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DEGRADATION OF ESTROGENS BY BACTERIA ISOLATED FROM ANIMAL FARM SOILS

Miss Chanoknad Pornsamrit

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By	Miss Chanoknad Pornsamrit
Field of Study	Environmental Management
Thesis Advisor	Assistant Professor Kanoktip Packdibamrung, Ph.D.
Thesis Co-Advisor	Assistant Professor Somporn Kamolsiripichaiporn, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Manaskorn Rachakornkij, Ph.D.)

.....Thesis Advisor
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Somporn Kamolsiripichaiporn, Ph.D.)

.....Examiner
(Tawan Limpiyakorn, Ph.D.)

.....External Examiner
(Sorawit Powtongsook, Ph.D.)

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สารเอสโทรเจนเป็นฮอร์โมนกลุ่มหลักในร่างกายสิ่งมีชีวิตเพศเมียทำหน้าที่ควบคุมการเจริญและลักษณะต่างๆ ทางเพศ สารเอสโทรเจนที่ถูกกำจัดออกจากร่างกายของสิ่งมีชีวิตจะปนเปื้อนสู่ดินและแหล่งน้ำ ซึ่งจะส่งผลไปรบกวนการทำงานของระบบต่อมไร้ท่อของสิ่งมีชีวิตในระบบนิเวศน์ ทำให้ระดับฮอร์โมนผิดปกติ และเกิดการเปลี่ยนแปลงสรีระทางเพศ งานวิจัยนี้มีวัตถุประสงค์ที่จะคัดเลือกแบคทีเรียจากดินในฟาร์มเลี้ยงสัตว์ที่สามารถย่อยสลายสารเอสโทรเจน 3 ชนิด คือ estrone (E1) 17β-estradiol (E2) และ 17α-ethinylestradiol (EE2) พร้อมทั้งศึกษารูปแบบในการย่อยสลายสารเอสโทรเจนและฮอร์โมนที่เกี่ยวข้องบางชนิด เพื่อนำแบคทีเรียที่แยกได้ไปใช้ในการกำจัด หรือลดปริมาณสารเอสโทรเจนที่ปนเปื้อนในสิ่งแวดล้อม แบคทีเรียในสารละลายดินตัวอย่าง ถูกนำมาเพิ่มปริมาณในอาหารเหลวที่มีสารเอสโทรเจนแต่ละชนิดเป็นแหล่งคาร์บอนและพลังงาน โดยเขย่าที่อุณหภูมิ 30 องศาเซลเซียส และนำไปแยกโคโลนีที่มีลักษณะแตกต่างกันลงบนอาหารแข็ง แล้วนำไปเลี้ยงในอาหารเลี้ยงเชื้อที่มีเอสโทรเจนชนิดเดิม หลังจากนั้นติดตามการสลายเอสโทรเจนด้วยการวัดปริมาณเอสโทรเจนที่เหลือและสารเมแทบอไลท์ ด้วย High-Performance Liquid Chromatography พบแบคทีเรียที่มีความสามารถในการย่อยสลาย E1 จำนวน 4 ไอโซเลท แบคทีเรียที่มีความสามารถในการย่อยสลาย E2 โดยมี E1 เป็นสารเมแทบอไลท์ จำนวน 2 ไอโซเลท และแบคทีเรียที่มีความสามารถในการย่อยสลาย E2 โดยไม่พบสารเมแทบอไลท์จำนวน 7 ไอโซเลท จากนั้นทำการจำแนกชนิดของแบคทีเรียที่มีความสามารถในการย่อยสลายเอสโทรเจนได้ดีจำนวน 8 ไอโซเลท ด้วยลำดับนิวคลีโอไทด์ของยีนส์บน 16S rRNA และสมบัติทางชีวเคมี พบว่า เป็นแบคทีเรียในวงศ์ Alcaligenaceae และสกุล *Microbacterium Planococcus* และ *Pseudomonas* เมื่อนำตัวแทนแบคทีเรียในกลุ่มที่สามามารถย่อยสลาย E1 และ E2 กลุ่มละ 1 ไอโซเลท มาทดสอบความสามารถในการย่อยสลายฮอร์โมน 4 ชนิดคือ E1 E2 EE2 และ methyltestosterone (MT) พบว่า แบคทีเรียทั้ง 2 ไอโซเลทสามารถย่อยสลายทั้ง E1 และ E2 ได้ แต่ไม่สามารถย่อยสลาย EE2 และ MT

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....ชนกนาฏ พรสัมฤทธิ์.....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5087058020: MAJOR ENVIRONMENTAL MANAGEMENT
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CHANOKNAD PORNSAMRIT: DEGRADATION OF ESTROGENS BY BACTERIA ISOLATED FROM ANIMAL FARM SOILS. THESIS ADVISOR: ASSISTANT PROFESSOR KANOKTIP PACKDIBAMRUNG, Ph.D., THESIS CO-ADVISOR: ASSISTANT PROFESSOR SOMPORN KAMOLSIRIPICHAIPORN, Ph.D., 93 pp.

Estrogens, a major group of hormone in female, are responsible for regulation of sexual characteristics. The excretion of estrogens from human or animals leads to contamination in soil and water resources. When contaminated to downstream water, estrogens can interfere with the hormonal system of aquatic livings resulting in unusual development. It is therefore needful to reduce the risk of endocrine disruption from these contaminants. This study aimed to screen for the bacteria capable of degrading estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) from animal farm soils and to study their degradation patterns. The bacteria from soil suspension were cultured in enrich media, containing E1 or E2 or EE2 as the carbon and energy source. Different colonies were picked and cultured in liquid medium containing each of estrogen at 30°C with shaking at 250 rpm. The remaining estrogens and their metabolites in the liquid media were determined by high-performance liquid chromatography. The results showed the existence of 4 bacterial isolates with E1 degradation activities. Two bacterial isolates were also found to degrade E2 to form E1, while other 7 isolates degraded E2 without any detected metabolite. Eight isolates having high degradation activities were identified by their 16S rRNA gene sequences and biochemical properties. They were distributed from family Alcaligenaceae, genus *Microbacterium*, genus *Planococcus* and genus *Pseudomonas*. The representative of E1-degrading bacteria and E2-degrading bacteria were selected to study their abilities to degrade E1, E2, EE2 and methyltestosterone (MT). Both of them showed degradation ability for E1 and E2, but not for EE2 and MT.

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Student's Signature.....Chanoknad Pornsamrit.....
Advisor's Signature.....Kanoktip.....
Co-Advisor's Signature.....Somporn K.....

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LIST OF ABBREVIATIONS

17α	=	17α-estradiol
A	=	2’-deoxyadenosine (in a DNA sequence)
AOB	=	ammonia-oxidizing bacteria
bp	=	base pairs
BLAST	=	Basic Local Alignment Search Tool
C	=	2’-deoxycytidine (in a DNA sequence)
°C	=	degree Celsius
C18	=	octadecyl
DAD	=	diode array detectors
DEO	=	10ε-17β-dihydroxy-1,4-estradien-3-one
DNA	=	deoxyribonucleic acid
dNTP	=	2’-deoxynucleoside 5’-triphosphate
E1	=	estrone
E2	=	17β-estradiol
E3	=	estriol
EDCs	=	endocrine disrupting chemicals
EDTA	=	ethylene diamine tetra acetic acid
EE2	=	17α-ethynylestradiol
G	=	2’-deoxyguanosine (in a DNA sequence)
GC	=	gas chromatography
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	=	high-performance liquid chromatography
IPTG	=	isopropyl-thiogalactoside
IS	=	inorganic salt
K_{ow}	=	octanol-water partitioning coefficient
kb	=	kilobase pairs
LB	=	Luria-Bertani
μg	=	microgram
μL	=	microliter

M	=	mole per liter (molar)
mA	=	milliampare
mg	=	milligram
mL	=	milliliter
mM	=	millimolar
MS	=	mass spectrometry
MT	=	methyltestosterone
MW	=	molecular weight
ng	=	nanogram
nm	=	nanometer
NMR	=	nuclear magnetic resonance
ODS	=	octadecyl silane
PCR	=	polymerase chain reaction
PTFE	=	polytetrafluoroethylene
RNase	=	ribonuclease
rRNA	=	ribosomal ribonucleic acid
SD	=	standard deviation
SDS	=	sodium dodecyl sulfate
T	=	2’-deoxythymidine (in a DNA sequence)
TB	=	Tris-borate buffer
TCA	=	tricarboxylic acid
TE	=	Tris-EDTA buffer
USEPA	=	United States Environmental Protection Agency
USGS	=	United States Geological Survey
UV	=	ultraviolet
V	=	voltage
v/v	=	volume by volume
X1	=	5-hydroxy-15-methyl-13-oxatetracyclo-heptadeca-2(7),3,5-trien-14-one
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER I

INTRODUCTION

1.1 Background and motivation

Existence of steroidal hormones in the environment due to discharges from municipal wastewater and land farming has recently attracted considerable attention for their high potency in causing abnormality to development of aquatic fauna. These hormones are categorized as endocrine disrupting chemicals (EDCs) that are potentially capable of modulating and/or disrupting the function of endocrine system in animals. In addition, many hormones are synthesized for using in pharmaceutical products such as contraceptives and for hormone therapy. This leads to wide spreading of steroidal hormones in ecological system.

