strain KC8 (with an optical density (OD_{600}) of 0.5), 0.5 mg/L of triclosan or bisphenol A, 1.5 mg/L of testosterone. The resting cell suspension was prepared as described in degradation kinetic tests. Acetone-free NMS medium was used. The flasks were incubated at room temperature at 180 rpm on the shaker for 7 d. Liquid samples were collected over time for triclosan, bisphenol A, and testosterone analysis. Autoclave-killed cells were used for controls. Concentrations of triclosan, bisphenol A, and testosterone were determined by GC/MS analyses.

3.2.4 Estrogen-Testosterone Utilization Kinetics

Substrate utilization kinetic tests were conducted in a series of EPA vials (40 mL) containing 10 mL of resting-cell suspension with OD_{600} of 0.5 and estrogens or testosterone. Initial concentrations ranged from 0.05 to 10 mg/L for 17β-estradiol, from 0.05 to 6 mg/L for estrone, and from 0.05 to 10 mg/L for testosterone. After adding cell suspension, the vial was vigorously shaken by hands for 30 sec before the first sample was taken. After 2 h of incubation at room temperature at 180 rpm on the shaker, the vials were sacrificed for estrogen and protein analyses. The incubation duration (2 h) was determined in the laboratory where initial degradation rates remained linear.

3.2.5 Effects of Complex Nutrients on Estrogen Degradation

Experiments were conducted to determine whether strain KC8 could retain its degradation ability toward estrogens after growing on a complex nutrient medium without exposure to 17β-estradiol for a period of time. The complex-nutrient-grown

cells were prepared as follows. Strain KC8 was grown and sub-cultured on 17β-estradiol-free-R2A agar plates every 3 d. After five transfers (i.e., 15 d), the cells were then grown in tryptic soy broth (TSB) overnight before harvested for 17β-estradiol degradation tests.

3.2.6 Development of Real-time-PCR Assay for Estrogen Degraders

Real-time *TagMan* PCR assays were developed for quantification of two known estrogen degraders, strains KC8 (256 bp long) and ARI-1 (206 bp long). The primer sets and *TaqMan* probes were designed to target the 16S rRNA gene sequences of KC8 and ARI-1. For strain KC8, the primers (KC8f (5'-ACCAGAGATGGTTTCCTTCAGT -3') and KC8r(5'-GCTTGCTG- CCCACTGTAGTT-3')) and probe (KC8 *Taq* (5'-[DFAM]ATAGGTGACAGGTGCTGCATGGCTGT[DHH1]-3')) were designed based on the alignments of 8 closely related bacterial 16S rRNA gene sequences (>92% similar) (AY771798, AY771797, AY771794, X94101, AJ009706, AJ292601, DQ177493 and AY509242) with KC8 16S rRNA gene sequence (DQ066438). Similarly, primers (ARI-1f (5'-GCTGTCCGGGCTCATGGAGT-3') and ARI-1r (5'-CCCGAAGGGAACACCCAATCT-3')) and *Taq*Man probe (ARI-1 *Taq*(5'-[6-FAM]ACCAGC- GTTTGACATCCCGCGCTAA[TAMRA-6-FAM]-3')) were designed to target the 16S rRNA gene sequence (AB070237) of ARI-1 strain. Twenty-eight closely related bacterial 16S rRNA gene sequences (EU127294, DQ985055, AB219359, AY690709, AB177883, AJ416411, AJ303009, AB025012-4, U20756, U20773-4, AJ001051, DQ840049, EF628247, EF421434, EF044233, AB023290, AJ009707,

AJ000920, AJ746092, AJ746094, AB110635, AF411072, AB362778, AB220123, AB220125) were used for the design.

The specificity of designed primers and probes were checked using the Basic Local Alignment Search Tool (BLAST) of GenBank. Based on the available sequences in GenBank (accessed on 9/29/2005), primers (KC8f and KC8r) and probe (KC8 *Taq*) had at least three total mismatches after comparing with more than 100 bacterial 16S rRNA gene sequences from BLAST as well as 8 closely related bacterial 16S rRNA gene sequences as mentioned above. Therefore, the designed primers and probe can discriminate 16S rRNA gene sequences of strain KC8 from closely related strains. Similarly, the primers (ARI-1f and ARI-1r) and probe (ARI-1 *Tag*) had also at least 3 of total mismatches after comparing with bacterial 16S rRNA gene sequences, based on the available sequences deposited in GenBank (accessed on 3/13/2008).

3.3 Analytical Methods

3.3.1 Estrogens and Testosterone Analysis

Estrogen concentrations in liquid samples (containing cells and growth medium) were determined by GC/MS analysis as described by Yu et al. (78). Briefly, the estrogens in liquid samples were extracted with ethyl ether, resuspended with DMF, and then derivatized with BSTFA. The derivatized samples were injected into an Agilent 6890 Network Gas Chromatograph equipped with a DB-5MS capillary column (J&W Scientific; 30 m x 0.25 mm i.d.; 0.25 μm film thickness) and an Agilent 5973 Network Mass Selective Detector. The analysis was performed in the SIM (selective ion

monitoring) mode. The primary ions selected for quantification were *m/z* 416 for 17β–estradiol and *m/z* 342 for estrone. The GC column temperature was initially set at 240 °C for 2 min, then ramped at 10 °C /min to 300 °C, and held constant for 3 min. Helium was the carrier gas and a flow rate of 1 mL/min was used. The detection limits for 17β-estradiol and estrone were 5 μg/L.

Similarly, testosterone concentrations in samples were measured by GC-MS analysis. Liquid samples containing testosterone were extracted with the same volume of ethyl ether. Then testosterone was resuspended with pyridine (250μL), and then derivatized with BSTFA (250μL) with 1% TMCS (trimethylchlorosilane). The derivatized samples were injected into the same GC/MS system using SIM mode. The column and the configurations of column temperature were same as used for estrogen measurement. The primary ion, *m/z* 432, was selected for testosterone quantification. The detection limit for testosterone was 50 μg/L. Concentrations of bisphenol A and triclosan were determined as described by Zhao (101) and Subramanya (102). The extraction and derivatization methods, and GC-MS equipments used for bisphenol A or triclosan were same as used for estrogen measurement. The injector temperature was 250 °C. The initial oven temperature was set at 80 °C, then increased to 280 °C with a rate of 30 °C/min and held for 3 min, then increased to 300 °C and held for 3 min. The SIM mode was used with the selected ions of 357 *m/z* and 372 *m/z* for bisphenol A and 200 *m/z* and 360 *m/z* for triclosan. The detection limits for triclosan and bisphenol A were 50 and 10 μg/L, respectively.

3.3.2 Protein Assay, Mixed Liquor Volatile Suspended Solids, and Optical Density

The cellular proteins in the samples were released by using a sonicator (Branson Sonifier-150) at a power of 50 watts for 10 sec (103). The treatment was repeated for three times before cooling the samples on ice for 45 sec. The released protein content was then determined using a BCA protein assay kit by following the manufacturer's instructions with a detection limit of 5 μg/mL. Mixed liquor volatile suspended solids (MLVSS) concentrations in liquid samples were determined by dividing the difference of weights after drying at 105 °C for 2-4 h and after burning at 550 °C for 25-40 min. The cell biomass in samples as optical density was determined by measurement of absorbance at 600 nm using a Hewlett Packard G1130A UV-visible spectrophotometer with a sensitivity of 0.0001 level.

3.3.3 Total Organic Carbon and Total Nitrogen

Collected liquid samples were filtrated with Whatman 0.2 μm membrane filters (Piscataway, NJ) before TOC, ammonia, nitrate, and nitrite analyses. TOC was determined using a Shimadzu Total Organic Carbon Analyzer TOC-VCSH (Shimadzu Corporation, Japan). Ammonia concentrations were measured by using an Accumet Ammonia Combination Ion Selective Electrode as followed by manufacturer's manual and ammonia-selective electrode method as described in Standard Methods (104). Concentrations of nitrite and nitrate were measured using a DX-80 Ion Chromatograph (IC) (Dionex Corporation, Sunnyvale, CA) equipped with an IonPac AS14A-5μm Analytical Column (3 x 150mm) for anion separation. Detection limits for TOC, ammonia, nitrite, and nitrate were 0.5, 0.1, 0.05, and 0.05 mg/L, respectively.

3.3.4 Activity Assay for Nonspecific Monooxygenases

Experiments were conducted similarly as described in degradation tests, except using estrogens and cell optical density (OD_{600}) of 0.2. Samples were collected every 15 min and divided into two subsets, one for estrogen analysis (5 mL) and the other one for the naphthalene oxidation assay (1 mL). Concentrations of estrogens were determined by GC/MS analysis. Naphthalene oxidation assays were conducted as described by Chu and Alvarez-Cohen (62). Since naphthol was produced from naphthalene oxidation, the naphthalene oxidation rates were measured as naphthol production rates. The produced naphthol quickly interacted with tetrazotized *o*-dianisidine solution (freshly made) to form purple naphthol-diazo complex. The quantity of the purple complex was determined by absorbance (at 530 nm) using a Hewlett Packard G1130A UV-visible spectrophotometer. Standard curves were constructed by using 1-naphthol over a range of concentrations. The curves were then used to determine the concentrations of naphthol in samples. *N. europaea*, an ammonia-oxidizing bacterium, was used as positive controls.

3.3.5 Activity Assay for Nonspecific Dioxygenases

The activities of nonspecific dioxygenases in strains KC6, KC7, and KC8 were determined by using an indole oxidation assay that was originally developed for determining the activity of toluene dioxygenases (63). Toluene dioxygenase can oxidize indole into dark red indoxyl. The production of indoxyl was monitored using a Hewlett Packard G1130A UV-visible spectrophotometer at 400 nm. Reaction mixtures excluding indole were used for negative controls. An *E.coli*. strain expressing

naphthalene dioxygenase (*E. coli.* TG1/pBS (Kan) NDO) (105) was used as a positive control for the indole oxidation assay.

3.3.6 DNA Extraction

FastDNA SPIN kit and FastDNA SPIN kit for soil (MP Biomedicals, LLC, Solon, OH) were used to extract genomic DNA of each bacterium (pure) and activated sludge samples (mixed) collected from SBRs and WWTPs, respectively. DNA extractions were conducted in accordance with the manufacturer's instructions with minor modifications; after ethanol wash procedures, the DNA complex (silica bound) was washed twice with ethanol (80% vol/vol) (78, 106, 107). DNA concentrations were determined using a Hoefer DyNa Quant 300 Fluorometer (Pharmacia Biotech, San Francisco, CA). Extracted DNAs were stored at -20 °C to use for molecular works such as real-time PCR and real-time-t-RFLP assays.

3.3.7 Real-time-PCR Assays for Estrogen Degraders, Ammonia-Oxidizing Bacteria, and *amoA* Gene

Real-time PCR assays were conducted as follows. The PCR mixture (25 µL of total volume) contained 600 nM forward and reverse primers (Table 3.2), 500 nM of probe, 12.5 µL of *Taq* Mastermix (or SYBR Green I for *amoA* gene), and 2-50 ng of DNA template. The PCR amplification protocol was listed in Table 3.2. The PCR amplification reactions were performed using a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The DNA concentrations in samples were determined by comparing to those of standard curves containing known DNA concentrations. Standard curves for quantifying total 16S rRNA gene copies in

samples were constructed by using plasmid #931, which carries a *Nitrospira* partial 16S rRNA gene (GenBank accession number AF420301). Standard curves (ranging from 2.3 x 10^1 to 2.3 x 10^8 copies of the 16S rRNA gene/mL) were generated parallel to the sample analysis. The standard curves for quantifying KC8 and ARI-1 gene copies in samples were constructed by using plasmid #931, which carries a strain KC8 partial 16S rRNA gene (GenBank accession number DQ066438) or the 16S rRNA gene of ARI-1 (GenBank accession number AB070237) (24, 27). Standard curves, ranging from 5.0 x $10^2$to 5.0 x 10^8 copies for the KC8 and 1.0x$10^1$to 1.0x10^9 gene copies/mL for ARI-1 partial 16S rRNA, were generated**.** Quantification of AOB was conducted using primers (CTO189fA/B and CTO189Fc) and a probe (TMP1) that were previously developed (72, 108). Primers (*amoA* 1f and *amoA* 1r) and SYBR Green I were used for quantification of *amoA* genes (73, 74). Standard curves for quantifying AOB gene and *amoA* gene copies were constructed by using plasmid #931, which carries a *Nitrosomonas europaea* 16S rRNA (GenBank accession number AB070938). The plasmid #931 was cloned into pCR2.1. Standard curves, ranging from 1.0x10^1 to 1.0x10^9 gene copies of plasmid #931 and *amoA* /mL, were generated.

Table 3.2. Primers and probes and PCR protocols used in real-time PCR assay

Target	Primer/ probe[1]	Sequence(5'-3')[2]	T_m (°C)[3]	Reference
Bacterial 16S rRNA	1055f	5'-ATGGCTGTCGTCAGCT-3'	57.7	(72)
	1392r	5'-ACGGGCGGTGTGTAC-3'	58.9	
	16S*Taq*1115f	5'-[6-FAM]-CAACGAGCGC-AACCC-[6-TAMRA]-3'	62.9	
	PCR protocol	95 °C for 15min; 45 cycles of 95 °C for 30s, 50 °C for 1min, and 72 °C for 2min		
Strain KC8	KC8 f	5'-ACCAGAGATGGTTTCCTTCAGT-3'	62.7	This study
	KC8 r	5'-GCTTGCTGCCCACTGTAGTT-3'	64.3	
	KC8*Taq*	5'-[DFAM]ATAGGTGACAGGTGCTGC-ATGGCTGT[DHH1]-3'	74.0	
	PCR protocol	95 °C for 15min, 40 cycles of 95 °C for 15s, 60 °C for 30s, 72 °C for 2min		
ARI-1	ARI-1 f	5'-GCTGTCCGGGCTCATGGAGT-3'	66.6	This study
	ARI-1 r	5'-CCCGAAGGGAACACCCAATCT-3'	64.5	
	ARI-1*Taq*	5'-[6-FAM]ACCAGCGTTTGACATCCCGCGCTAA-[TAMRA-6-FAM]-3'	67.9	
	PCR protocol	95 °C for 15min, 40 cycles of 95 °C for 15s, 63 °C for 30s, 72 °C for 2min		
AOB	CTO189fA/B	5'-GGAGRAAAGCAGGGGATCG-3'	63.4	(72, 108, 109)
	CTO189fC	5'-GGAGGAAAGTAGGGGATCG-3'	62.3	
	RT1r	5'-CGTCCTCTCAGACCARCTACTG-3'	65.5	
	TMP1	5'-[6-FAM]-CAACTAGCTAATCAGRCATCRGCC-GCTC[TAMRA]-3'	69.0	
	PCR protocol	95 °C for 10min, 40 cycles of 95 °C for 30s, 58 °C for 1min, 72 °C for 1min		
AmoA	*amoA* 1f	5'-GGGGTTTCTACTGGTGGT-3'	59.9	(73, 74, 76, 109)
	amoA 1r	5'-CCCCTCKGSAAAGCCTTCTTC-3'	65.5	
	PCR protocol	94 °C for 5min, 50 cycles of 94 °C for 30s, 60 °C for 1min, 72 °C for 30s		

1) f = forward primer, r = reverse primer, *Taq* = *Taq*Man probe
2) 6-FAM = 6-carboxyfluorescein, 6-TAMRA = 6-carboxytetramethylrhodamine, 6-DFAM = 6-carboxyfluorescein, DHH1 = [ ], R=A or G; K=G or T; S=G or C
3) T_m (°C), melting temperature was calculated by the oligo calculator of Sigma Genosys

3.3.8 Real-time-t-RFLP Assay for Characterizing Microbial Community Structure

Real-time terminal restriction fragment length polymorphism (real-time-t-RFLP), a quantitative fingerprinting method, was used for determining the microbial diversity and abundance in samples of SBRs. A region (352 bp long) of the 16S rRNA gene sequence was used for the real-time-t-RFLP assay (72, 78, 107). A fluorescence-labeled forward primer 16SHex1055f (5'-hexachlorofluorescein-ATGGCTGTCGTCAGCT-3'), reverse primer 16S1392r (5'-ACGGGCGGTGTGTAC-3'), and TaqMan probe 16STaq-1115f (5'-[6-carboxyfluorescein]-CAACGAGCGCAACCC-[6-carboxytetramethylrhod-amine]-3') were used to amplify the target region as follows. The PCR mixture (25 μl) contained 10 x PCR buffer A (2.5 μl), *Taq* polymerase (1.25 U, Fisher Scientific, Fair Lawn, NJ), bovine serum albumin (BSA, 0.5 μg), each of four deoxynucleoside triphosphates (DTP, 200 μM), $MgCl_2$ (4.5 mM), forward and reverse primer (600 nM), TaqMan probe (250 nM), and DNA (10 ng). The amplification reactions were performed using a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 2 min, and a final incubation at 4 °C. The same standard as used in 3.4.2 was used. The DNA bands (352 bp) of the PCR products were excised from the 1.5 % agarose gel in 1 x Tris-acetate-EDTA buffer, and the DNA bands of the expected size. The PCR products were purified by using MicroSpin columns (Amersham Biosciences, Piscataway, NJ), precipitated by ethanol, and then digested with restriction enzyme *MspI* at 37 °C for 16 h following 20 min of incubation at 70 °C. The digestion

mixture (30µl) contained the purified PCR product (60-90 ng), and *MspI* (10 U).