Natural steroidal hormones released from human and livestock through excretion are accumulated in the aquatic environment. These hormones are constantly discharged and are not controlled by legal regulation. The primary steroidal hormones are progesterone, estrone, 17β-estradiol, testosterone and cortisol, which are lipophilic and poorly soluble in water. The steroids of major concern are estrone and 17β-estradiol, since they exert their physiological effects at a lower concentration than other steroids and can be found in the environment in concentrations above their lowest observable level for fish and plants (10 ng/L) (Shore *et al*. 2003).

Estrone (E1) and 17β-estradiol (E2) are two common forms of estrogens, a group of sex hormones produced primarily by female ovaries. Estrogens are normally synthesized to stimulate the secondary sex characteristics and also to regulate the function of menstrual cycle. Exposure to estrogen in the environment causes feminization and sexual disruption in many aquatic organisms even at extremely low concentration of nanogram per liter. For example, E2 was found to cause production of egg-yolk protein, vitellogenin, in male fish at the environmental concentration of 1.0 ng/L (Purdom *et al*. 1994).

Recently, some bacteria were found to be able to degrade estrogens such as *Novosphingobium tardaugens* ARI-1 (Fujii *et al*., 2002), *Sphingomonas* sp. strain D12 (Chao *et al.*, 2004), *Rhodococcus zopfii* and *Rhodococcus equi* (Yoshimoto *et al.*,

2004). These bacteria were isolated from activated sludge and municipal wastewater, and had different degradation characteristics. Research works related to degradation of estrogens, especially EE2, by ammonia-oxidizing bacteria from nitrifying activated sludge have been reported (Khongkham, 2008, Likitmongkonsakun, 2008 and Sermwaraphan, 2005).

Animal farms have been reported to release large amount of estrogen into soil and outflow (Lange *et al.,* 2002 and Lorenzen *et al.,* 2004). For example, Lorenzen *et al*. (2004) showed that the estrogen amount in swine manure was as high as 6 mg estrogenic activity, expressed as 17β-estradiol equivalent, per kg dry weight. At this high amount of contamination, the endocrine disrupting effect is naturally occurring in the environment.

The agricultural sector has played an important role in economic development for Thailand. According to the Food and Agriculture Organization, Thailand is one of the largest exporters of agricultural products among Asian countries. Although Thailand is assumed to have a well managed and globalized agricultural sector, the government has been fairly lax in dealing with the negative environmental and biological impact. A major problem can arise in the areas where animal farming are concentrated, mostly in the central region of the country. Not much work has been done so far to find out the extent of estrogen contamination in the environment and the associated impact, as well as the means for environmental control, protection and mitigation.

Bio-remediation is a well accepted technique to get rid of the contaminants in soil and water. It is possible that the bacteria from the estrogen contaminated areas are suitable for estrogen bio-remediation. This study isolated bacteria from soil samples obtained from different animal farms. Three types of estrogen were used as the main carbon source for screening and isolation of estrogen-degrading bacteria. The isolates showing high estrogen-degrading activity were selected for detailed study of estrogen transformation and bacterial identification.

1.2 Hypotheses

The following hypotheses were examined:

1. Many estrogen-degrading bacteria species exist in animal farm soils.
2. These bacteria have different biodegradability for different forms of estrogens.

1.3 Objectives

The objectives of the study were:

1. To screen and isolate the estrogen-degrading bacteria from animal farm soils.
2. To compare the estrogen-degrading ability and estrogen transformation characteristics of the selected bacteria.
3. To study the ability to degrade other related hormones.
4. To check the novelty of the isolated estrogen-degrading bacteria.

1.4 Scope of the study

Investigation in the current study encompassed the followings:

1. Screening of estrogen-degrading bacteria from 5 samples of animal farm soils using 3 types of estrogen: estrone, 17β-estradiol and a synthetic estrogen, 17α-ethynylestradiol.
2. Time course study of estrogen degradation of each isolated bacteria.
3. Examination of the ability of the isolated bacteria to degrade various hormones, i.e. estrone, 17β-estradiol, 17α-ethynylestradiol and methyltestosterone, a synthetic androgen.
4. Identification of bacteria exhibiting high estrogen-degrading activity using biochemical properties and sequence analysis of their 16S ribosomal RNA gene.

CHAPTER II

LITERATURE REVIEW

2.1 General information on steroid hormones

Hormones are chemicals released by one or more cells and exert effects on cells in other parts of the organism. Hormones are grouped into three classes based on their main structure: steroids, peptides or amides.

Steroid hormones are a group of biologically active compounds that are synthesized from cholesterol and have in common a cyclopentan-*o*-perhydrophenanthrene ring (Ying *et al.,* 2002). Five classes of steroid hormones are derived from cholesterol, i.e. progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens (Berg *et al.,* 2002). These hormones are powerful signal molecules that regulate a host of organism functions. Progesterone, a progestagen, is an important hormone in pregnancy and also involved in the female menstrual cycle and embryogenesis of human and other species. Androgens and estrogens are the primary sex hormones; they regulate the development of secondary sex characteristics. Cortisol, the principal glucocorticoid in human, induces enzymes needed for gluconeogenesis. Mineralocorticoids influence the salt and water balance in the body. These hormones exert their actions by passing through the plasma membrane and binding to intracellular receptors that serve as transcription factors to regulate gene expression. These signal compounds regulate metabolism, growth and reproduction in vertebrates.

2.1.1 Synthesis of steroid hormones

The major sites of synthesis of each steroid hormone are different: the corpus luteum for progestagens; the ovaries for estrogens; the testes for androgens; and the adrenal cortex for glucocorticoids and mineralocorticoids. Since cholesterol is the precursor of these five classes of hormones, the carbon atoms in steroids are numbered as shown for cholesterol in Figure 2.1. The rings in steroids are denoted by the letters A, B, C, and D. The synthetic relations of steroid hormones and cholesterol are shown in Figure 2.2. Steroid hormones contain 21 or fewer carbon atoms, whereas