The lengths of T-RFs of digested PCR products were determined by using Genescan ROX 500 size standards with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, Calif.) at DNA Technologies Laboratory in Texas A&M University (College Station, TX). The lengths of T-RFs were automatically determined by comparison with the internal standards by using the GeneScan software, version 3.1. Data acquisition and analysis were done as described by Yu et al. (78, 107).

3.4 Data Analysis

3.4.1 Quantitative Characterization of Microbial Community Structure Using Results Obtained from Real-time-t-RFLP

The abundance and microbial diversity of samples in SBRs operating under three different SRTs were determined simultaneously by using real-time-t-RFLP profiles (78, 107). The 16S rRNA gene copy # of the *i*th T-RF per unit volume (*Ci*) in a microbial community was calculated using the following equation:

$$C_i = C_0 \times \frac{({}^{j}A_i)}{\sum_{i=1}^{n} ({}^{j}A_i)} \qquad (3\text{-}1)$$

where, C_i = 16S rRNA gene copy #

C_o = initial 16S rRNA gene copy #/ml in the sample

${}^{j}A_i$ = peak area of the *i*th T-RF of *j* length (bases)

n = total number of T-RFs

The relative abundance of the *i*th T-RF in a microbial community was estimated by the

ratio of each peak area to the total peak area, $\frac{({}^{j}A_i)}{\sum_{i=1}^{n}({}^{j}A_i)}$. T-RFs less than 25 bp long were excluded from the data analyses. The peak area that contributed less than 1% of the total area was regarded as background noise and was excluded from the analysis (27).

3.4.2 Microbial Community Structure: Diversity Indices

After quantitative analysis of real-time-t-RFLP profiles as described in 3.4.1, microbial structural diversities were analyzed with respect to following indices: (i) richness (*S*), (ii) the Shannon-Weaver diversity index (*H*), (iii) evenness (*E*), and (iv) the reciprocal of Simon's index (*1/D*) (99, 110, 111). The richness (*S*) of the microbial community is determined based on the number of unique T-RFs in a community profile. Shannon-Weaver diversity index (*H*) was calculated using the equation, $H = -\sum (p_i)(\log_2 p_i)$, where p_i is the relative abundance of fragment *i*. Evenness (*E*) was estimated by the equation, $E = H/(\log_2[S])$. The reciprocal of Simon's index (*1/D*) was determined by the equation, $1/D = 1/(\Sigma p_i^2)$.

4. DEGRADATION OF ESTROGENS BY ESTROGEN-DEGRADING ISOLATES*

4.1 Introduction

Natural (17β-estradiol and estrone) and synthetic (17α-ethinyl estradiol, a major ingredient in contraceptives) estrogens in treated wastewater are considered to contribute the most to the estrogenic activity (19, 20) since estrogens have three orders of magnitude higher estrogenic potencies than other identified EDCs in wastewater. Estrogens produced naturally by humans and animals or used for personal care are excreted in urine and feces as inactive polar conjugates. The conjugates can be converted back to unconjugated estrogens (active forms) by bacterial enzymes in the raw wastewater and during the wastewater treatment processes. Estrogens survived from the treatment processes are subsequently released into the environment through effluent. Thus, treated wastewater is considered one of the most likely estrogenic sources released into the environment. Numerous laboratory and field studies have focused on the fate of estrogens in wastewater treatment plants in the past decades (see review (9, 21, 50-53, 55, 112)). The estrogen concentrations in the effluent of WWTPs ranges 0.1-1 ng/L (22, 113).

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* Reprinted with permission from "17β-estradiol-degrading bacteria isolated from activated sludge" by Chang-Ping Yu, Hyungkeun Roh, and Kung-Hui Chu, 2007. *Environmental Science & Technology*, 41, 486-492, Copyright [2007] by American Chemical Society.

Removal of estrogens by WWTPs was observed to different degrees − ranging from 19-94 % for estrone, 76-92 % for 17β-estradiol, and 83-87 % for 17α-ethinyl estradiol (9).

While estrogens were suggested to be mainly removed via biodegradation during wastewater treatment, little is known about the microorganisms responsible for estrogen degradation. Early studies reported that several human intestinal bacteria and oral microorganisms are capable of converting estradiol to estrone and *vice versa* (114-116). Shi et al. (23) reported that biodegradation of estrogens by nitrified activated sludge and *Nitrosomonas europaea* and suggested that other heterotrophic bacteria might be involved in 17β-estradiol degradation. Recently, Fujii et al. (24) isolated the first 17β-estradiol-degrading bacterium, *Novosphingobium* species (ARI-1), from activated sludge. Lately, six estrogen-degrading isolates (four *Rhodococcus* strains, an *Achromobacter* strain, a *Ralstonia* strain) from activated sludge were reported by Yoshimoto et al. (25) and Weber et al.(26).

Based on the hypothesis that estrogen-degrading cultures are widespread in activated sludge, fourteen phylogenetically diverse 17β-estradiol-degrading cultures (strains KC1 through KC14) were isolated from activated sludge in this laboratory (27). Results of 17β-estradiol degradation tests indicated that all fourteen isolates could convert 17β-estradiol to estrone within 7 d. However, only three strains (strains KC6, KC7, and KC8) were capable of degrading estrone. Based on the extent of estrogen being transformed and the estrogenicity of metabolites and/or end-products of estrogen degradation, three different degradation patterns (Patterns A, B and C) were observed.

Isolates in the group of Pattern A were capable of degrading 17β-estradiol but not estrone within 7 d. One-to-one stoichiometric conversion of 17β-estradiol to estrone was observed and the sum of molar concentrations of 17β-estradiol and estrone remained unchanged for 7 d. Complete transformation of 17β-estradiol to estrone occurred rapidly within 1 d (Figure 4.1) or slowly within 7 d. However, as the concentrations of 17β-estradiol decreased, the concentrations of estrone increased and accumulated over the tested period, suggesting that estrone was a metabolite during 17β-estradiol transformation. Strains KC1 through KC5 and KC14 exhibited degradation Pattern A.

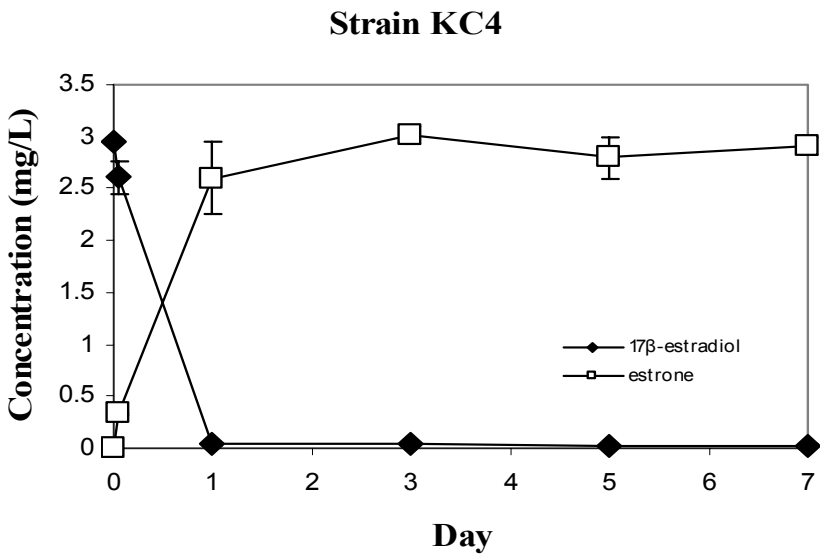
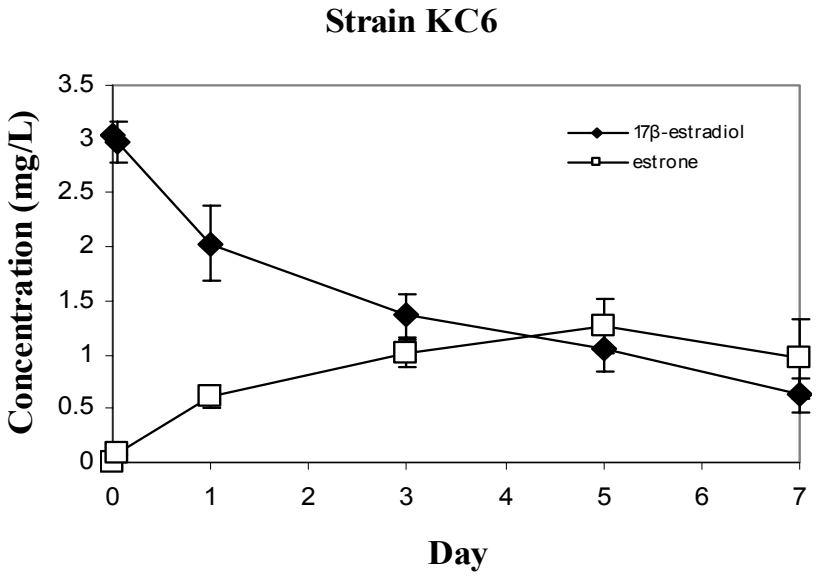


Figure 4.1 Estrogen degradation Pattern A. 17β-Estradiol was completely converted to estrone that was later accumulated in liquid medium. Solid diamonds (17β-estradiol) and open squares (estrone). The bars indicate the ranges of duplicate experiments (27).

Isolates in the group of Pattern B exhibited the ability to transform estrone within 7 d. In Pattern B, the transformation of 17β-estradiol to estrone occurred much slower compared to those observed in Pattern A. After the initial accumulation of estrone during 17β-estradiol degradation, the concentrations of estrone began to decline after 5 d. Strains KC6 and KC7 showed this degradation trend (Figure 4.2). Their abilities to degrade estrone were consistent with results observed in the 7-d estrone degradation tests. Approximately 21 ± 5% and 27 ± 4% of estrone were degraded by strains KC6 and KC 7, respectively. Degradation of 17β-estradiol and estrone were much improved in Pattern C. 17β-estradiol was rapidly transformed into non-estrogenic compounds within 7 d. Only strain KC8 showed this degradation pattern (Figure 4.3). Interestingly, an increase of estrone concentration was observed in the first 20 min of 17β-estradiol degradation. Within 24 h, 17β-estradiol was no longer detected, and estrone was measured at an average concentration of 48μg/L. No estrogenic activity was observed in day-5 and day-7 samples as determined by YES assays, suggesting the absence of estrogenic metabolites and/or end products from 17β-estradiol degradation.

The overall results indicated that strain KC8 could rapidly degrade 17β-estradiol and estrone into non-estrogenic compounds. Three isolates (strains KC6, KC7, and KC8) that showed an ability to degrade estrone were further characterized in this section.

(a)



(b)

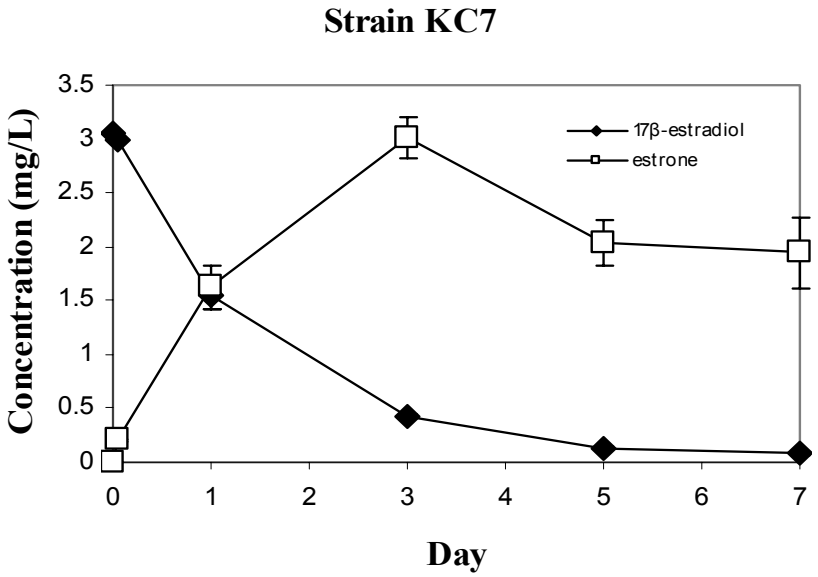


Figure 4.2. Estrogen degradation Pattern B. 17β-Estradiol and estrone were degraded at slower rates, leading reduction of total estrogenic activity and estrogen concentrations. Solid diamonds (17β-estradiol) and open squares (estrone). The bars indicate the ranges of duplicate experiments (27).

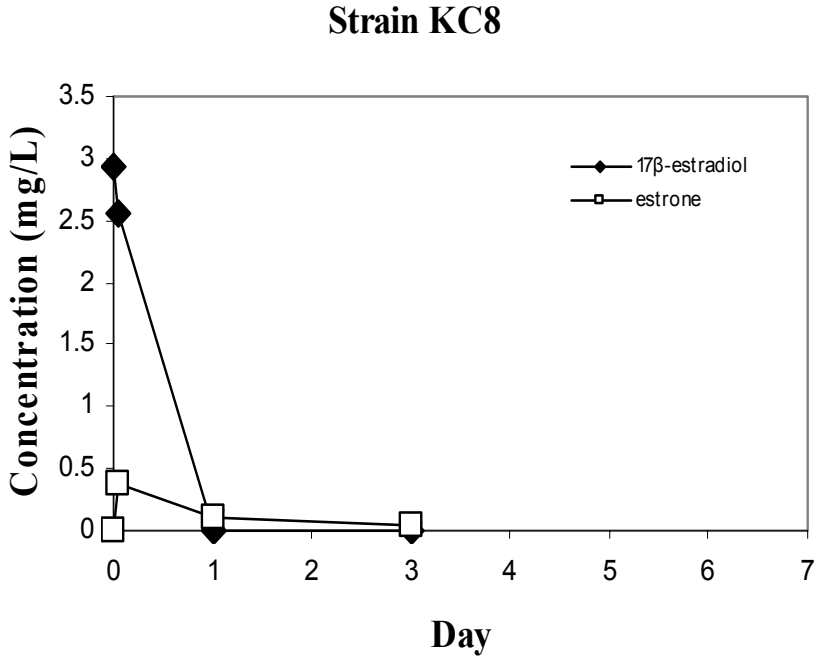


Figure 4.3 Estrogen degradation Pattern C. 17β-Estradiol and estrone were degraded rapidly into none estrogenic compounds in 7 d. Solid diamonds (17β-estradiol), and open squares (estrone). The bars indicate the ranges of duplicate experiments (27).

4.2 Results and Discussion

4.2.1 Estrogen Degradation

While the ability to transform 17β-estradiol to estrone was observed in all fourteen isolates, the ability to degrade estrone was observed only in three strains (strains KC6, KC7, and KC8) (27). The experiments for estrogen degradation by strains KC6 and KC7 were conducted with a different initial biomass (OD_{600}=0.5). The trends

of 17β-estradiol degradation by strains KC6, KC7, and KC8 (data not shown) were very similar to Pattern B and C, respectively, as described in Yu et al. (27). Along with the production and/or accumulation of estrone during 17β-estradiol degradation, three points could be made. First, the ability to oxidize the secondary alcohol on the C17 position of 17β-estradiol to ketone might be a common feature among these isolates. Second, estrone was a major metabolite during 17β-estradiol biodegradation. Third, the step to degrade estrone might be the rate-limiting step for converting estrogens (17β-estradiol and estrone) to non-estrogenic metabolites or end products. These suggestions were supported by many previous studies (9, 52, 112, 117). A good removal of 17β-estradiol but a less satisfactory removal of estrone by wastewater treatment plants was reported (9, 52, 112, 117). In some cases, much higher concentrations of estrone were observed in effluents than in influents of wastewater treatment plants (9, 117). Laboratory batch tests using ^{14}C-labeled estrogens revealed a better removal for 17β-estradiol than for estrone (21).

4.2.2 Ability to Use 17β-estradiol as a Sole Carbon Source

After 25 d of incubation, the amended 17β-estradiol was depleted in flasks inoculated with strain KC8 (Figure 4.4). Protein contents in the flasks increased from 6 mg/L to 63 mg/L. The average growth yield was estimated to be 0.23 mg of protein/mg of 17β-estradiol. The doubling time of strain KC 8 using 17β-estradiol was estimated to be 27 h. However, no significant degradation of 17β-estradiol or increase in protein content was observed for strain KC6 (Figure 4.5) or KC7 (Figure 4.6) over 36 d,

suggesting that strains KC6 and KC7 were not able to use 17β-estradiol as a sole carbon source for growth.

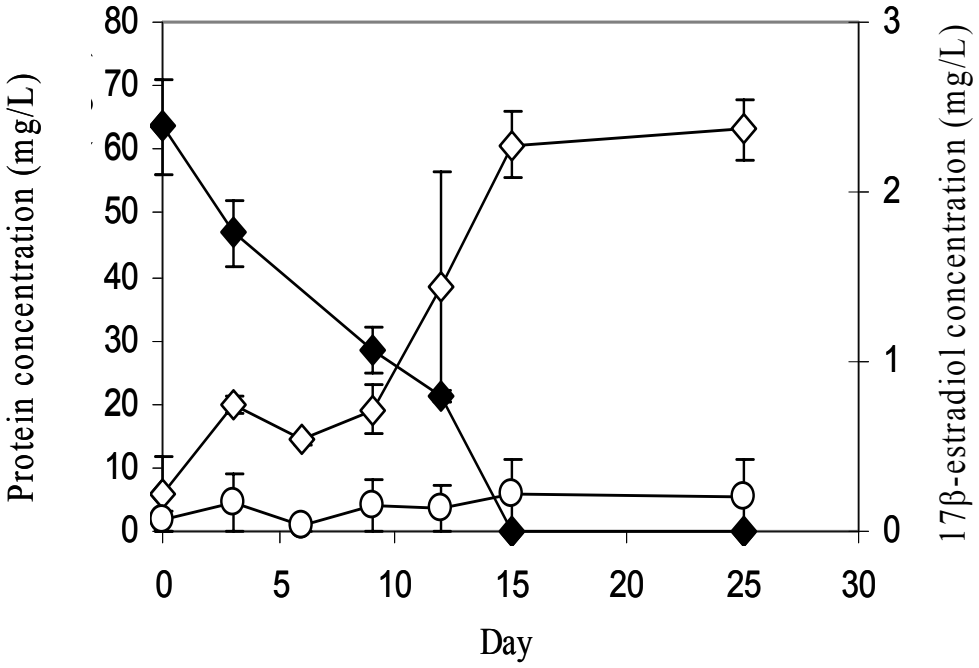
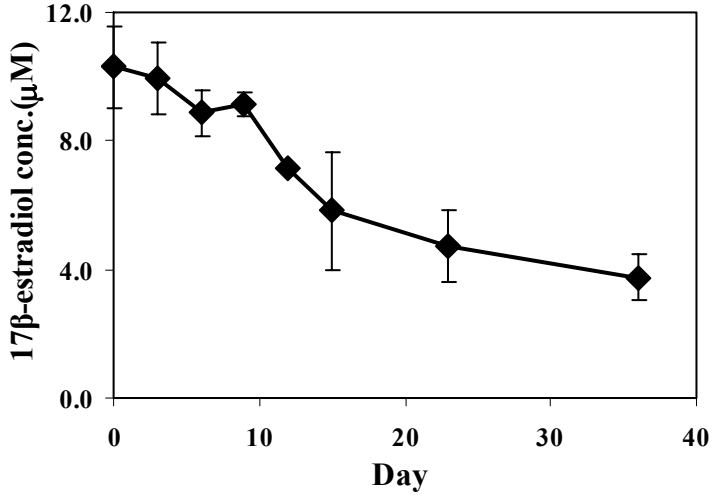


Figure 4.4 Degradation of 17β-estradiol and growth of strain KC8 in NMS-estrogen medium. The growth was monitored by measuring the protein concentration of the cultures. Solid diamonds (17β-estradiol), open diamonds (protein content, with 17β-estradiol), and open circles (protein content, without 17β-estradiol (growth controls)). The bars indicate the ranges of duplicate experiments (27).

(a)



(b)

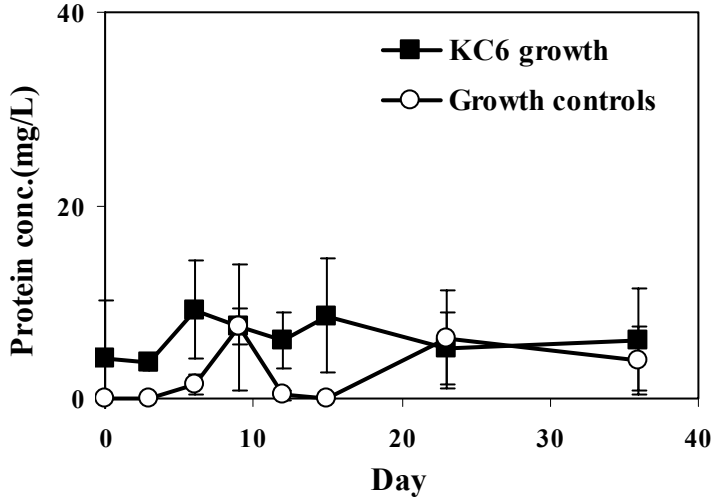
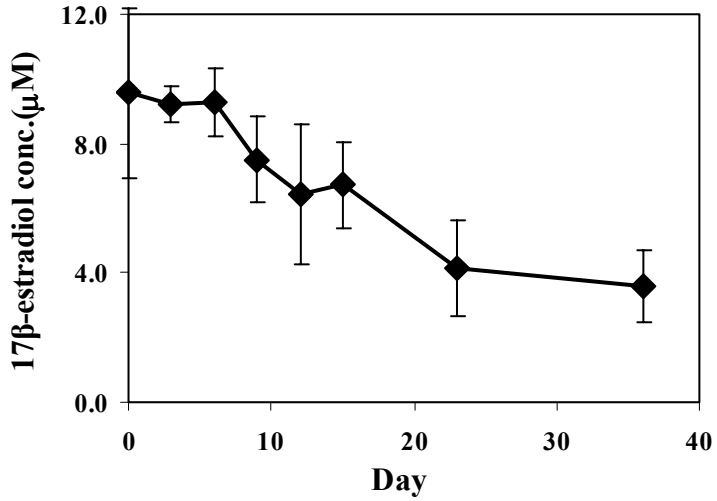


Figure 4.5 Degradation of 17β-estradiol (a) and growth of strain KC6 (b) in NMS-estrogen medium. The growth was monitored by measuring the protein concentration of the cultures. Solid diamonds (17β-estradiol), solid squares (protein content, with 17β-estradiol), and open circles (protein content, without 17β-estradiol (growth controls)). The bars indicate the ranges of duplicate experiments.

(a)



(b)

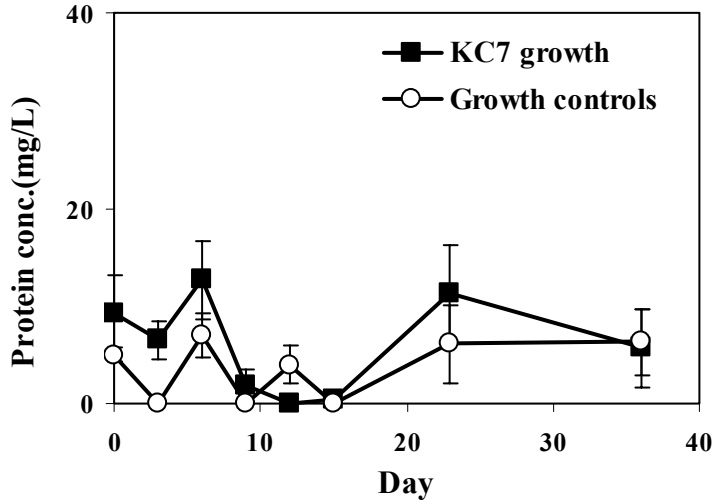


Figure 4.6 Degradation of 17β-estradiol (a) and growth of strain KC7 (b) in NMS-estrogen medium. The growth was monitored by measuring the protein concentration of the cultures. Solid diamonds (17β-estradiol), solid squares (protein content, with 17β-estradiol), and open circles (protein content, without 17β-estradiol (growth controls)). The bars indicate the ranges of duplicate experiments.

The different degradation abilities of these isolates might be explained by two possible degradation mechanisms, non-growth linked (cometabolic) and/or growth-linked (metabolic) reactions. Cometabolic reactions ─ non-beneficial reactions catalyzed by existing enzymes that are designed specific for other purposes ─ might be responsible for estrogen degradation resulting in degradation Pattern A. This hypothesis was supported by several lines of evidence observed in this study. Strains showing the ability to convert 17β-estradiol to estrone in a rapid and stoichiometric matter (Pattern A1) strongly suggested that degradation of 17β-estradiol might be due to cometabolic reactions using enzyme(s) already present in these strains. The slower transformation of 17β-estradiol to estrone observed in degradation pattern A2 might be due to a weaker affinity of enzyme to 17β-estradiol. Furthermore, several attempts to grow these strains with 17β-estrodial as a sole carbon source were unsuccessful (data not shown).

These observations of partial removal of estrone (20-30 % in 7 d) were insufficient to indicate which degradation mechanism was responsible for estrogen degradation in strains KC6 and KC7. A recent study reported estrone was converted to 17α-estradiol under nitrate-reducing conditions (118). In our 17β-estradiol and estrone degradation tests, no 17α-estradiol was detected. Yet, more tests conducted with a longer experimental duration are needed to clearly illustrate the biodegradation of estrone. Nonetheless, the results of growth tests for strains KC6 and KC7 suggested that estrogens were most likely degraded by strains KC6 and KC7 via cometabolic reactions.

Growth-linked, metabolic reactions ─ commonly involved in energy or carbon sources for microbial growth ─ might explain the degradation Pattern C shown by strain KC 8. This hypothesis was supported by the results of growth tests. As shown in Figure 4.4, strain KC 8 was capable of growing in the acetone-free NMS-estrogen medium (with 20 mg of 17β-estradiol) for two weeks, with an average growth yield of 0.23 mg of protein/mg of 17β-estradiol and a doubling time of 27 h.

The implication of the widespread ability to transform 17β-estradiol to estrone among fourteen phylogenetically diverse isolates is important, since the total estrogenic activity is readily reduced. Interestingly, only three strains were found to degrade estrone. Since estrone still has a relatively high estrogenic potency (119) and higher average effluent concentrations (about 3.5 times higher than 17β-estradiol) (9, 117), these results suggested that effective removal of estrone in wastewater is probably the key to reduce total estrogenicity in the effluent. Three strains (KC6, KC7, and KC8) exhibited degradation of estrone and thus hold a great promise for completely mineralizing estrogens.

4.2.3 Enzyme Activity of Nonspecific Oxygenases in Strains KC6, KC7, and KC8

Positive results of naphthalene oxidation assays were observed for all three strains, suggesting the presence of nonspecific monooxygenase enzymes. Negative results of indole oxidation assays were observed for strains KC6, KC7, and KC8, implicating that these strains might not have nonspecific dioxygenase enzymes.

The results of enzymatic characterization showed that strain KC8 exhibited nonspecific monooxygenase activity but not dioxygenase activity. Similar findings were

also obtained for strains KC6 and KC7. Nonspecific monooxygenases are known to be responsible for metabolic and/or cometabolic reactions of a wide range of organics (120, 121). Shi et al. suggested that ammonia monooxygenase (a nonspecific monooxygenase enzyme produced by ammonia-oxidizing bacteria) might be responsible for estrogen degradation (23). In this study, it is likely that nonspecific monooxygenase enzymes were responsible for estrogen degradation by strain KC8. However, more experiments are needed to test this hypothesis.

5. CHARACTERIZATION OF *SPHINGOMONAS* STRAIN KC8, AN ESTRADIOL- UTILIZING BACTERIUM

5.1 Introduction

Conventional WWTPs are designed to remove bulk organics not micropollutants like estrogens. As a result, estrogens remaining in the treated wastewater are subsequently released into the environment via effluent discharge. Not surprisingly, estrogens are detected in effluent, rivers and even in drinking water (18, 19, 122-124).

Previous studies have suggested that biodegradation is an important removal mechanism for estrogens during wastewater treatment processes (17, 112, 117), despite incomplete removal of estrogen in WWTPs. Layton et al. reported the first evidence of estrogen mineralization in activated sludge samples (21). Since then, several estrogen-degrading bacteria were isolated from activated sludge, including *Novosphingobium tardaugens* (ARI-1) (24), *Rhodococcus zopfii* and *Rhodococcus equi* (25), and *Achromobacter xylosoxidans* and *Ralstonia* sp. (26), and strains KC1 through KC14 (27). Nevertheless, the degradation kinetics of estrogens for the known estrogen-degrading isolates have not been investigated. The knowledge of estrogen utilization kinetics is essential for determining the fate of estrogen in wastewater and for formulating effective biological treatment processes for estrogen removal.

Strain KC8 has the potential to be used for effective estrogen removal because it can (i) degrade both 17β-estradiol and estrone (within 3 d) to non-estrogenic compounds, (ii) use 17β-estradiol as a sole carbon source, and (iii) grow rapidly on complex nutrients

(27). However, one potential limitation is that low levels (ng/L) of estrogens in wastewater might not be sufficient to support the growth of strain KC8. Given that there are a wide range of organics in wastewater, it would be important to know whether strain KC8 can grow on other wastewater organics while retaining its degradation ability toward estrogens. Furthermore, strain KC8 exhibited the nonspecific monooxygenases activity (i.e., oxidizing naphthalene to naphthols), but not nonspecific dioxygenases activity (27). However, it was unclear if the nonspecific oxygenases were involved in estrogen degradation and/or capable of degrading other emerging micropollutants in wastewater.

The objective of this study is to further characterize strain KC8 with respect to its potential to be used for effective estrogen removal in engineered biological systems. Experiments in this study were designed to address the following questions: (i) Does strain KC8 have an ability to degrade and/or utilize macro- and micro-pollutants in wastewater? (ii) Can strain KC8 grow on complex nutrients and retain its ability to degrade 17β-estradiol and estrone? (iii) What are the utilization kinetic parameters for estrogens (17β-estradiol and estrone)? and (iv) Can strain KC8 degrade and/or utilize male hormone like testosterone? If yes, what is the utilization/degradation kinetics?