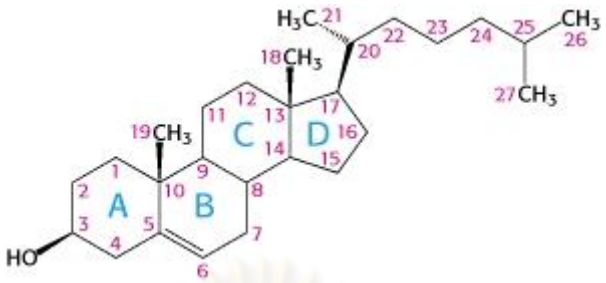


Figure 2.1: Cholesterol carbon numbering

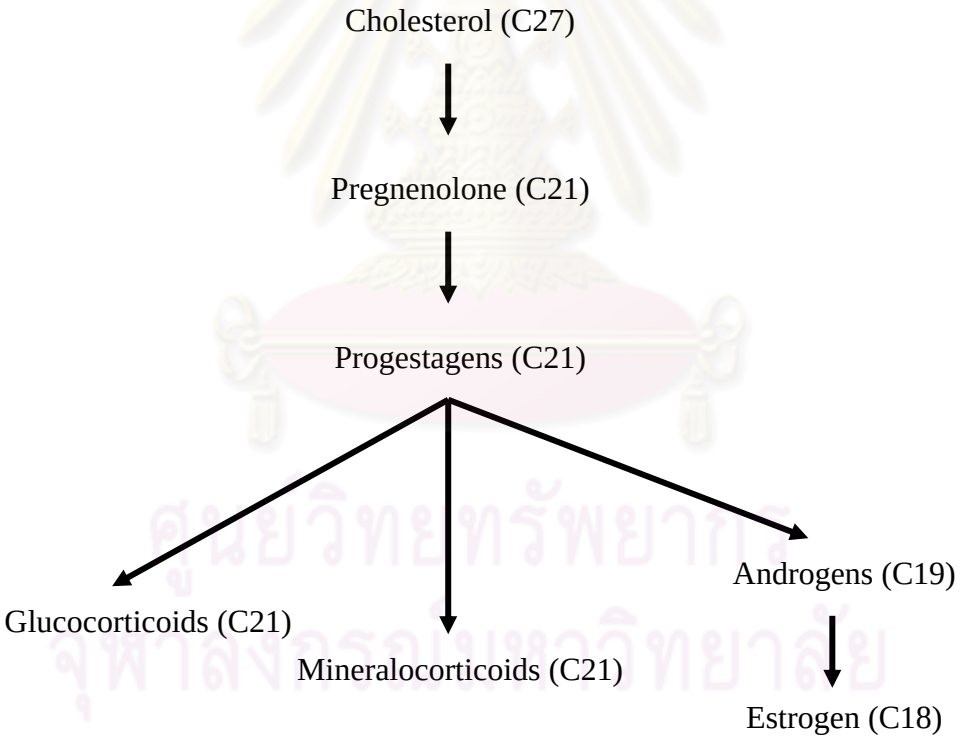


Figure 2.2: Synthetic relations of steroid hormones and cholesterol

cholesterol contains 27 atoms. One main difference between cholesterol and steroid hormones is the absence of the aliphatic side chain in steroid hormones (Figure 2.3). Chloresterol is hydroxylated and shortened to give the C21 intermediates, pregnenolone and progestagen, by removal of the hydrophobic side chain. The other 4 steroid hormones are further synthesized from progestagen. Cortisol, the major glucocorticoid, is synthesized by hydroxylations of progestagen at C11, C17, and C21. The synthesis of aldosterone, the major mineralocorticoid, is initiated by hydroxylation of progestagen at C11 and C21. The following oxidation of the C18 angular methyl group to an aldehyde then yields aldosterone. The synthesis of androgens, which contain 19 carbon atoms, starts with hydroxylation of progestagen at C17. The side chain at C20 and C21 are then cleaved to yield androstenedione. Testosterone, an androgen, is formed by the reduction of the 17-keto group of androstenedione. Estrogens are synthesized from androgens through the loss of the C19 angular methyl group. Estrone, an estrogen, is derived from androstenedione, whereas estradiol, another estrogen, is formed from testosterone (Berg *et al.*, 2002).

2.1.2 Steroid hormones in the environment

Steroid hormones, especially estrogens and testosterone, are frequently detected in the environment and are likely to exert endocrine disrupting effects on aquatic wildlife at concentrations in the nanogram per liter range (Hanselman *et al*., 2003, Sumpter and Johnson, 2005). They are categorized to be endocrine disrupting chemicals (EDCs), which are defined as chemicals that can induce adverse health effects by disruption of an organism's endocrine system or normal development *in vivo* (Ashby *et al.*, 1997). The potential endocrine disrupting effects of estrogens, such as vitellogenin production and feminization of male fish, have been well documented (Jobling *et al.*, 1998, Panter *et al.*, 1998). Testosterone has also been found to cause intersex gonad in newly hatched medaka (*Oryzias latipes*) (Koger *et al.*, 2000).

Human and animals have been the main sources of steroid hormones released into the environment. In human, females normally excrete natural estrogens, estrone and estradiol, each at about 5 μg/day while males excrete androgens, primarily testosterone and androstenedione, each at about 10 mg/day (Hoffmann and Evers, 1986). In addition, substantial amounts of natural and synthetic hormones consumed

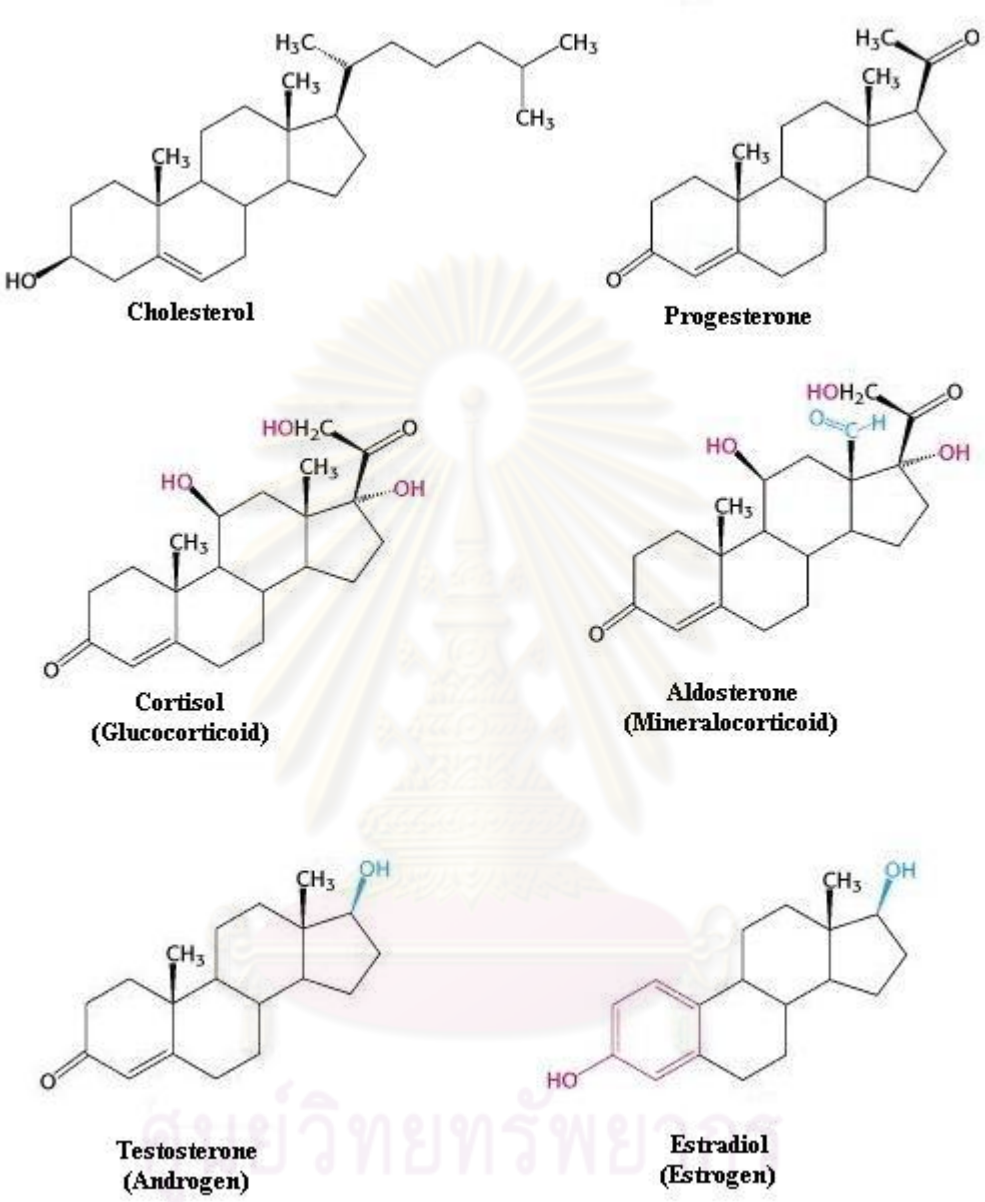


Figure 2.3: Cholesterol and steroid hormones

as pharmaceuticals are excreted by human. Animal manure has also been referred as a major source of natural steroidal estrogen and testosterone in the environment. The use of animal manures for fertilization in the fields and the process of concentrated animal feeding operation have increased the impact of estrogen containing manures on watersheds (Shore *et al.*, 1992). Run-off from the fields following a rain event contained substantial amounts of estrogen and testosterone (1–3 μg/L) (Nichol *et al.*, 1998). With these high amounts of contamination, the concerns over the potentially negative ecological effects of steroid hormones have increased and many researches have focused on this phenomenon.

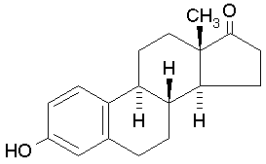
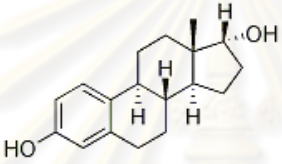
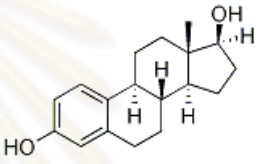
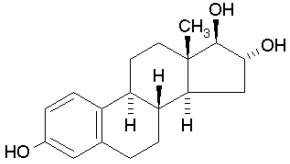
2.2 Estrogens in the environment

The main naturally occurring estrogens in all classes of vertebrates are estrone (E1), 17β-estradiol (E2), and estriol (E3). The other estrogens excreted by animals are 17α-estradiol (17α) from cattle, and equilin from pregnant horses (Shore *et al.*, 2003). Natural steroidal estrogens share the same tetracyclic molecular framework, which is composed of a four ring structure, a phenol, two cyclohexanes, and a cyclopentane. The differences among these compounds are the configuration of the D-ring at position of C16 and C17. For example, E1 has a carbonyl group on C17, E2 has a hydroxyl group on C17, and E3 has two alcohol groups on C16 and C17. E1 and E2 are found to be able to interchange by 17-ketoreductase enzyme. Table 2.1 shows the structures, some physical and chemical properties as well as the biological potency of free estrogens. Estrogens are moderately hydrophobic, poorly soluble in water and have low vapor pressure, hence deemed chemically stable.