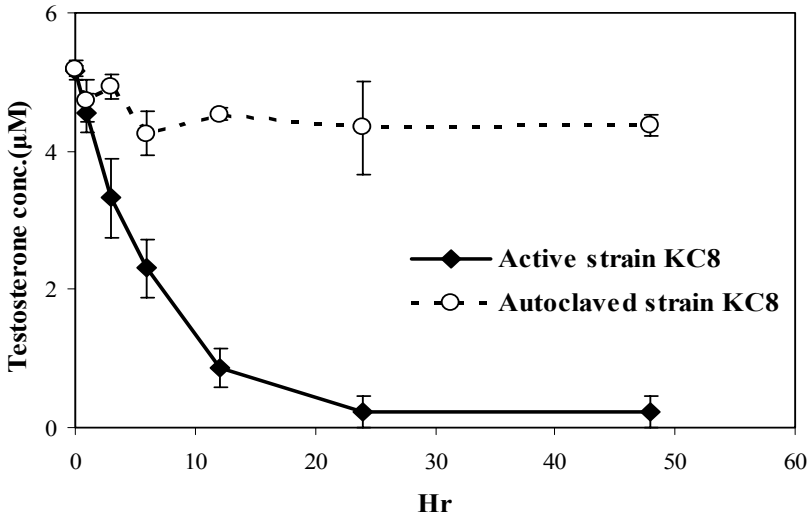
5.2 Results

5.2.1 Ability to Utilize and/or Degrade Macro- and Micro-pollutants in Wastewater

Results of substrate degradation tests showed that strain KC8 could degrade testosterone, but not triclosan or bisphenol A**.** Approximately 95 % of added testosterone was degraded within 1 d (Figure 5.1a). Strain KC8 could also grow on testosterone. After 25 d of incubation, the biomass of strain KC8 increased twelve times, from 4 to 48 mg-protein/L (Figure 5.1b). The average growth yield was estimated to be 0.33 mg-protein/mg-testosterone and the doubling time was estimated to be 61 h.

Strain KC8 was able to utilize all organic substrates tested to support its growth, except for phenol. The biomass of strain KC8 (expressed as protein content) increased from 9 to 45 mg/L for glucose in 4 d; from 9 to 38 mg/L for sodium succinate in 2.5 d; and from 11 to 47 mg/L for sodium acetate in 5 d (Figure 5.2). Assuming each substrate was completely depleted when the maximum optical densities were reached and the cellular decay was insignificant, the average cellular yields were estimated to range from 0.04 to 0.4 mg-VSS/mg-BOD_L. The doubling times were estimated ranging from 20 to 29 h when using different substrates (Table 5.1).

(a)



(b)

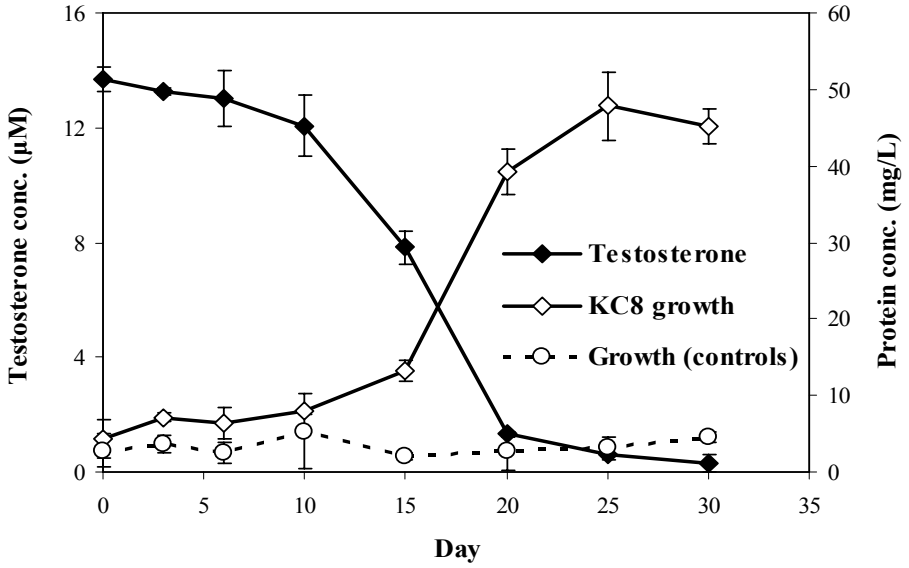


Figure 5.1 Degradation of testosterone by strain KC8 over time (a) and utilization of testosterone as a growth substrate by strain KC8 in NMS medium (b). Solid diamonds (testosterone). Open circles (controls (a)), open diamonds (growth of strain KC8 expressed as protein content (b)), and open circles (protein content of strain KC8 in controls (b))). The bars represent the ranges of duplicate experiments.

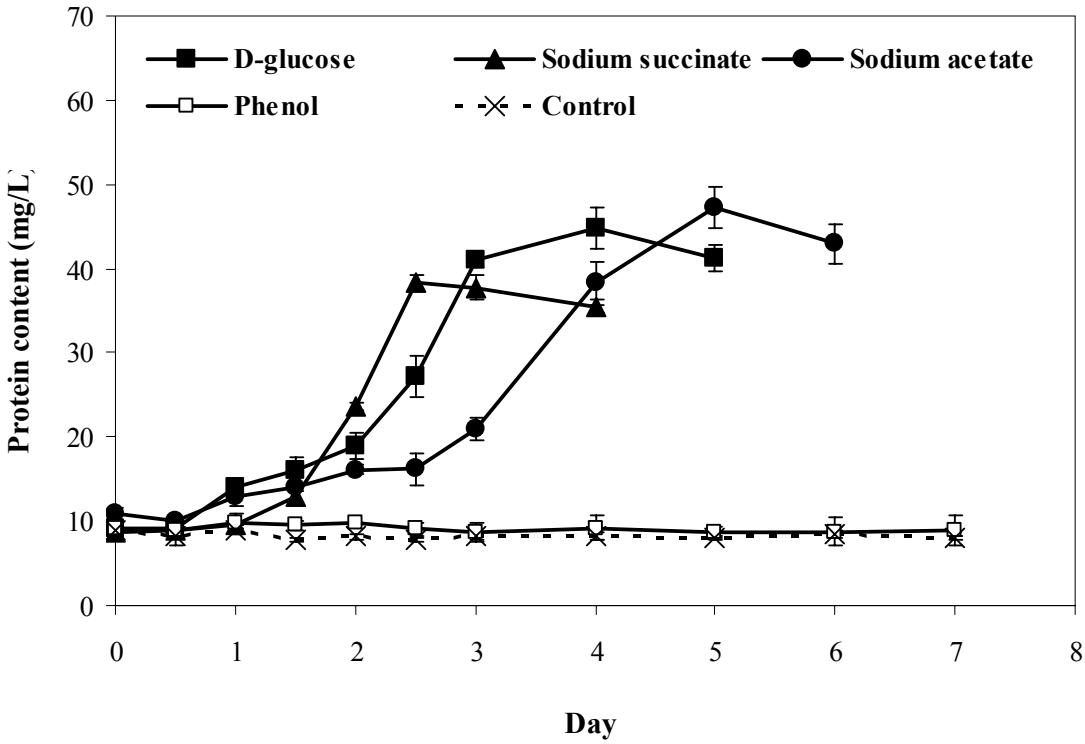


Figure 5.2 Cell growth (as protein content) of strain KC8 over time by using organics as a growth substrate. Strain KC8 utilized glucose, sodium succinate, and sodium acetate, but not phenol, as a sole carbon source. Initial protein contents were around 14 mg/L. Solid squares (glucose), solid triangles (sodium succinate), solid circles (sodium acetate), and open squares (phenol), "X" (negative controls). The bars indicate the ranges of duplicate data points.

Table 5.1 Substrate utilization kinetic parameters of strain KC8

Parameters \ Substrate	17β-est-radiol	Estrone	Testos-terone	Glucose	Sodium succinate	Sodium acetate	Typical value for activated sludge process[a]
q_m (mg-substrate/ mg-protein/d)	0.37 ± 0.02	0.50 ± 0.02	0.17 ± 0.01	–	–	–	–
q_m (mg-BOD_L/mg -protein/d)[b]	1.00 ± 0.05	1.35 ± 0.05	0.48 ± 0.03	–	–	–	52-70 [d]
K_m (mg-substrate/ L)	1.9 ± 0.2	2.7 ± 0.3	2.4 ± 0.4	–	–	–	–
K_m (mg-BOD_L/L)[b]	5.1 ± 0.5	7.3 ± 0.8	7.0 ± 1.2	–	–	–	>10
Y (g-VSS/g-BOD_L) [b,d]	0.22[c]	_	0.30	0.05	0.04	0.4	0.42-0.49
Doubling time (hr)	27[c]	_	61	20	24	29	_

q_m = maximum specific substrate utilization rate, K_m = half velocity constants

Y= yield coefficient - = not available

[a] data from Rittmann and McCarty (64)

[b] 1 mg-substrate/L = 2.7, 2.8, 1.1, 0.6, or 0.5 mg-BOD_L /L for 17β-estradiol, testosterone, glucose, sodium succinate, and sodium acetate, respectively, based on their theoretical oxygen demand.

[c] data from Yu et al.(27).

[d] The reported q_m values range from 20-27 mg-BOD_L /mg-VSS/d. For comparison, the unit of these values was converted to mg-BOD_L / mg -protein/d. The conversion was done by using VSS (g) = 2.6 x protein (g) as measured in the laboratory.

5.2.2 Estrogen-Testosterone Utilization Kinetic Parameters

The Monod equation described estrogens and testosterone degradation kinetics well (Figure 5.3). The maximum specific substrate utilization rate (q_m) were estimated to be 0.37 ± 0.02, 0.50 ± 0.02, and 0.17 ± 0.01 mg-substrate/mg-protein/d for 17β-estradiol, estrone, and testosterone, respectively. The estimated half velocity constants (K_m) are 1.9 ± 0.2 mg-17β-estradiol/L, 2.7 ± 0.3 mg-estrone/L, and 2.4 ± 0.4 mg-testosterone/L (Table 5.1). Changes of cellular protein content were negligible (less than 5 %) during the kinetic experiments.

(a)

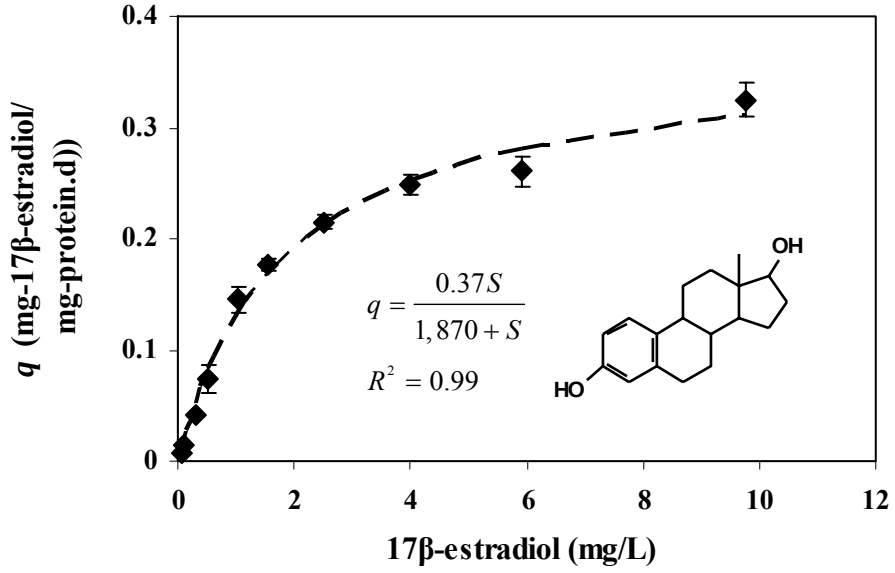
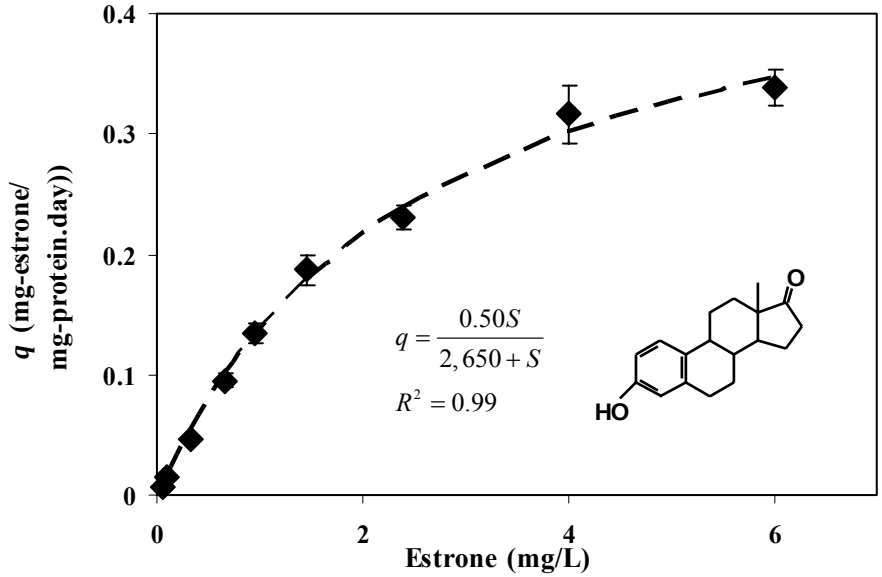


Figure 5.3 Monod degradation kinetic of 17β-estradiol (a), estrone (b), and testosterone (c) by strain KC8. The changes of biomass concentrations were negligible within 2 h (less than 5 %). Solid symbols represent experimental data and the dash line represents fitted Monod kinetic curve. The bars indicate the ranges of duplicate data points.

(b)



(c)

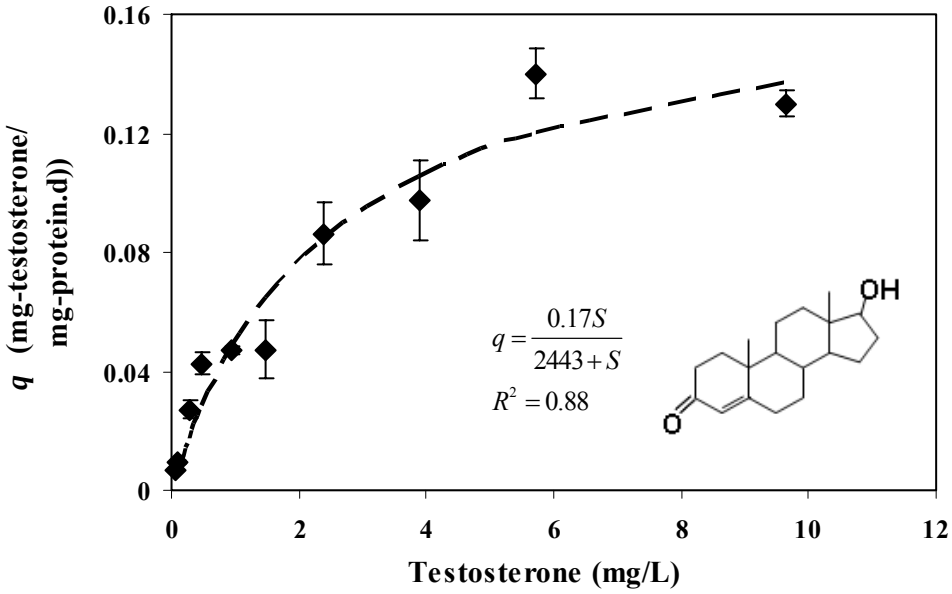
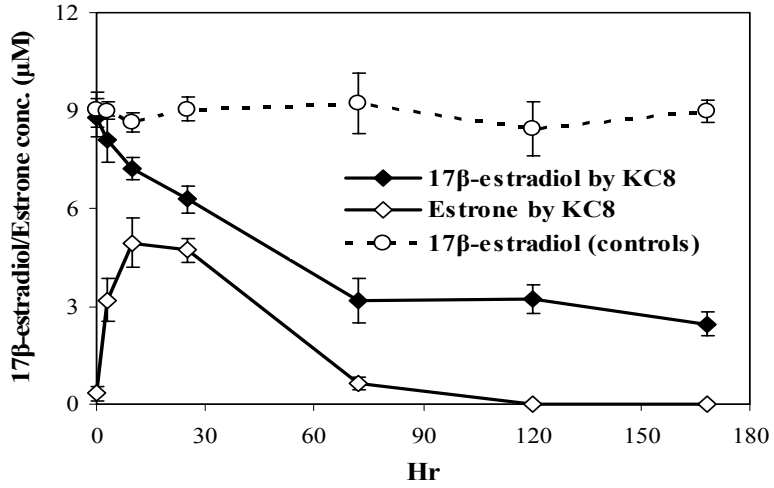


Figure 5.3 Continued.

5.2.3 Effects of Complex Nutrients on Estrogen Degradability

While *Novosphingobium tardaugens* (ARI-1) could degrade estrogens when grown on nutrient-rich medium containing estrogen (Figure 5.4d), ARI-1 lost its degradability toward estrone after growth on nutrient-rich, estrogen free medium for 7 d (Figure 5.4b) (31). Similar experiments were conducted to examine the effects of complex nutrients on estrogen degradability of strain KC8. Strain KC8 retained its ability to degrade 17β-estradiol and estrone after growth on R2A agar plates without 17β-estradiol for 15 d (Figure 5.4a). Approximately 62 % of the initial 17β-estradiol was degraded. Meanwhile, estrone concentrations increased in the first 30 h and then decreased to non-detectable level after 5 d. The results were different from those obtained from the strain KC8 grown with a complex nutrient medium containing 17β-estradiol (Figure 5.4c), where 17β-estradiol was rapidly degraded to near zero within 24 h.

(a)



(b)

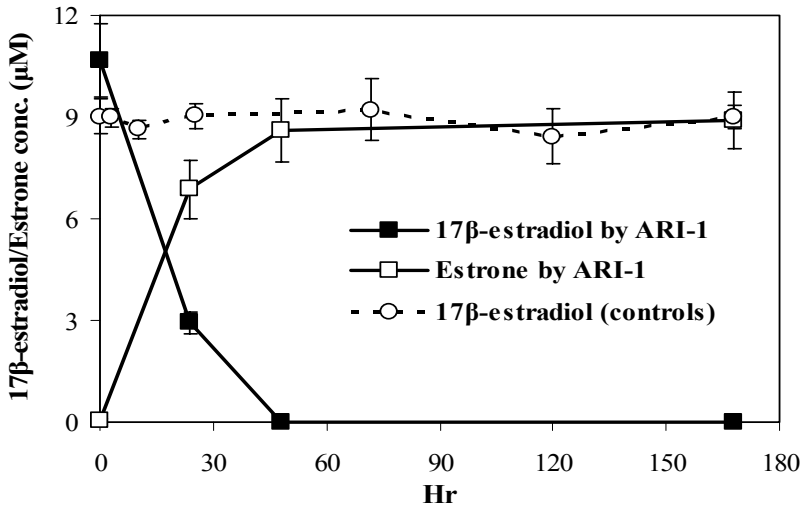
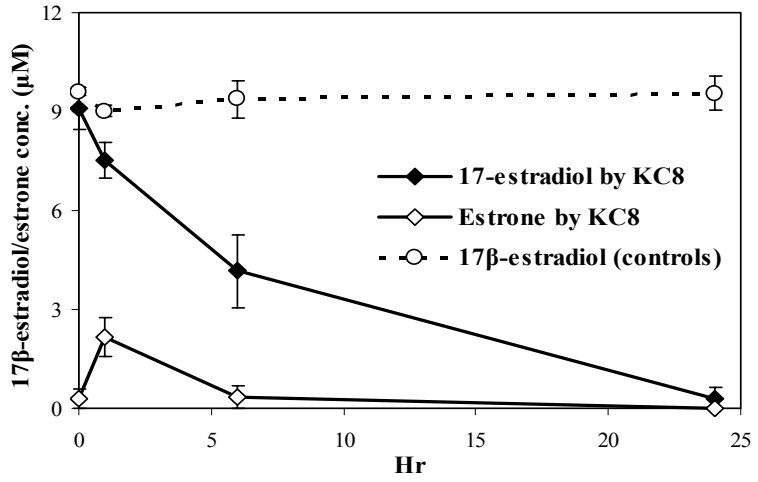


Figure 5.4 Effects on 17β-estradiol degradation of strain KC8 (a) and ARI-1 (b) after growth in complex nutrient medium over a period of time. Biodegradation of 17β-estradiol by strains KC8 (c) and ARI-1 (d) when 17β-estradiol presented in the complex nutrient medium. Solid diamonds (17β-estradiol by KC8), open diamonds (estrone by KC8), solid squares (17β-estradiol by ARI-1), open squares (estrone by ARI-1)and open circles (controls). The bars represent the ranges of duplicate data points. Figure (b) and (d) were from Yang et al. (31).

(c)



(d)

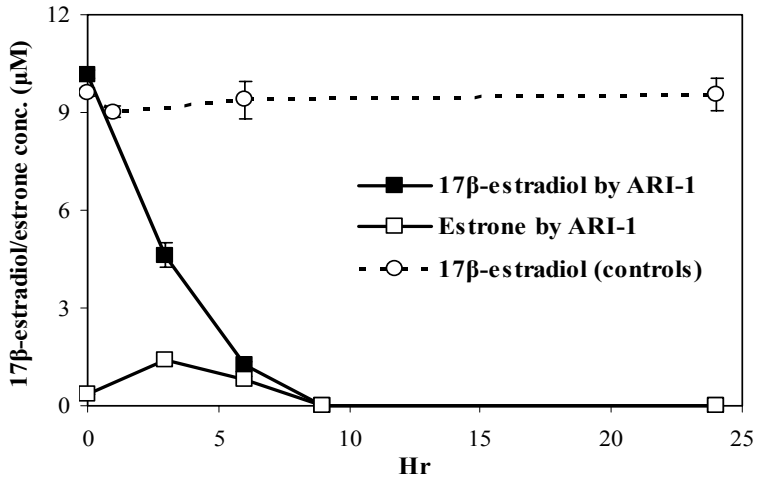


Figure 5.4 Continued.

5.3 Discussion

The 17β-estradiol-utilizing bacterium *Sphingomonas* strain KC8 was characterized from many different aspects. One important finding of this study was that strain KC8 could degrade and further utilize testosterone as a growth substrate. Testosterone is a micropollutant that co-exists with estrogens in wastewater. Testosterone has been detected in 2.8 % of 139 US rivers with a medium concentration of 116 ng/L (125). Such an ability of strain KC8 is favorable, since strain KC8 would remove testosterone and estrogens (17β-estradiol and estrone) simultaneously from wastewater.

The utilization kinetics of estrogens and testosterone by strain KC8 is an important factor to evaluate whether strain KC 8 can be applied practically for effective estrogen and testosterone removal in biological systems. For easy comparison, the units of utilization kinetic parameters were converted to BOD_L basis (see Table 5.1). The K_m values (5.1-7.3 mg-BOD_L/L) for strain KC8 are smaller than those for heterotrophs in activated sludge (K_m > 10 mg-BOD_L /L). The measured q_m values (0.5-1.4 mg-BOD_L /mg-protein/d) are also smaller than the typical value for heterotrophs in activated sludge (reported as 20-27 mg-BOD_L/mg-VSS/d (64) or 52-70 mg-BOD_L/mg-protein/d as shown in Table 1). Based on these kinetic values and the small growth yield of strain KC8 (0.23 mg of protein/mg of 17β-estradiol) (27), an impractical long solid retention time (SRT) will be required for completely mixed activate sludge systems to remove estrogens down to ng/L levels

The need of the long SRTs might be overcome if strain KC8 can grow rapidly on other common organic constituents (macro-pollutants like glucose and other organics) in wastewater without losing its degradation ability toward estrogenic compounds. To investigate this aspect, strain KC8 was examined for its ability to grow on glucose, sodium succinate, or sodium acetate. Glucose is a representative carbohydrate in wastewater, with a typical influent concentration 10^4-fold times higher than the concentrations of estrogens in wastewater. Sodium succinate and sodium acetate are compounds which are able to be involved in TCA (tricarboxylic acid) cycle responsible for generating energy and carbon sources in many microorganisms. As shown in Figure 5.2 and Table 5.1, strain KC8 can utilize these three organics with short doubling times ranging from 20 to 29 h, suggesting that strain KC8 can thrive in existing activated sludge systems. This hypothesis can be easily tested by measuring the abundance of stain KC8 in various biological wastewater treatment processes.

Effective removal of estrone might be a key to reduce total estrogenicity in wastewater (27), as many field studies reported elevated estrone in treated wastewater and not many known estrogen-degrading isolates can degrade estrone effectively. To our knowledge, both strains ARI-1 and KC8 could degrade 17β-estradiol and estrone rapidly when grown on complex nutrients with 17β-estradiol ((Figures 5.4c and 5.4d). A previous study has shown that *Novosphingobium tardaugens* ARI-1 (a known estrogen degrader) was unable to retain its degradation ability toward estrone after growing on nutrient-rich, estrogen-free medium for 7 d (31) (Figure 5.4b). Unlike strain ARI-1, strain KC8 was still able to degrade 17β-estradiol without accumulation of estrone, when

17β-estradiol was removed from the growth medium for 15 d (Figure 5.4a). These results implied that strain KC8 would play an important role in degrading estrogen to non-estrogenic metabolites/end product even in wastewater with low, and/or fluctuating concentrations of estrogens.

Little is known about the biodegradation pathway of estrogen. The positive results from the naphthalene oxidation tests suggest that nonspecific monooxygenases might be involved in estrogen degradation by strain KC8 in Section 4 (27). In addition, strain KC8 was able to degrade and utilize testosterone and estrogens in this study. As a meta-cleavage enzyme has been identified as a key enzyme for the testosterone degradation by a Gram-negative bacterium *Comamonas testosteroni* TA441 (61, 126), it is possible that nonspecific monooxygenases and/or meta-cleavage enzyme is involved in the degradation of estrogens by strain KC8. Further studies are needed to identify the degradation pathway of estrogen degradation by strain KC8.

6. EFFECTS OF SOLIDS RETENTION TIMES ON ESTROGEN-DEGRADING MICROBIAL COMMUNITY STRUCTURE IN BIOREACTORS

6.1 Introduction

The presence of endocrine disrupting compounds (EDCs) in the environment is of a concern due to their ability to cause reproductive abnormalities in wildlife (1, 5, 9). Incomplete removal of estrogens by wastewater treatment plants (WWTPs) contributes to environmental EDCs through the discharge of estrogen-containing effluent (18, 19, 123, 124). Therefore, improving estrogen removal by WWTPs is essential to minimize estrogens entering the environment.

Solid retention time (SRT) is an important operating parameter used to create unique microbial communities capable of removing organic (BOD) and/or nitrogen during biological wastewater treatment. A short SRT (from 5 to 10 d) is commonly used to achieve high BOD removal; while a longer SRT (10 to 20 d) is required for slow growing microorganisms (64, 127, 128) like nitrifying microorganisms during nitrification. Biodegradation of estrogen by both pure ammonia-oxidizing bacteria and by nitrifying activated sludge have been recently reported (23, 129, 130). Additionally, several heterotrophic estrogen-degrading microorganisms have been recently isolated from nitrifying activated sludge (21, 24-27). Even though many studies suggested that a long SRT is needed for enhanced estrogen (124, 127, 131, 132), no correlations between SRTs and estrogen removals have been established and/or verified.

The objective of this study is to examine whether one can enhance estrogen removal by controlling SRT in bioreactors. As a change in SRT will subsequently change function and structure of the microbial community in bioreactors, this study examined the changes of estrogen-degrading microbial community in lab-scale sequencing batch reactors (SBRs) operated under different SRTs (5, 10, and 20d). An effective estrogen degrader, *Sphingonmonas* strain KC8 reported from our previously study (27), was used as a model estrogen degrader in the SBRs to examine for its application potential for estrogen removal. The strain KC8 can degrade estrogen into non-estrogenic products. The performances of SBRs in terms of estrogen removal, BOD removal, and nitrification, were evaluated. Molecular techniques were developed and applied to quantify the total microbial population, estrogen degraders (strain KC8, ARI-1, ammonia oxidizing bacteria (AOB), and *amoA* gene) in lab-scale SBRs and three full-scale WWTPs. Effects of SRT on microbial community structure in SBRs were examined using quantitative fingerprinting assay, real-time-t-RFLP (real time terminal restriction fragment polymorphism) (78).

6.2 Results

6.2.1 Validation of Real-Time PCR Assays

Real-time PCR assay for strain KC8 was validated using axenic KC8, mixed culture of SBR-5d, and thirteen other estrogen degraders (strains KC1 through KC7 and KC9 through KC14). A linear relationship ($r^2 = 0.998$) was observed between the Ct values and the amounts of DNA of strain KC8 in the templates, ranging from 0.2 ng to

100 ng (APPENDIX A-1). Similarly, a linear relationship (r^2 = 0.999) was observed for the mixed culture in the templates (ranging 0.5 ng to 150 ng) (APPENDIX A-2). No signals (Ct > 40 cycles) were detected when the assay was challenged against all thirteen other 17β-estradiol-degrading bacteria. Validation of real-time PCR assay for ARI-1 was conducted similarly using known amounts of DNA of strain ARI-1. A linear range (r^2 = 0.994) for templates containing 0.25 ng to 250 ng of DNA of strain ARI-1 was observed. For DNA of SBR mixed culture, the linear range was from 0.25 ng to 250 ng (r^2 = 0.993) (APPENDIX B). Again, no signals (Ct > 40 cycles) were detected when the real-time PCR assay for ARI-1 was challenged against eight KC strains (KC1 through KC2 and KC9 through KC14).

6.2.2 Performance of Bioreactors

The initial biomass concentrations (expressed as MLVSS) in all three SBRs decreased rapidly during the startup phase and then reached steady state after the reactors were operated for two times of each operating SRT. The steady biomass concentrations were 650, 750, and 905 mg/L for SBR-5d, SBR-10d, and SBR-20d, respectively (Figures 6.1A(a), 6.1B(a), and 6.1C(a)). After the first cycle of operation, TOC removal rapidly reached 87 % for SBR-5d, 89 % for SBR-10d, and 92 % for SBR-20d (Figures 6.1A(b), 6.1B(b), and 6.1C(b)). Initial ammonia concentration (25 mg/L) was reduced to less than 1 mg/L in effluent in all three SBRs. The steady ammonia removals (after operating for 2 times of each SRT) were 91-99 % for SBR-5d, 96-99 % for SBR-10d, and 97-99 % for SBR-20d (Figures 6.1A(c), 6.1B(c), and 6.1C(c)). No nitrite was detected in influent and effluent. Influent concentrations of nitrate ranged

Fig 6.1A. Fig 6.1B.