2.2.1 Sources and occurrence of estrogens in the environment

Natural estrogens are contributed to the environment predominantly by human and livestock through feces and urine. The excretion rates and types of estrogens in different species have been reported to vary, for example, swine and poultry manures contained high E2 while cattle wastes contained E2 less than 17α (Hanselman *et al*., 2003). Of all types of estrogens, E2 and E1 accounted for more than 95% of the total estrogenic potency of natural steroidal hormone excreted by human and livestock.

Table 2.1: Structures and properties of natural steroidal estrogens

Estrogen Hormone	Estrone (1)	17α-estradiol (2)	17β-estradiol (3)	Estriol (3)
Acronyms	E1	17α	E2	E3
Chemical structure				
Molecular weight (g/mole)	270.37	272.38	272.38	288.38
Solubility in water (mg/L)	0.8-12.4	3.9	5.4-13.3	3.2-13.3
Log K_{ow}*	3.1-3.4	3.94	3.8-4.0	2.6-2.8
Vapor pressure	$3x10^{-8}$	-	$3x10^{-8}$	$9x10^{-13}$
E2 Equivalent**	0.1-0.2	-	1	0.02

Sources: (1) Ternes *et al*., 1999 (2) Wishard *et al.*, 2009 (3) Lai *et al*., 2002

* octanol-water partition coefficient

** The estrogenic activity derived from the bioassay analysis was expressed as the equivalent quantity of E2.

Johnson *et al*. (2000) reported that on an average, 1.6 μg/day of E2 was excreted by human male and 2.3-2.5 μg/day of E2 was excreted by female. Human excretion of E2 can reach as high as 5 mg/day in case of pregnant women (Duguet *et al.*, 2004). Higher excretion was also reported in pregnant animals. A non-pregnant dairy cow excreted approximately 0.8-1.2 mg/day of 17α as compared to 11.4 mg/day in pregnant cow. E1 excretion of a non-pregnant sow was approximately 0.6-1.4 mg/day whereas a pregnant sow excreted up to 10.8 mg/day (Lange *et al.*, 2002 and Lorenzen *et al.*, 2004). Estrogens excreted by animals are usually associated with solids such as municipal sludge and livestock manure. A review by Lange *et al.* (2002) reported the respective annual contribution by cattle, swine, and chicken manure to be 45, 0.8, and 2.7 metric ton in the United States. According to the United States Environmental Protection Agency (USEPA), confined animal feeding operations do not require waste treatment as long as the waste is not disposed directly into the water bodies. However, surface runoff and land application of manure can carry contamination to downstream water including groundwater. Nichols *et al.* (1998) reported an average E2 concentration of 3,500 ng/L in surface runoff following poultry litter application to grassland. Furthermore, an E2 concentration of 37.6 ng/L was detected in aquifers underlying areas where animal manure was applied (USEPA, 2002). A survey of 139 streams from 30 states conducted by United States Geological Survey (USGS) in 1999-2000 revealed that these water bodies contained E1 and E2 as high as 112 and 200 ng/L. Moreover, estrogenic hormones are frequently administered to livestock as growth promoters. This may increase their urine output of estrogens (Herschler *et al.*, 1995). Callantine *et al.* (1961) found that giving E2 to livestock resulted in 5-6 fold increase in urine estrogen production.

2.2.2 Effects of environmental estrogens

Normally estrogens are excreted in the conjugated forms after esterification of free estrogens with glucuronide and/or sulfate groups at the position(s) of C3 and/or C7. The conjugated parts increase the solubility as compared to the free forms. Panter *et al*. (1999) showed that the conjugated forms (estrogenically inactive forms) excreted from humans and animals were converted back into free estrogens (active forms) by bacterial enzymes in the raw wastewater and during the wastewater

treatment processes. These free estrogens were reported to cause endocrine disruption. As shown in Table 2.1, E2 was the most potent estrogenic compound and has been defined as the reference value of 1.0, while E1 and E3 had lower potency. E2 was likely to be responsible for the majority of the estrogenic effects found in the environment (Khanal *et al*., 2006). In addition, the use of a synthetic estrogen, 17α-ethynylestradiol (EE2), as an oral contraceptive and hormonal supplement led to contamination through human excretion. EE2 was reported to be capable of inducing biological effects at the environmentally relevant concentrations (Haiyan *et al.,* 2006).

Human and animal waste-borne steroidal estrogens are referred as endogenous steroidal EDCs, which is characterized by extremely high estrogenic potency, 10,000-100,000 times higher than exogenous EDCs, or synthetic chemical. A number of aquatic species were reported to be sexually reversed by the presence of estrogens. In 1998, Routledge *et al.* reported that exposure of E2 at the level of 10 ng/L significantly induce production of vitellogenin, a female protein, in male fathead minnows, *Pimephales promelas*. E2 at a concentration of 5 ng/L induced the production of female specific proteins in male Japanese medaka (Tabata *et al*., 2001). Less than 1 ng/L of EE2 has been shown to stimulate the vitellogenin production in male fathead minnows (Purdom *et al.*, 1994). Similarly, a concentration of 4 ng/L of EE2 can cause failure in male fathead minnows to develop normal secondary sexual characteristics (Lange *et al.*, 2001). With these negative effects on the environment, many studies focused on fate and transport of estrogens as well as removal of hormone from contaminated sites (Casey *et al*., 2003 and Khanal *et al*., 2006).

2.2.3 Removal of environmental estrogens

Removal of estrogen compounds from aqueous phase could be achieved through three major pathways: volatilization, adsorption and degradation (Schoenberg *et al.*, 1994).

2.2.3.1 Volatilization

Volatilization of natural estrogens from liquid phase into gas phase could be judged theoretically by Henry's law constants. It is a relative concentration between aqueous phase and gas phase. The higher the Henry's law constant value, the more

partial pressure in the gas phase the chemical tends to have. Table 2.1 indicates low vapor pressures of estrogens, thus, the Henry's law constants are relatively low. The removal of estrogens by volatilization was likely to be ineffective under normal temperature and pressure conditions and their loss from the aqueous phase through volatilization tended to be limited (Khanal *et al.*, 2006).