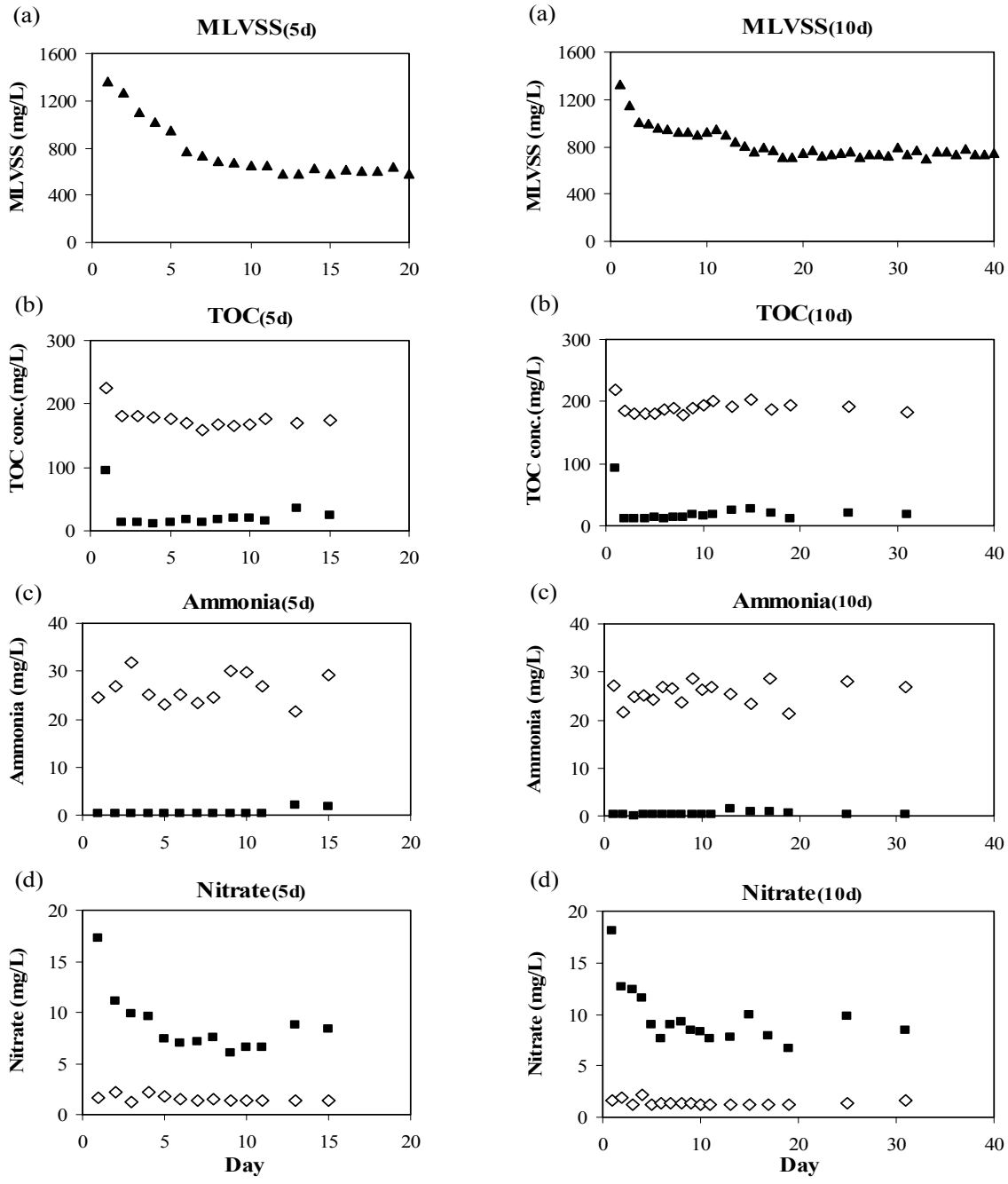


Figure 6.1. MLVSS (a), TOC (b), ammonia (c), and nitrate (d) concentrations in SBRs under 5d (Fig 6.1A), 10d (Fig 6.1B), and 20d (Fig 6.1C) of SRT. Solid triangles for (MLVSS). Open diamonds (influent) and solid squares (effluent) for TOC, nitrate and ammonia concentration.

Fig 6.1C.

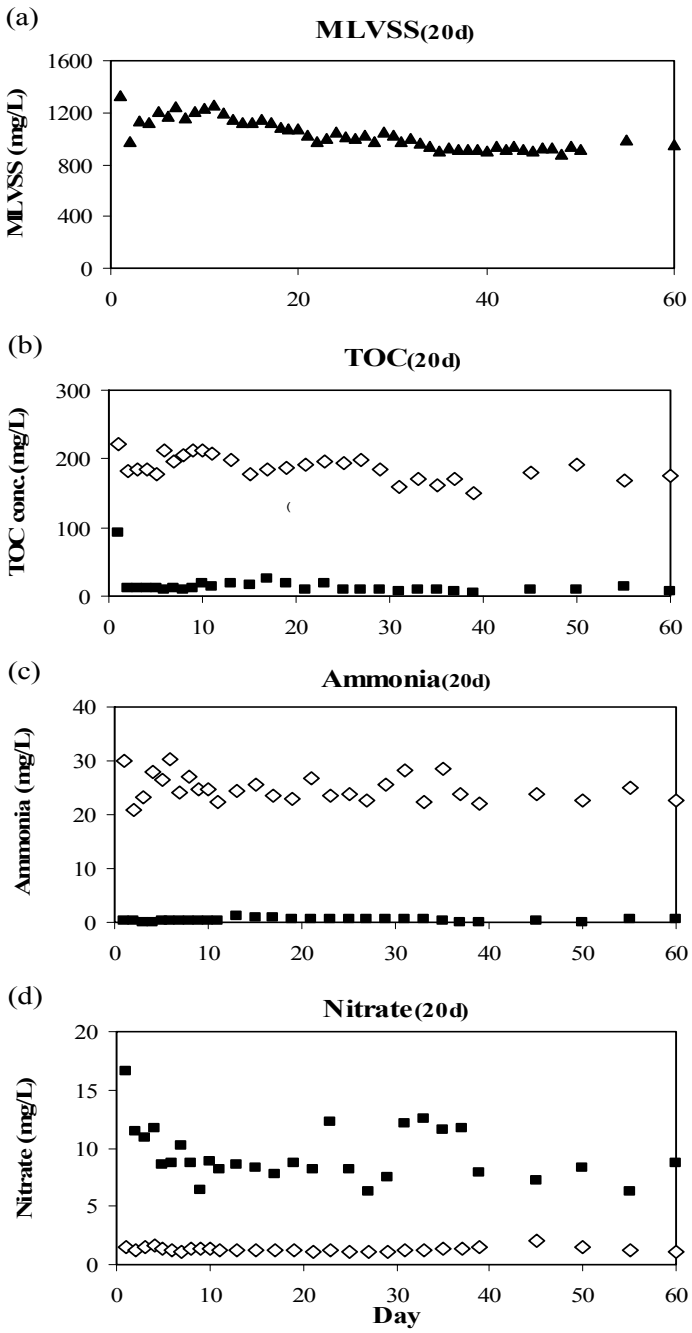
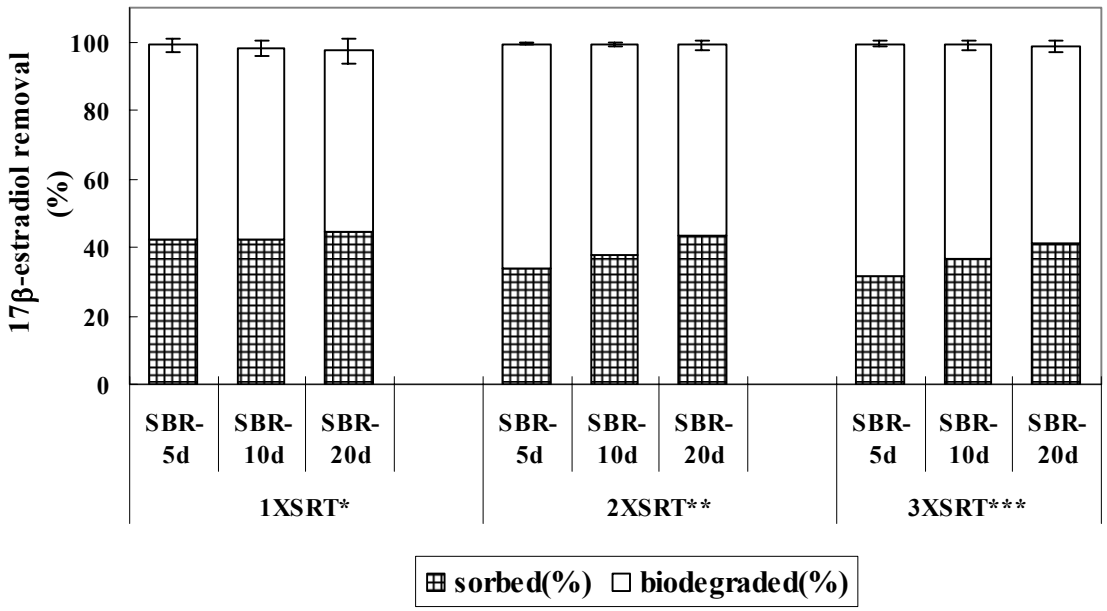


Figure 6.1. Continued.

from 1 to 2 mg/L. The range of nitrate concentrations in the effluent was 6.0-17.0 mg/L for SBR-5d, 7-18 mg/L for SBR-10d, and 6-17 mg/L for SBR-20d (Figures 6.1A(d), 6.1B(d), and 6.1C(d)).

The overall removals of 17β-estradiol were more than 98.7 % for all three SBRs (details are available in APPENDIX B). The fraction of estrogen removed due to sorption onto biomass was estimated using sorbed fraction = (MLSS x K_d) / (1 + MLSS x K_d) and a sorption coefficient (K_d = 476 l/kg) (133). The percentages of 17β-estradiol sorbed onto biomass were estimated to be 32 % in SBR-5d, 37 % in SBR-10d, and 42 % in SBR-20d. Accordingly, the percentages of 17β-estradiol removed due to biodegradation were 67 % for SBR-5d, 62 % for SBR-10d, and 57 % for SBR-20d. In contrast to results from previous studies (124, 127, 131, 132), our results suggested that a shorter SRT (5d) was more favorable for 17β-estradiol biodegradation than longer SRTs (10 and 20d) (Figure 6.2).



* 1XSRT indicates the time period of 0-5d (SBR-5d), 0-10d (SBR-10d), and 0-20d (SBR-20d)

** 2XSRT indicates the time period of 6-10d (SBR-5d), 11-20d (SBR-10d), and 21-40d (SBR-20d)

*** 3XSRT indicates the time period of 11-15d (SBR-5d), 21-30d (SBR-10d), and 41-60d (SBR-20d)

Figure 6.2. 17β-estradiol removals for 3 x SRT in the bioreactors operating under 5, 10, and 20d of SRT. 17β-estradiol removals by sorption (small grid) and by biodegradation (open). Error bars indicate the standard deviation of results of 17β-estradiol removals.

6.2.3 Microbial Community Structure in Sequencing Batch Reactors

Changes in microbial community structure in three SBRs were observed (Figure 6.3). The species richness (*S*) of microbial community structures in three SBRs were observed – the number of ribotypes (i.e., terminal-restricted fragments T-RFs) increased from nine to twelve as STR increased from 5 to 20 d. Among these ribotypes, six T-RFs (i.e., T-RF= 84 bp, 104 bp, 106 bp, 305 bp, 326 bp, and 328 bp) appeared in the microbial communities of all three SBRs. Theoretical T-RFs of known estrogen degraders by using *in silico* analysis with a restriction enzyme *Msp*I were shown in Table 6.1. Based on the values of evenness (*E*), there was not much difference in the evenness of microbial communities in three SBRs. However, the values of Shannon-Weaver diversity index (*H*) and the reciprocal of Simon's index (*1/D*) increased as SRT increased (Table 6.2).

Figure 6.3A

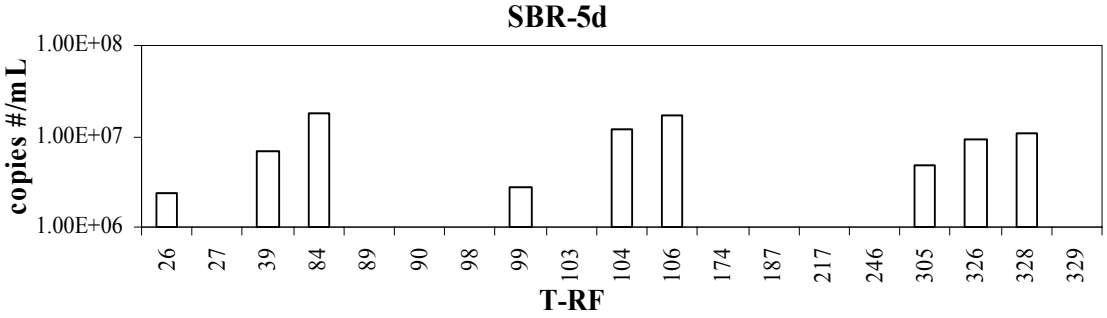


Figure 6.3B

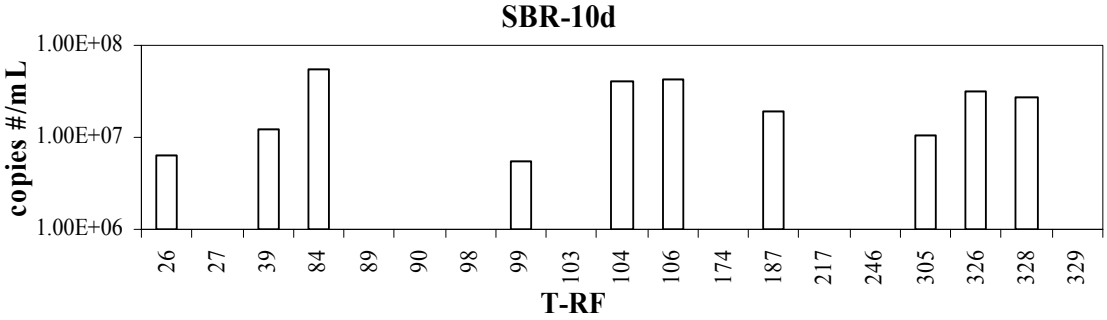


Figure 6.3C

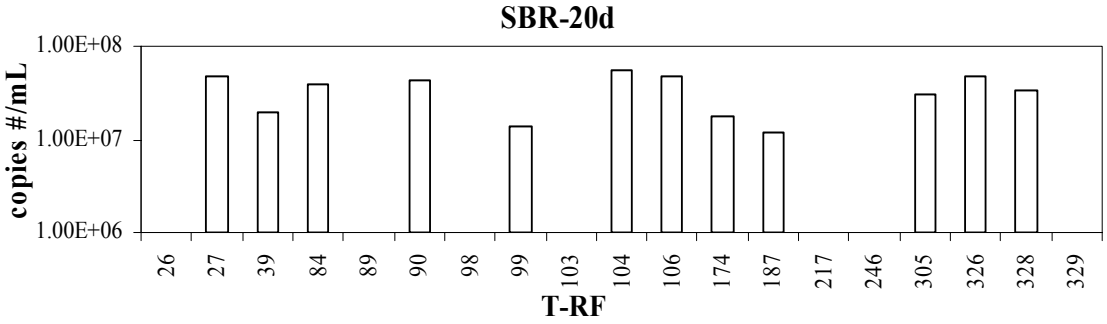


Figure 6.3 Real-time-t-RFLP profiles in SBRs after operating three times of SRT. Real-time-t-RFLP profiles in samples of SBRs operating under 5d (Fig 6.3A), 10d (Fig 6.3B), and 20d (Fig 6.3C) of SRT. Below tables contained the list of the known estrogen degraders which might contribute to the T-RFs based on restriction enzyme (*MspI*) analysis

Table 6.1 Theoretical T-RFs of known estrogen degraders by using *in silico* analysis with a restriction enzyme *Msp*I

Theoretical T-RFs (bp)	Known estrogen degraders to be contributed to T-RFs
84	KC3
99	*Achromobacter xylosoxidans, Ralstonia picketii*
104	KC2, 6-12, 14, ARI-1
106	AOB, KC4-5, *Rhodococcus zofii, Rhodococcus equi*
187	KC1

Table 6.2 Analysis of microbial community structure in SBRs

SRT	Sample	S (1)	H (2)	E (3)	$1/D$ (4)
5 d	1 x SRT (5d)	8	1.939	0.646	6.109
	2 x SRT (10d)	10	2.049	0.617	6.458
	3 x SRT (15d)	9	2.015	0.636	6.674
10 d	1 x SRT (10d)	11	2.180	0.630	7.644
	2 x SRT (20d)	10	2.037	0.613	6.278
	3 x SRT (30d)	10	2.082	0.627	7.026
20 d	1 x SRT (20d)	14	2.511	0.659	11.276
	2 x SRT (40d)	12	2.349	0.655	9.300
	3 x SRT (60d)	12	2.386	0.665	10.155

(1) S : richness

(2) H: Shannon-Weaver Index

(3) E : Evenness

(4) $1/D$: Reciprocal of Simpson's Index

6.2.4 Estrogen Degrading Bacteria in Sequencing Batch Reactors

The relative abundance of estrogen degrading bacteria (strains KC8, ARI-1, and *N. europaea*) in three SBRs was estimated. The initial concentrations of total bacteria in three SBRs were 4.1-17.1 $x10^8$ copies of 16S rRNA gene /mL (Figure 6.4). The concentrations of total bacteria decreased rapidly during the startup phase in each bioreactor. After three times of SRTs, the total bacterial concentration reached steady, approximately 8.4E+07, 2.5E+08, and 4.1E+08 copies of 16SrRNA/mL for SBR-5d, SBR-10d, and SBR-20d, respectively. Similar trend was observed for strain KC8, from initial concentrations of 1.9-3.2$x10^8$ copies of 16SrRNA gene/mL to steady concentrations of 2.2E+05 for SBR-5d, 7.6E+04 for SBR-10d, and 1.0E+04 copies/mL for SBR-20d of SRT.