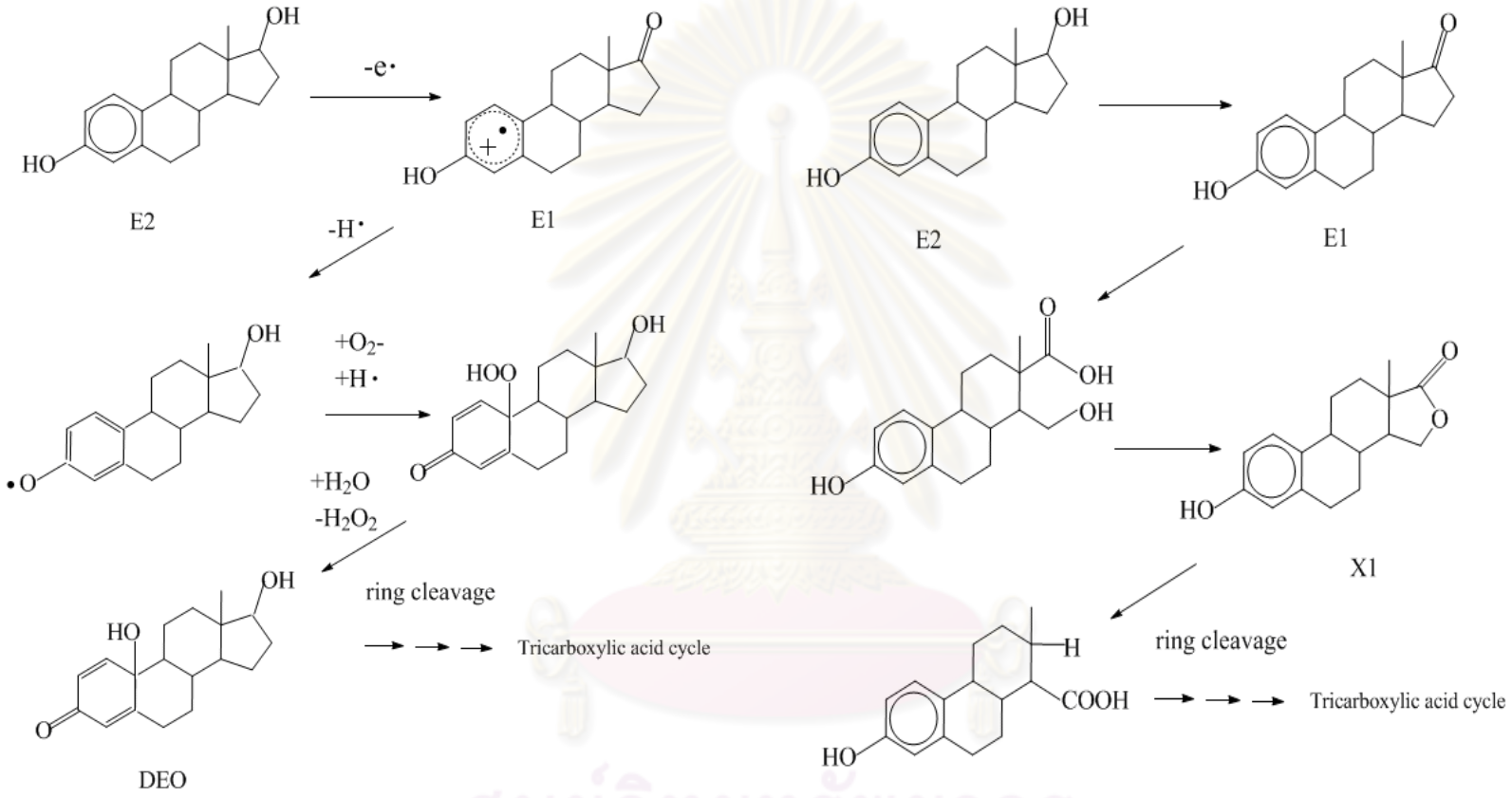
2.2.3.2 Adsorption

Due to the hydrophobic property of estrogen, these compounds are mainly removed by adsorption onto associated solid phase, such as sludge in wastewater treatment or soil in case of land application. It has been reported that during the sewage wastewater treatment processing, 50-90% of E2 was removed by adsorbing onto activated sludge and by other mechanism independent of microbial degradation. However, the proportion of steroidal estrogens remaining in the effluent was still capable of inducing the estrogenic effects (Routledge *et al.*, 1998).

2.2.3.3 Degradation

A study by Danish Environmental Protection Agency (2004) indicated that degradation of free estrogens was achieved mainly through a biotic route, whereas under abiotic conditions, the estrogen level remained fairly constant at an initial estrogen level of 500 ng/L E2 equivalent. Colucci and Topp (2001) and Colucci *et al.* (2001) found that E2 could be abiotically degraded but E1 and 17α were only biotically degraded. Schlenker *et al.* (1998) observed 80% estrogen removal in cattle manure following 12 weeks of incubation at 20-23°C. Waterborne algae and some aquatic bacteria were also capable of enhancing E2 oxidation into E1 (Lai *et al.*, 2001 and Matsuoka *et al.*, 2005). However, the oxidation of E2 into E1 is considered incomplete in terms of estrogenicity removal because E1 still retains estrogenicity level at 0.1-0.2 of E2 equivalent. Ohko *et al*. (2002) reported the TiO_2-assisted photocatalytic degradation pathway as shown in Figure 2.4 (A). At first, E2 was chemically degraded at the phenol ring A to intermediate product DEO (10ε-17β-dihydroxy-1,4-estradien-3-one) and finally to carbon dioxide through the TCA cycle.

Bacteria present in wastewater have been found to be capable of completely degrading estrogenic compounds into harmless products. Therefore, estrogens are



(A) Degradation by TiO_2 photocatalyst (B) Biodegradation by sewage bacteria

Figure 2.4: Pathways of estrogen degradation

Sources: (A) Ohko *et al.*, 2001 (B) Lee *et al.*, 2002

suggested to be mainly removed via biodegradation during wastewater treatment (Khanal *et al.*, 2006). The study of estrogen-biodegradation has mostly focused on the degradation of E2 due to its high estrogenicity. Lee *et al*. (2001) studied the biodegradation pathway of estrogen by sewage bacteria as illustrated in Figure 2.4-B. E2 was oxidized from the cyclopentane ring D at C17 into E1 during enzymatic degradation and then further degraded into metabolite X1 (5-hydroxy-15-methyl-13-oxatetracyclo-heptadeca-2(7),3,5-trien-14-one) and finally to carbon dioxide through the TCA cycle.

Several estrogen-degrading bacteria were isolated from activated sludge and municipal wastewater; each showed different degradation characteristics. The first reported 17β-estradiol-degrading bacterium, *Novosphingobium tardaugens* ARI-1, was isolated by Fujii *et al.* (2002) from activated sludge of a sewage treatment plant in Tokyo, Japan. ARI-1 took 480 hours to degrade 5 mg of E2 in 30 mL of medium. ARI-1 was also found to degrade E1 and E3. In determining the metabolites from E2 degradation using GC-MS and ^{1}H-NMR, they suggested that E2 was degraded by ARI-1 to simple organic acids or compounds with very low molecular mass. Thus, no toxic or accumulative metabolites of E2 were produced from the degradation pathway.

Ammonia-oxidizing bacterium (AOB), *Nitrosomonas Europaea,* isolated from nitrifying activated sludge was found to degrade estrogens (Shi *et al*., 2004). AOB are a group of bacteria usually responsible for the oxidation of ammonia to nitrite, which exclusively produced energy used for bacterial growth. They are also known to be capable of oxidizing various hydrocarbons. *N. Europaea* degraded 95% of 0.4 mg/L E1 and E2 within 187 hours. It also degraded E3 and EE2. Chao *et al*. (2004) isolated *Sphingomonas* species (D12) from soil. D12 could degrade 800 mg of E2 in 4 mL of medium completely in 8 days (the detection limit was 0.25 mg/L). During degradation of E2 by D12, E1 was present as a metabolite and was degraded with higher rate than that of E2.

Yoshimoto *et al*. (2004) performed the experiment using enrichment culture of activated sludge from wastewater treatment plants; four strains of bacteria were found to degrade estrogens, namely Y50155, Y50156, Y50157 and Y50158. Y50158 was identified as *Rhodococcus zopfii*, while the other three strains were similar to *Rhodococcus equi*. These four strains degraded 100 mg/L of E2 and E1 in 10 mL of

medium completely in 24 hours, and E3 was degraded about 80% in 24 hours. They further tested the estrogenic activity of the degradative products, using MVLN human breast cancer cell. It was suggested that these four strains degraded E2 to a substance with loss of estrogenic activity.

Recently, Yu *et al.* (2007) isolated fourteen phylogenetically diversed E2-degrading bacteria (strain KC1-14) from activated sludge. These isolates widely distributed among eight genera; *Aminobacter* (strains KC6 and KC8), *Brevundimonas* (strain KC12), *Escherichia* (strain KC13), *Flavobacterium* (strain KC1), *Microbacterium* (strain KC5), *Nocardioides* (strain KC3), *Rhodococcus* (strain KC4), and *Sphingomonas* (strains KC8, KC11 and KC14). All strains were capable of converting E2 to E1, but only three strains (KC6, KC7 and KC8) showed the ability to degrade E1. Based on the degree of estrogen transformation, three different degradation patterns were observed (pattern A-C). Eleven out of fourteen isolates showed degradation pattern A, where E2 was stoichiometrically converted to E1, but E1 was not further degraded. Strains KC6 and KC7 exhibited degradation pattern B, where both E2 and E1 were degraded although E2 degradation was slower than that observed in pattern A. Strain KC8 was the only strain exhibiting degradation pattern C, where both E2 and E1 were rapidly degraded within 3 days.

A few studies investigated further the metabolic pathway of EE2 degradation. EE2 was shown to be removed by co-metabolism of AOB (Shi *et al.*, 2004). In 2006, Haiyan *et al.*, isolated a bacteria from activated sludge in wastewater treatment plant of an oral contraceptive producing factory in China. The bacteria, *Sphingobacterium* sp. JCR5, grew on EE2 as sole carbon and energy source and metabolized up to 87% of EE2 added (30 mg/L). In Thailand, Sermwaraphan (2006) studied the AOB from nitrifying activated sludge capable of degrading EE2 via co-metabolism. These bacteria could also degrade E2 which was found to be competitive in the degradation of EE2.