The gene copy numbers for strain ARI-1, AOB, and *amoA* genes in SBRs were also measured (Figure 6.5). The 16SrRNA gene of strain ARI-1 was detected at low quantities in all three SBRs, ranging from 1.4E+00 to 1.4E+01 copies /mL. These low values were expected since strain ARI-1 was not spiked during the startup of the bioreactors. Ammonia oxidizing bacteria and *amo*A genes were detected in all three SBRs, ranging from 7.7E+03 to 3.6E+04 copies/mL, and 1.5E+02 to 3.7E+03 copies/mL, respectively.

Figure 6.4A

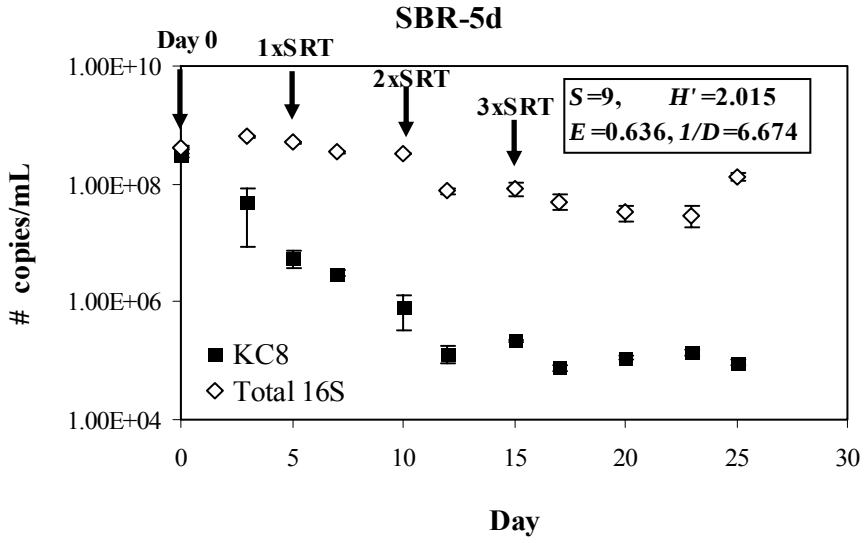


Figure 6.4B

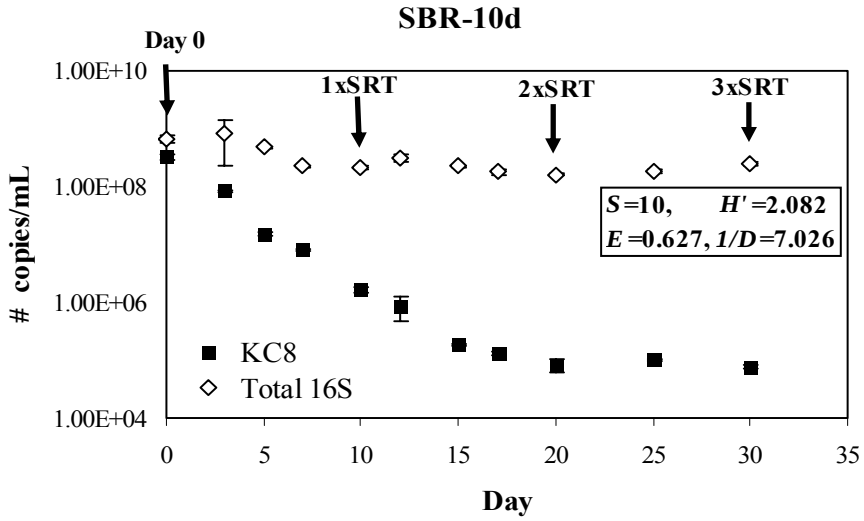


Figure 6.4 Quantification of total bacteria (16S rRNA) and strain KC8 in SBRs operating under 5d (Figure 6.4A), 10d (Figure 6.4B), and 20d (Figure 6.4C) of SRT. Microbial diversity indices in samples at steady state (at 3 x SRT) under each SRT condition were described in text boxes. Open diamonds (total 16S rRNA) and solid squares (strain KC8). Error bars indicate the standard deviation of the results of real-time PCRs.

Figure 6.4C

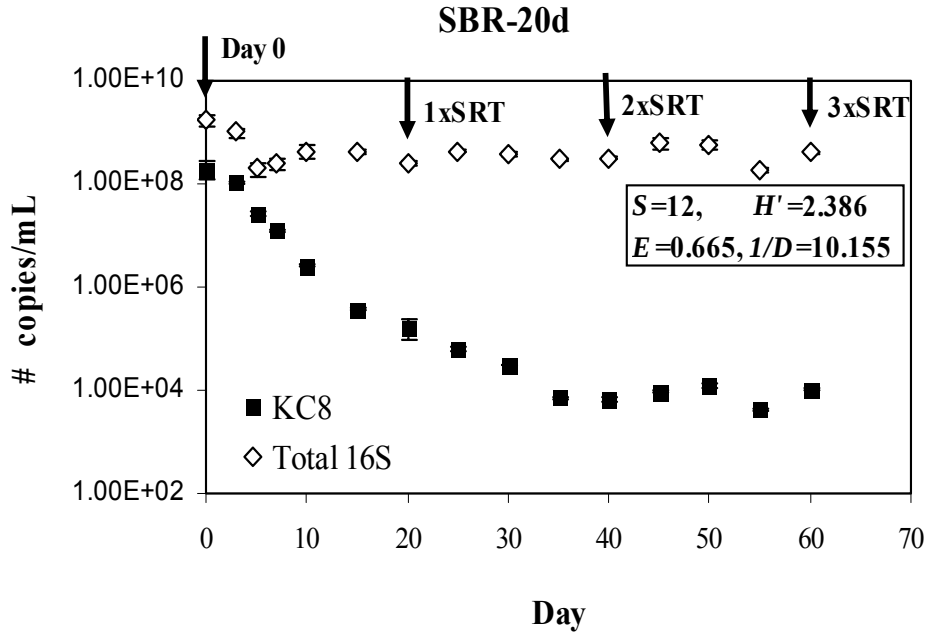
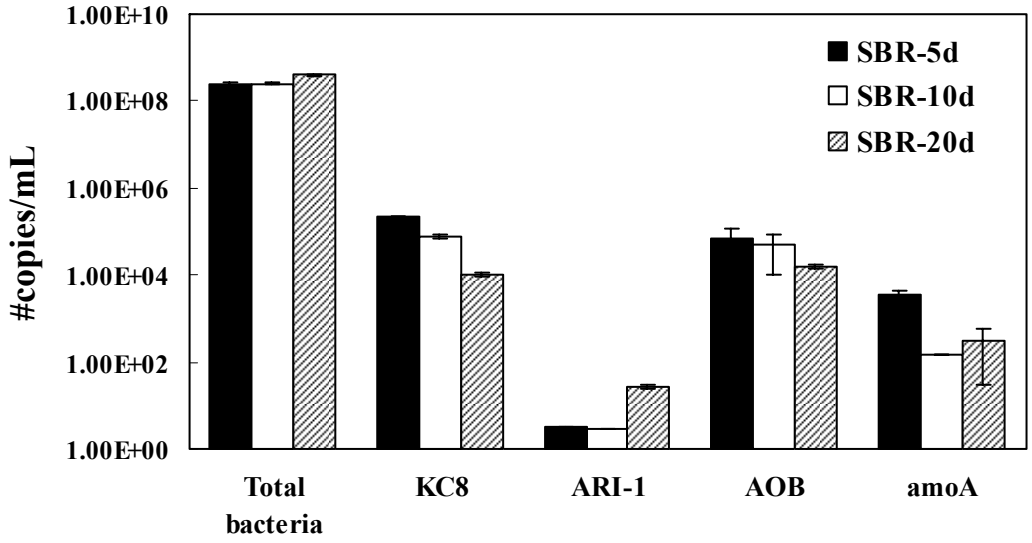


Figure 6.4 Continued.



(1) The values were measured for samples of SBRs at steady state under each SRT condition.

(2) After operating three times of SRTs in each SRT conditions, MLVSS concentrations were 0.45, 0.71, and 0.92 g/L for 5, 10, 20d of SRTs, respectively.

Figure 6.5 Quantification of total bacteria (16S rRNA), estrogen degraders, and *amoA* gene in SBRs at steady state under three different SRT conditions. SBR-5d (black), SBR-10d (open), and SBR-20d (upward diagonal). Error bars indicate the standard deviation of results of real-time PCRs.

6.2.5 Prevalence of Estrogen Degraders in Wastewater Treatment Plants

The quantities of estrogen degraders and *amo*A gene in WWTP samples were quantified using real-time PCR assays. Despite the differences in operating conditions and treatment process configurations among the WWTPs, the quantity of strain KC8 was not much different between 2.8E+04 and 2.8E+05 copies/mL. However, much lower gene copies of strain ARI, ranging from 2.1E+02 to 6.3E+03 copies/mL, were observed

in all three WWTP samples. The concentrations of AOB ranged from 7.6E+04 to 3.7E+05 copies/mL. The *amo*A gene copy numbers were measured from 1.5E+04 to 2.1E+04 copies/mL (Table 6.3).

Table 6.3 Quantification of estrogen degrading bacteria and *amo*A gene in WWTPs

WWTPs		WWTP #1	WWTP #2	WWTP #3	
				1st stage	2nd stage
Real-time PCR assay, #copies/mL (cells/mL) (1)	Total bacteria (16S rRNA)	7.98E+09 (2.22E+09)	1.43E+10 (3.98E+09)	1.72E+10 (4.77E+09)	
	Strain KC8	7.22E+04 (7.22E+04)	2.84E+05 (2.84E+05)	2.78E+04 (2.78E+04)	20-30
	ARI-1	6.34E+03 (6.34E+03)	2.14E+02 (2.14E+02)	5.16E+02 (5.16E+02)	1.10
	AOB	1.51E+05 (1.51E+05)	1.93E+05 (1.93E+05)	7.42E+05 (7.42E+05)	
	amoA	1.68E+04 (8.38E+03)	2.12E+04 (8.38E+03)	1.51E+04 (7.57E+03)	

1) cells/mL = (#copies/mL) / (gene copy number/cell). Gene copy number/cell is assumed to be 3.6 (total 16S rRNA), 1.0 (strainKC8, ARI-1, and AOB), and 2 (*amoA* gene) (72)

6.3 Discussion

In this study, the effects of SRT (5, 10, and 20 d) on estrogen removal, TOC removal, and nitrification were investigated in three lab-scale SBRs. All SBRs reached pseudo-steady state conditions after operated for three times of SRT, based on the monitoring results of MLVSS, TOC, ammonia, nitrite, and nitrate (Figure 6.1). The three times of SRT are consistent with previous reports that it requires 1.5 to 5 times of SRT for bioreactors to reach steady state conditions (128, 134, 135). The removals of TOC and ammonia in all three SBRs are also consistent with findings of previous studies on the performance of bioreactors operated under similar SRT conditions (136-139). Long SRTs are required for effective nitrification because the key players, AOB and nitrite-oxidizing bacteria, are slow growers. Not surprisingly, a slightly higher ammonia removal was observed in SBR-20d. Surprisingly, in this study, the high ammonia removal did not correspond to the decline ratios of AOB or *amoA* gene to total bacterial population as SRT increased (Figure 6.5). To our knowledge, this trend was not expected nor reported previously. On the other hand, results of samples from WWTPs are consistent with the results reported by Kuo et al that the ratios of AOB or *amoA* gene to total bacteria increased as SRT increased (73). However, due to small sample numbers of WWTPs surveyed in this study, effects of SRT on AOB or *amo*A can not be established.

Similar declining trend was observed for the ratios of strain KC8 to total bacterial population when SRT increased from 5 d to 20 d. The concentrations of strain KC8 decreased more than 1000 times, from 1.9~3.2 x 10^8 to 1.0~22 x 10^4 gene copies/mL,

corresponding to a decline ratio of strain KC8 to total bacterial population (KC8/total bacteria) from 0.27 % to 0.003 %. The decline of strain KC8 concentration as SRT increased can be explained by the characteristics of strain KC8. Based on our estrogen degradation and growth kinetics study (27), a minimum SRT of 12 d would be needed when strain KC8 uses 17β-estradiol as a sole carbon source. Since strain KC8 can grow much faster when utilizes common organics (glucose, succinate, acetate) in wastewater, it is expected that strain KC8 can survive under a SRT as short as 5 d. Not surprisingly, in this study, strain KC8 was detected in all three SBRs operated under 5 to 20 d. The decline over SRT might be due to a high decay coefficient of estrogen degraders. The concentrations of strain ARI-1 were much lower than the concentrations of AOB and strain KC8 in SBRs and three WWTPs, suggesting strain ARI-1 might not be important for estrogen removal in the bioreactors.

Several studies reported that high estrogen removals in WWTPs operating under long SRTs (124, 127, 131, 132, 140). However, no significant differences in estrogen removals (>98.7 %) were observed in three SBRs as shown in Figure 6.2. In fact, our results of estrogen degraders (Figure 6.5) and the biodegraded percentages of 17β-estradiol (Figure 6.2) suggested an opposite finding - biodegradation of estrogen was higher when SBR operated under a short SRT (5 d) than a long SRT (20 d). This finding is also supported by the declining trends of the ratios of strain KC8, AOB, and *amo*A genes, as discussed above. However, it remains unclear which estrogen degraders, mainly AOB and strain KC8, were actively involved in 17β-estradiol degradation.

Based on real-time-t-RFLP profiles, more T-RFs were observed in SBR-20d than in SBR-5d and six ribotypes (T-RF = 84, 104, 106, 305, 326, and 328 bp) might coexist in three SBRs (Figure 6.3). The ranges of evenness (*E*) were not too big (0.64-0.67) in all three SBRs, implying no significant differences of variations in communities between the species. The higher values of *S*, *H*, and *1/D* were observed for SBR-20d (Table 6.2), suggesting that higher diversity of microbial community structure in SBR-20d than in SBR-5d. These results are consistent with previous reports (127, 128) that longer SRT might lead to more diverse microbial community structure.

However, as more than one species might contribute to one single T-RF, the diversity indices might change if different restriction enzyme (other than *Msp*I) was used for real-time-t-RFLP analysis. This aspect was explored by Saikaly et al. (99). In their study, they reported that diversity indices would depend on the types of restriction enzyme used and that higher degree of diversity might not correspond to higher SRT. In this study, we further examined this aspect by conducting an exercise of *in-silico* digestion with restriction enzyme *MspI* on known estrogen degraders. As shown in Table 6.1, many known estrogen degraders would contribute to T-FRs= 84, 99,104, 106, and 187 bp. Accordingly, it will not be an easy task to determine the true diversity of a given microbial community without the knowledge on the identity of each microorganism and its abundance.

7. SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

7.1 Overview

Due to the potential adverse effects of environmental estrogens on the reproductive system of aquatic wildlife and humans, the effective estrogen removal by biodegradation in wastewater treatment plants is essential. Several bacteria and activated sludge degrade estrogens. This study is the first to examine the characteristics of estrogen degraders (strains KC6, KC7, and KC8) and the effects of an operating parameter (SRT) on estrogen removals and the microbial community structure in bioreactors.

The overall hypothesis of this study, "Enhanced estrogen degradation can be achieved by promoting the growth of estrogen-degraders in engineered bioreactors", was tested by the characterization of estrogen degraders (Sections 4 and 5) and the applications of estrogen degraders in bioreactors (Section 6). The results (Section 4) of growth tests exhibited that strain KC8 was capable of growing in the acetone-free NMS-estrogen medium for two weeks, with an average growth yield of 0.23 mg of protein/mg of 17β-estradiol and a doubling time of 27 h; however, no significant growth of strains KC6 and KC7 was observed. Since estrogens were degraded aerobically by strains KC6, KC7, and KC8, it was assumed that oxygenase enzymes have a key role in estrogen degradation. Strain KC8 contains nonspecific monooxygenase enzyme activities. Strain KC8 can degrade and grow with 17β-estradiol and testosterone, suggesting that the degradation pathway for 17β-estradiol and testosterone in strain KC8 might be similar or

even the same. In this study, the enzymes responsible for estrogen degradation were unclear. More studies are needed to elucidate the degradation pathway.