In the previous studies, activated sludge seemed to be a good source of the estrogen-degrading bacteria. In some cases, soil and sediment were used as starting materials. Bacteria in animal manure were also reported to be capable of degrading estrogens. Raman *et al.* (2001) tested estrogen degradation in swine and cattle manures. They found that E2 concentration dropped sharply during the first 24 hours

of incubation under aerobic conditions while E1 was accumulated and reached a peak concentration in 48 hours. The total estrogenic activity measured by yeast screen decayed following first-order kinetics and the rate constants increased with temperature from 0.03 per day at 3°C to 0.12 per day at 5°C. The manure microorganism *Cornybacterium* spp. was believed to be responsible for the biodegradation of both E1 and E2. In this study, the different types of animal farm soil were used as sources of the bacteria. The new estrogen-degrading bacteria isolated from these animal farm soils are expected to support further application to prevent and control contamination of estrogen in the environment and can be applied in wastewater treatment to decrease the potential risk of estrogen to human and ecosystem.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental framework

This experiment aimed to screen the estrogen-degrading bacteria and study their degradation time course and their ability to degrade the related forms of hormone. The experimental framework of this study is shown in Figure 3.1. Animal farm soils at the Demonstration Farm, Department of Animal Husbandry, Faculty of Veterinary Sciences, Chulalongkorn University in Nakhonpathom Province were used as bacterial sources. In primary screening, bacteria were enriched in liquid media containing various estrogens as the carbon and energy source. Each single colony was isolated and screened by replica plating on IS-agar plate with and without estrogen. The positive colonies, growing on IS-agar plate with estrogen but not on the control plate, were then separately cultured in each estrogen medium for secondary screening. The bacterial isolates showing high estrogen-degrading ability were studied for their degradation time course and were identified using their morphologies as well as 16S rRNA gene sequences and their biochemical properties. The bacterium having the best degrading activity of each estrogen was investigated whether it could degrade other hormones of similar structure, such as E1, E2, EE2 and MT (Figure 3.2). In addition, in order to check the photolysis of E2 and EE2, two control sets with and without light irradiation were carried out.

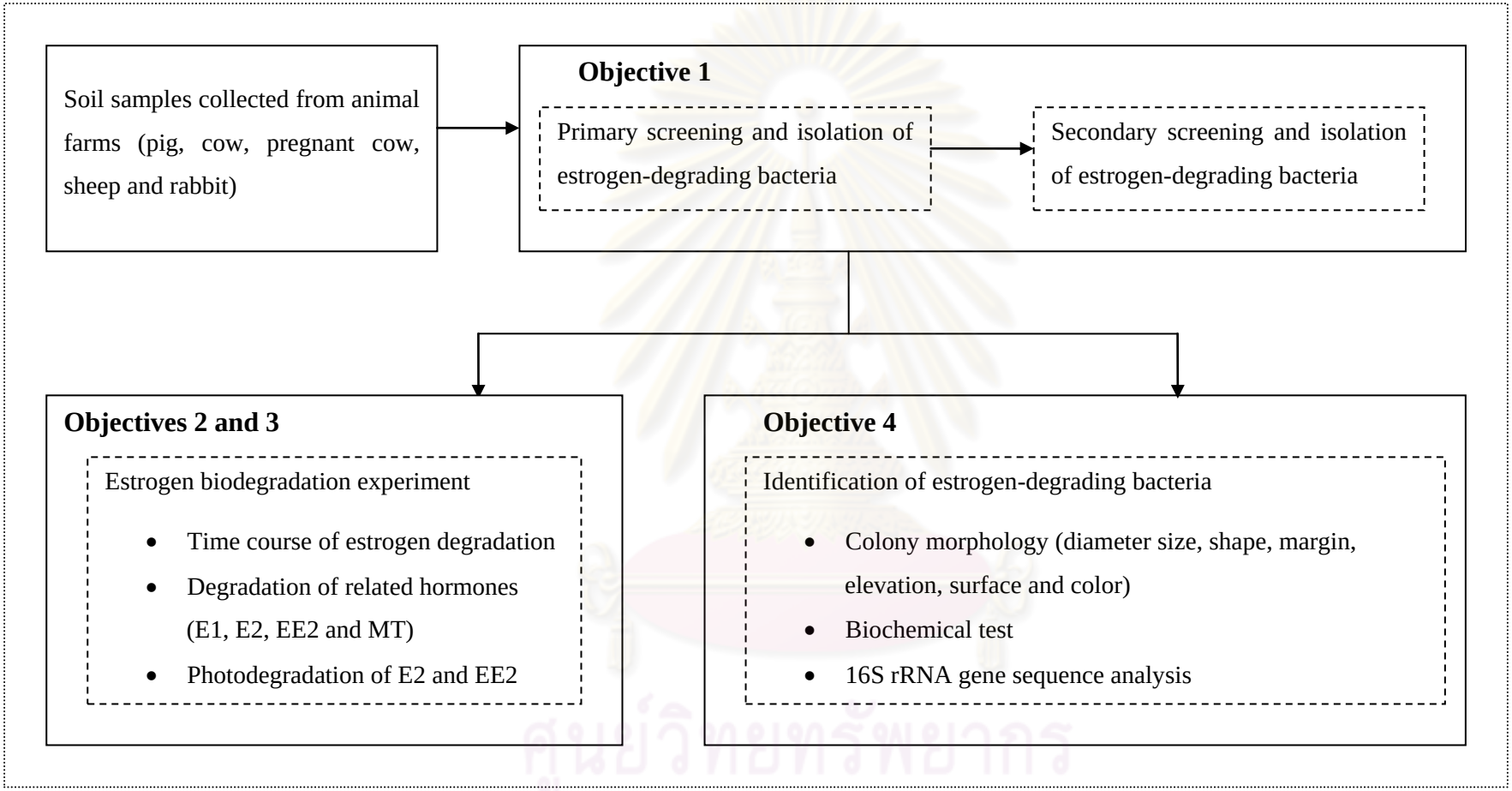
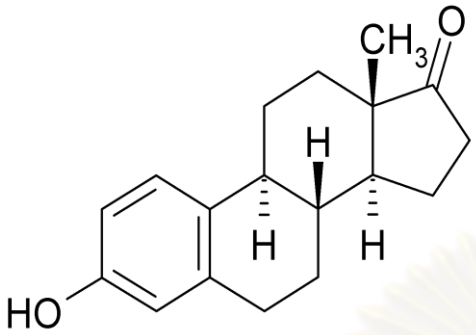
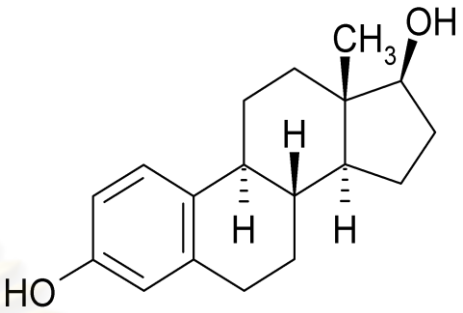


Figure 3.1: Experimental framework

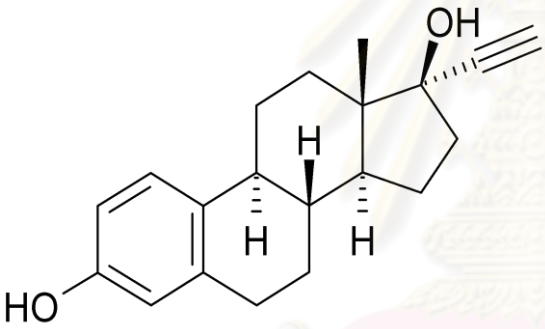
(A) Estrone



(B) 17β-Estradiol



(C) 17α-Ethynylestradiol



(D) Methyltestosterone

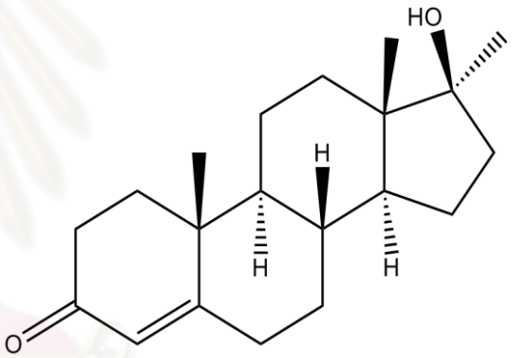


Figure 3.2: Structure of steroidal hormones

3.2 Materials

3.2.1 Equipments and accessories

Autoclave: Model MLS-2420, Sanyo Electric Co., Ltd, Japan

Autopipette: Pipetman, Gilson, France

Gene Pulser^{R}/*E. coli* Pulser^{TM} Cuvettes: Bio-Rad, USA

Gel Documentation: BioDoc-It^{TM} Imaging system, UVP, USA

Heating box: Type 17600 Dri-Bath, Thermolyne, USA

High-Performance Liquid Chromatography (HPLC): Class 20AD, SHIMADZU, Kyoto, Japan)

HPLC column: Reversed phase HPLC Inertsil ODS-3, 250 mm x 4.6 mm x 5 μm column, GL Sciences Inc., Japan

Horizontal electophoresis: Gelmate 2000, Toyobo, Japan

Incubator shaker: Model E24R, New Brunswick Scientific, USA

Incubator waterbath: Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc.,USA

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

Membrane filter: 0.2 μm VertiClean polytetrafluoroethylene (PTFE) syringe filter, Vertical Chromatography Co., Ltd., Thailand