The Monod kinetic parameters of estrogens and testosterone degradation by strain KC8 estimated in Section 5 were smaller than those for heterotrophs in activated sludge processes, implying that relatively longer solids retention time is needed in activated sludge processes to remove estrogens. If strain KC8 can grow rapidly in other common organic compounds, the need for long SRT would be overcome. This hypothesis was verified by examining for strain KC8's ability to grow on glucose (representative carbohydrate in wastewater) and two major compounds in TCA cycle (sodium succinate and sodium acetate). The range of doubling times for those 3 compounds is small, suggesting that strain KC8 might be abundant in activated sludge systems. The hypothesis was tested by quantifying strain KC8 in bioreactors (Section 6). Since after having grown on nutrient-rich, estrogen-free medium for 7d, ARI-1 (a known estrogen degrader) lost its degradation ability toward estrone, a major metabolite of 17β-estradiol in a previous study, strain KC8 was examined to see if it retained the ability to degrade 17β-estradiol and estrone after growth on complex nutrient containing no-estrogen for 15 d.

The effects of normal ranges of SRT (5, 10, and 20 d) on the estrogen removals and the changes of estrogen-degrading microbial community structures in lab-scale SBRs were investigated (Section 6). Since strain KC8 utilized succinate as a growth substrate, synthetic wastewater containing succinate was added as a carbon source in bioreactor operations. Total organic carbons and ammonia removals in SBRs were

consistent with the results of previous studies, and both removals under higher SRT (20 d) are slightly higher than those under shorter SRTs (5 and 10 d). Effective removals (>98.7 %) of 17β-estradiol with no significant differences were observed in SBRs under three SRTs. The biodegraded percentages of 17β-estradiol were higher when SBR operated under a short SRT (5 d) than a long SRT (20 d). This finding was supported by the declining trends of the ratios of strain KC8, AOB, and *amo*A genes to total bacteria as SRT increased in SBRs. However, it remains unclear which estrogen degraders were actively involved in 17β-estradiol degradation. The microbial diversity based on the variations of solids retention time and other parameters in activated sludge processes was analyzed by real-time-t-RFLP assay. There were 9, 10, and 12 ribotypes in samples of 5, 10, and 20 d of SRTs at steady state in SBRs, respectively. Some of those ribotypes might be contributed by known estrogen degraders including strain KC8, AOB, and ARI-1. The higher richness in SBR at the longer SRT (20 d) indicated that the microbial community structure is more diverse than those in SBRs at 5 and 10 d of SRT. The values of diversity indices imply that the operating parameter of SRT might be an important factor to diversify microbial community structure.

7.2 Summary and Conclusions

1) Strain KC8 could utilize 17β-estradiol as a growth substrate. The average growth yield was estimated to be 0.23 mg-protein/mg-17β-estradiol, and the doubling time was 27h. However, no significant degradation of 17β-estradiol or no cell growth were observed for strains KC6 and KC7, suggesting that strains KC6 and KC7 could not utilize 17β-estradiol as a growth substrate. It is likely that the degradation of 17β-estradiol by strain KC8 is growth-linked (metabolic) reactions, while the 17β-estradiol degradations by strains KC6 and KC7 might be due to nongrowth-linked (cometabolic) reactions.
2) Strain KC6, KC7, and KC8 exhibited nonspecific monooxygenase enzyme activity, but not nonspecific dioxygenase enzyme activity.
3) Strain KC8 could degrade testosterone as a growth substrate, but not triclosan or bisphenol A. The average growth yield was estimated to be 0.33 mg-protein/mg-testosterone, and the doubling time was 61 h. Strain KC8 was able to utilize organic substrates (glucose, succinate, and acetate) for its growth, except for phenol. the average cellular yields were estimated to range from 0.04 to 0.4 mg-VSS/mg-BOD_L. The doubling time was estimated to range from 20 to 29 h when different substrates were used, which suggests that strain KC8 is very likely thrive in activated sludge systems.
4) The Monod equation described estrogens and testosterone degradation kinetics well. The maximum specific substrate utilization rate (q_m) were estimated to be 0.37 ± 0.02, 0.50 ± 0.02, and 0.17 ± 0.01 mg-substrate/mg-protein/d and the estimated half

velocity constants (K_m) are 1.9 ± 0.2, 2.7 ± 0.3, and 2.4 ± 0.4 mg-substrate/L for 17β-estradiol, estrone, and testosterone.

5) Strain KC8 retained its ability to degrade 17β-estradiol and estrone after growth on complex nutrients without 17β-estradiol for 15 d, unlike another estrogen degrader, ARI-1. These results imply that strain KC8 would play an important role on degrading estrogen into non-estrogenic metabolites/end product even in wastewater with low, and/or fluctuating concentrations of estrogens.

6) TOC removals were 87, 89, and 92 % under 5, 10, and 20 d of SRTs, respectively. The ranges of ammonia removals in SBRs were 91-99 % for 5 d, 96-99 % for 10 d, and 97-99 % for 20 d of SRT. The higher ammonia removals at long SRT (20 d) were consistent with the information that long SRTs are required since the key players (AOB and nitrite-oxidizing bacteria) for nitrification, are slow growers. Surprisingly, in this study, the high ammonia removal did not correspond to the decline ratios of AOB or *amoA* gene to total bacterial population as SRT increased.

7) Regardless of SRTs (5, 10, or 20 d), similar overall removals of total organic content (TOC, >87 %), ammonia (>91 %) and 17β-estradiol (> 98.7 %) in SBRs were observed. The ratios of known estrogen degraders (strains KC8 and ammonia-oxidizing bacteria (AOB) and *amo*A gene (associated with ammonia oxidation) to total bacteria population decreased as SRT increased in SBRs. These observations correspond to the decreasing percentages of 17β-estradiol biodegraded in SBR when SRT increased from 5 d to 20 d, when the sorption of 17β-estradiol onto biomass was considered.

8) Base on real-time-t-RFLP (using the restriction enzyme *Msp*I), more ribotypes were observed in SBRs operated under 20 d than 5 d. The species evenness (*E*) in microbial community structures in three SBRs was not affected by SRT. However, diversity indices (Shannon-Weaver diversity index (*H*) and the reciprocal of Simpson's index (*1/D*)) suggested that longer SRT might lead to more diverse microbial community structure.

7.3 Future Studies

1) This study reported that nonspecific monooxygenase enzyme activities are present in strains KC6, KC7, and KC8; however, the roles of the enzymes in estrogen degradation pathway are still unclear. More studies are needed to describe the roles of the enzymes in the degradation pathway and to identify any metabolites of estrogen degradation by strain KC8. These efforts will help to propose a degradation pathway of estrogens.
2) The effects of solids retention times on estrogen removals and microbial community structures in lab-scale bioreactors were studied in this study. Additional optimization studies are needed for enhancing estrogen removals in bioreactors. The operation parameters (dissolved oxygen concentrations, carbon/nitrogen ratios, temperatures, and pH) can be optimized for estrogen removals in wastewater treatment processes.
3) The application studies of molecular technologies (real-time PCR and real-time-t-RFLP) in various WWTPs using different operating parameters as well as the fate of

estrogens in the plants are necessary to better understand the relationships between microbial community structures or the prevalence of estrogen degraders and their operating conditions.

4) The estrogenic activity needs to be measured with samples of SBRs by using an estrogenic activity assay even though estrogen appeared to be removed in SBRs.

5) The correlations between the population of estrogen degraders and the estrogen degradation need to be investigated in the future. In addition, further investigations about which estrogen degraders in mixed culture are more actively involved in estrogen degradation will be necessary.

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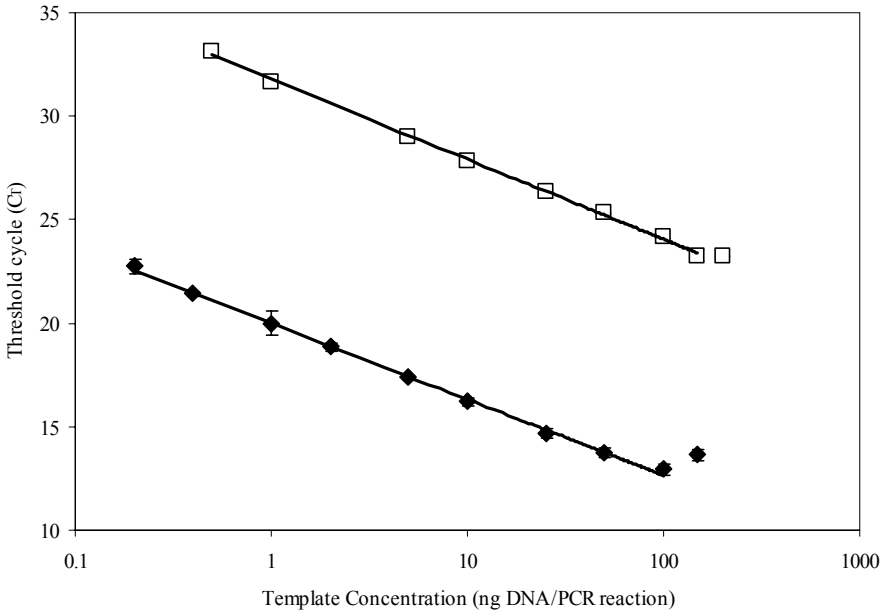
APPENDIX A

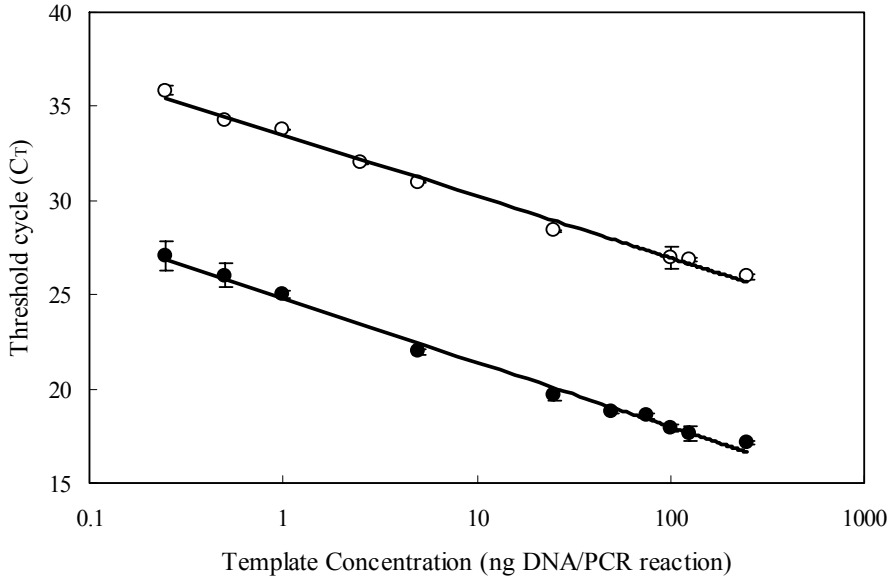
VALIDATION OF REAL-TIME PCR ASSAYS

A-1. Validation of Real-time PCR Assay for Strain KC8



Linear range of template concentrations for real-time PCR assays specific for strain KC8. Total DNA extracted from strain KC8 (solid diamonds); total DNA extracted from mixed strain KC8 and activated sludge samples collected from SBR bioreactor (open circles). Equations of the regressions are as follows: $y= 31.78 - 3.86x$, $r^2 = 0.999$ (mixture); $y= 20–3.67x$, $r^2 = 0.998$ (strain KC8). Error bars indicate the range of duplicate real-time PCRs. C_T, threshold cycle.

A-2. Validation of Real-time PCR Assay for Strain ARI-1

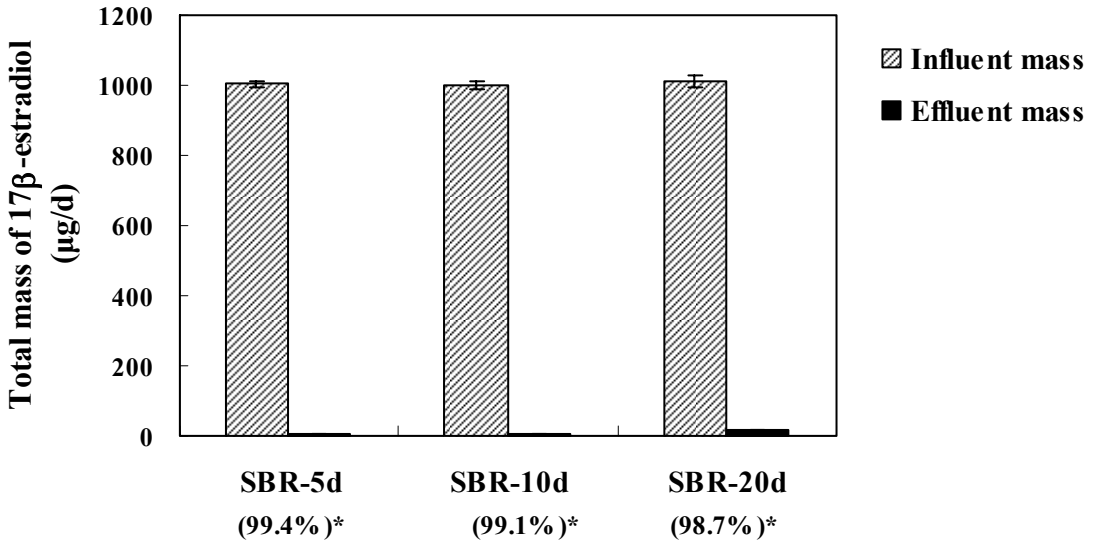


Linear range of template concentrations for real-time PCR assays specific for strain ARI-1. Total DNA extracted from ARI-1 (solid circles); total DNA extracted from mixed strain KC8 and activated sludge samples (open circles). Equations of the regressions are as follows: $y= 33.50–1.42x$, $r^2 = 0.993$ (mixture); $y= 24.83 – 1.49x$, $r^2 = 0.994$ (ARI-1) (S2). Error bars indicate the range of duplicate real-time PCRs. C_T, threshold cycle.

APPENDIX B

REMOVAL OF 17β-ESTRADIOL IN SBRs

B-1. Total Mass of 17β-Estradiol and Percentage Removal in SBRs under 5, 10, and 20d of SRTs



* 17β-estradiol removals

Total mass of 17β-estradiol added and removed of each cycle (24 hr) in SBR-5d, SBR-10, and SBR-20d. Steady state data (i.e. after 2 times of SRT) was used for calculation. Average removal of 17β-estradiol for each SBR was listed in the parenthesis. Influent mass of 17β-estradiol (upward diagonal) and effluent mass of 17β-estradiol (black). Error bars indicate the standard deviation of 17β-estradiol removals, based on 5, 10, and 20 data points for SBR-5d, SBR-10, and SBR-20d, respectively. Influent mass of 17β-estradiol (upward diagonal) and effluent mass of 17β-estradiol (black). Error bars indicate the standard deviation of results of 17β-estradiol removals.

B-2. Sample Calculation of Mass Balance of 17β-estradiol after 3xSRT in SBRs Operating after 3xSRT

Description*	Day 15 at SBR-5d	Day 30 at SBR-10d	Day 60 at SBR-20d
$Mass_{in}$ (ng/d)	$1{,}0x10^6$	$1{,}0x10^6$	$1{,}0x10^6$
$Mass_{out}$ (ng/d)	$5.7x10^3$	$9.9x10^3$	$1.4x10^4$
P_x** (Sludge production,mg-sludge produced /L-treated wastewater	18.9	17.0	16.9
Removal (%)	99.5	99.1	98.7

* The equations (eq 1-2, 4-5) in Pholchan et. al. (140) were used for these calculations.

** $$P_X = \frac{1}{Q}\left[\frac{QY(COD_{\inf} - COD_{eff})}{1+(k_d)SRT} + \frac{(f_d)(k_d)QY(COD_{\inf} - COD_{eff})SRT}{1+(k_d)SRT}\right]$$

where, Q: influent flow (L/d), Y:heterotrophic biomass yield
COD_{inf} and COD_{eff} : influent and effluent COD (mg/L)
k_d : heterotrophic endogenous decay coefficient (0.12gVSS/gVSS-d)
f_d : cell debris fraction (=0.15)

VITA

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