Membrane filter: 0.2 μm polyesthersulfone membrane, Whatman, USA

Microcentrifuge: Microfuge 22R, Beckman Instrument Inc., USA

Microcentrifuge tubes 0.5 and 1.5 mL, Axygen Hayward, USA

pH meter: Model S200, METTLER TOLEDO Co.,Ltd., Switzerland

Refrigerated centrifuge: J-30I, Beckman Instrument Inc., USA

Thermo cycler: Mastercycler gradient, eppendorf, Germany

Thin-wall microcentrifuge tubes 0.2 mL, Axygen Hayward, USA

Vortex: Model K-550-GE, Scientific Industries, Inc, USA

3.2.2 Chemicals

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, USA

Ammonium chloride: M&B, England

Ampicillin: Sigma, USA

Boric acid: Merck, Germany

5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal): Sigma, USA

Bromphenol blue: Merck, Germany

Calcium chloride 2-hydrate: Scharlau, Spain

Chloroform: BDH, England

Cobalt chloride 6-hydrate: Ajax Finechem, New Zealand

Copper sulfate 6-hydrate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylene diamine tetra acetic acid: M&B, England

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., USA

100 base pair DNA ladder, Promega Co., USA

17β-Estradiol (E2): Sigma, USA

Estrone (E1): Sigma, USA

Ethidium bromide: Sigma, USA

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

17α-Ethynylestradiol (EE2): Sigma, USA

Ferric ethylene diamine tetraacetic acid (FeEDTA): Sigma, USA

Geneaid gel/PCR DNA fragments extraction kit: Geneaid, USA

High-speed plasmid mini kit: Geneaid, USA

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): Sigma, USA

Isopropyl-β-D-thiogalactopyranoside (IPTG): US Biological, USA

Methanol: Lab-Scan, Thailand

17α-Methyltestosterone (MT): Fluka, Switzerland

Magnesium sulphate 7-hydrate: BDH, England

Manganese chloride 4-hydrate: Carlo Erba Reagenti, Italy

Nickle chloride 6-hydrate: Ajax Finechem, New Zealand

Peptone from casein pancreatically digested: Merck, Germany

Phenol solution: Sigma, USA

Potassium hydroxide: Scharlau, Spain

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate (SDS): Sigma, USA

Sodium hydroxide: Merck, Germany

Sodium molybdate 2-hydrate: Ajax Finechem, New Zealand

Sodium nitrate: Sigma, USA

Yeast extract: Scharlau microbiology, European Union

Zinc chloride: Ajax Finechem, New Zealand

3.2.3 Enzymes and restriction enzymes

Lysozyme: Sigma, USA

Proteinase K: Sigma, USA

Restriction enzymes: *Eco*RI, New England BioLabs, Inc., USA

RNaseA: Sigma, USA

Taq DNA polymerase: New England BioLabs, Inc., USA

T_4 DNA ligase: New England BioLabs, Inc., USA

3.2.4 Bacterial strain and plasmid

Escherichia coli strain JM109

pGEM®-T Easy Vectors: Promega, USA

3.2.5 Media

3.2.5.1 Inorganic salt medium

Inorganic salt medium (IS medium) was used as minimal medium in this experiment. It was prepared using the protocol described by Chao *et al*. (2004). IS medium contains 2 g of NH_4Cl, 1 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of FeEDTA, 0.05 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of K_2HPO_4, 0.6 mg of $MnCl_2 \cdot 4H_2O$, 0.5 mg of H_3BO_3, 0.1 mg of $ZnCl_2$, 0.1 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.6 mg of $CoCl_2 \cdot 6H_2O$, 0.12 mg of $NiCl_2 \cdot 6H_2O$, 0.12 mg of $CuSO_4 \cdot 5H_2O$ and 4 g of HEPES in 1 liter of milli-Q

water at pH 7.0. In preparing 1 liter of IS-medium, the chemicals were dissolved in 50 mL of autoclaved water and the solution was filtered through a 0.2 μm pore-size polyestersulfone membrane filter into a flask containing 950 mL of autoclaved water. For plate preparation, 15 g of agar was added in 1 liter of IS medium.

3.2.5.2 Luria-Bertani medium

Medium for enrichment or rich medium used in this experiment was Luria-Bertani medium (LB medium). LB medium contains 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 liter of milli-Q water at pH 7.2. The medium was sterilized by autoclaving. For plate preparation, 15 g of agar was added in 1 liter of LB medium.

3.2.5.3 LB Plate with ampicillin/IPTG/X-gal

LB plate with ampicillin was prepared by autoclaving 1 liter of LB with 15 g of agar. After the medium was cooled down to 50°C, ampicillin was added to a final concentration of 100 μg/mL. This medium was then poured into Petri dishes. The LB-ampicillin plate was spread over by 100 μL of 100 mM IPTG and 20 μL of 50 mg/mL of X-gal, respectively.

3.3 Methods

3.3.1 Sample collection

Soil samples were collected from drainage areas, where animal excretions are concentrated (Figure 3.2), at various animal farms (pig, cow, pregnant cow, sheep and rabbit) of the Department of Animal Husbandry, Faculty of Veterinary Sciences, Chulalongkorn University in Nakhonpathom Province. The soil samples were stored in the refrigerator at 4°C until used.

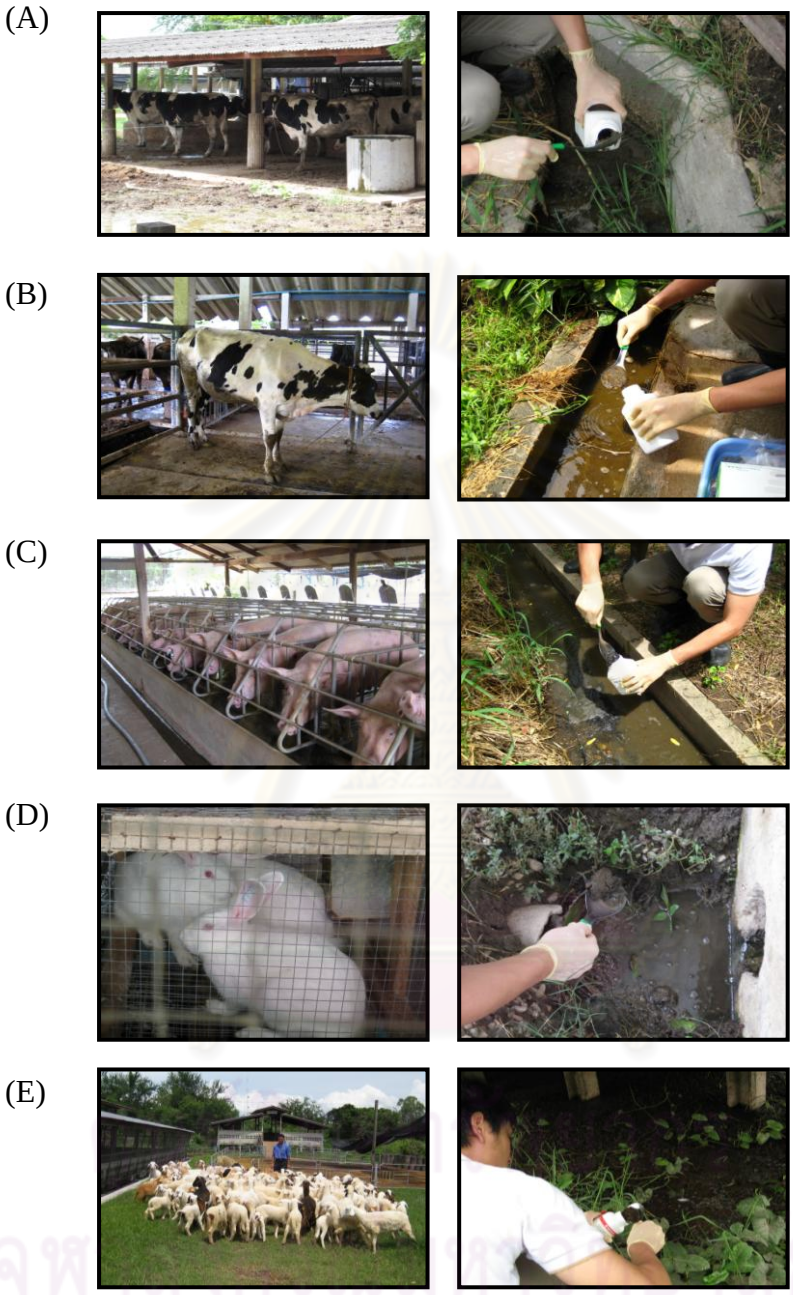


Figure 3.3: Soil sample collection: (A) cow farm; (B) pregnant cow farm; (C) swine farm; (D) rabbit farm; and (E) sheep farm

3.3.2 Screening and isolation of estrogen-degrading bacteria

3.3.2.1 Primary screening and isolation of estrogen-degrading bacteria

To screen bacteria possessing estrogen-degrading activity, 10 g of soil samples were first suspended in 10 mL of tap water, 2 mL of each soil suspension was then added into 18 mL of IS medium supplemented with 100 mg/L of each estrogen (E1, E2 or EE2). The samples were incubated at 30°C with rotation at 250 rpm. Estrogen utilizing bacteria were enriched by sub-culturing 5 mL of each sample to a new sterile medium at day 5 and day 10. At day 15, the cultures were diluted with IS medium by 10^{-4}, 10^{-6} and 10^{-8} folds and spread onto the LB-agar plate. After incubation at 30°C for 2 days, the bacterial colonies of different morphology observed on each plate were picked and each colony was streaked separately onto a new LB-agar plate. The obtained colonies were then confirmed for their abilities to utilize each estrogen by replica plating onto IS-agar plate, as a control, and onto IS-agar plate coated with 200 μg of each estrogen on the surface. Colonies that grew well only on the plate with estrogen were used for secondary screening.

3.3.2.2 Secondary screening and isolation of estrogen-degrading bacteria

Single colony of each isolate obtained from section 3.3.2.1 was separately cultured in 3 mL of IS medium supplemented with 20 mg/L and 100 mg/L of each estrogen at 30°C with rotation at 250 rpm. At day 10 of cultivation, the amount of estrogen left in the medium was analyzed by HPLC. An equal volume of methanol was added to the sample to completely solubilize estrogen. In case of sample culturing in 100 mg/L of estrogen, two volumes of methanol was added. The mixture was centrifuged and the supernatant was filtered through a 0.2 μm pore-size PTFE filter prior to application onto HPLC. In the HPLC analysis, 40 μl of sample was separated on reverse phase C18 column at 40°C using 60% v/v acetonitrile/water as mobile phase with the flow rate of 0.5 mL/min. Absorbance at wavelength of 210 nm was read using diode array detector (DAD) (SPD-20A, SHIMADZU, Kyoto, Japan). The absorbance value was transformed into μg by comparing with the standard curve (see Appendix A).

The bacterial strains showing high estrogen-degrading activity were selected for further study in section 3.3.3.

3.3.3 Estrogen degradation experiment

3.3.3.1 Time course of estrogen degradation

Time course studies were carried out for estrogen degradation. Seed culture was prepared by culturing single colony of each selected bacterium in 5 mL of LB medium at 30°C with shaking at 250 rpm for 18 hours and was then transferred into a flask containing 100 mL of LB medium for further 3 hours cultivation. Cells were collected by centrifugation and washed twice with 2 volumes of IS medium and re-suspended in 15 mL of IS medium. Five mL of cell suspension were added to 95 mL of IS medium containing 15 mg/L of estrogen to yield approximately 10^9 colony forming unit/mL before further cultivation at 30°C with rotation at 250 rpm. The amount of estrogens left in the medium was measured by HPLC as described in section 3.3.2.2, every 24 hours for 4 days. The bacteria showing high estrogen-degrading activity were used for further study of their ability to degrade related hormones degradation as described in section 3.3.3.2.

3.3.3.2 Degradation of related hormones

To investigate whether the selected bacteria were capable of degrading the hormones of similar structure, time course studies of various hormones (E1, E2, EE2 or MT) were carried out. Concentration of hormones in IS medium were 15 mg/L. Cell collection and culturing condition were performed as described in section 3.3.3.1. In this experiment, control set was prepared using IS medium supplemented with 15 mg/L of each hormone without bacterial inoculation.

3.3.3.3 Photodegradation of estrogens

Photolysis of E2 and EE2 were reported by Mazellier *et al.* (2008) in aqueous solution. Degradation time course of E2 and EE2 without bacterial inoculation were performed to check if any natural degradation took place in the experimental samples. A flask containing 100 mL of IS medium with 15 mg/L of each estrogen, E2 or EE2, was incubated at 30°C with rotation at 250 rpm as a normal condition set. Another flask with the same amount of IS medium and estrogen was covered with aluminium foil before incubation was used as a control set without any light irradiation. The

amount of estrogen left in the medium was measured by HPLC every 24 hours for 4 days.

3.3.4 Identification of estrogen-degrading bacteria

3.3.4.1 Colony morphology

Each estrogen-degrading bacterium was streaked on an LB agar plate. The colony morphologies were observed for their diameter size, shape, margin, elevation, surface and pigment. Single colony of each bacterium was further analyzed for its biochemical properties.

3.3.4.2 Biochemical properties of estrogen-degrading bacteria

Biochemical properties of estrogen-degrading bacteria strain were analyzed by the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Thailand.

3.3.4.3 Analysis of 16S rRNA gene sequence

3.3.4.3.1 Chromosomal DNA extraction

Chromosomal DNA was extracted by the method of Frederick *et al.*, (1995). A single colony was inoculated into 10 mL of LB medium and incubated at 30°C for 24 hours with shaking. Cell culture of 1.5 mL was centrifuged in a microcentrifuge tube at 8,000xg for 2 minutes. The cell pellet was resuspended in 550 μL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. The cell solution was then treated with 3 μL of 5 mg/mL lysozyme, 2 μL of 10 mg/mL RNaseA, 30 μL of 10 % SDS followed by 3 μL of 20 mg/mL proteinase K and incubated for 1 hour at 37^{o}C. After incubation, the DNA was extracted by an equal volume of phenol-chloroform (1:1 v/v) and centrifuged at 12,000xg for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube. DNA was precipitated by addition of 5 M NaCl to the final concentration of 1 M and 2 volumes of absolute ethanol, before keeping at -20^{o}C for at least 30 minutes. Afterwards, the DNA solution was centrifuged at 12,000xg for 10 minutes. DNA pellet was collected and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer. Finally, DNA concentration was

estimated by submarine agarose gel electrophoresis in comparison with known amount of λ/*Hin*dIII marker. Preparation of reagents is described in Appendix B.

3.3.4.3.2 Agarose gel electrophoresis

The 0.8 g of agarose was added to 100 mL electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in Erlenmeyer flask and heated until complete solubilization in a microwave oven. The agarose solution was left at room temperature to 50^{o}C before pouring into an electrophoresis mould. When the gel was completely set, the DNA samples were mixed with gel loading dye and loaded onto agarose gel. Electrophoresis was performed at constant voltage of 8 volt/cm until the loading dye migrated to appropriate distance on the gel. The gel was stained with 2.5 μg/mL ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was determined by comparison of band intensity and relative mobility with those of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

3.3.4.3.3 PCR amplification

Extracted DNA was used as a template for PCR amplification of 16S ribosomal RNA gene using the universal primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGC TAC CTT GTT ACG ACT T-3'). The total volume of PCR reaction was 50 μL consisting of 2.5 units of *Taq* polymerase, 1x standard *Taq* reaction buffer, 0.2 μM of each primer and 0.2 mM of dNTPs mixture. PCR amplification was performed in Eppendorf mastercycler gradient under the conditions of predenaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing temperature (from 42 to 58.6°C) for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. After 30 cycles of amplification, the product from PCR was purified by Geneaid gel/PCR DNA fragments extraction kit.

3.3.4.3.4 Cloning for sequencing

PCR products obtained from section 3.3.4.2.3 were cloned into pGEM®-T Easy Vectors. The total volume of ligation reaction was 20 µL consisting of 25 ng of PCR product (1.5 kb), 50 ng of pGEM®-T Easy Vectors and 4,000 units of T4 DNA ligase and made up the volume with 1x rapid ligation buffer. The reaction mixture was chilled at 4°C for 18 hours. The recombinant plasmids obtained were further used for transformation.

3.3.4.3.5 Transformation

The recombinant plasmids were transformed into competent cells of *E. coli* JM109 by electroporation. In the electroporation step, cuvette and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set at 25 µF capacitor, 2.5 kV, and the pulse controller unit was set at 200Ω. Competent cells were gently thawed on ice. Two microliters of recombinant plasmid was mixed with 40 µL of competent cells and placed on ice for 1 minute. This mixture was transferred to a cold cuvette and the cuvette was applied to the Gene Pulser apparatus with one pulse. Subsequently, 1 mL of LB medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette and transferred to new tube, incubated at 37^{o}C for 1 hour with shaking. Cell culture was centrifuged to eliminate 800 µL of the medium and then resuspended to 200 µL. Finally, cell suspension was spread onto the LB plate with ampicillin/IPTG/X-gal (section 3.2.5.3) and incubated at 37^{o}C for 12 hours. White colonies growing on the selective plate were picked and plasmids were extracted using high-speed plasmid mini-kit. The extracted plasmids were digested with restriction enzyme *Eco*RI to determine the fragment size of insert.

3.3.4.3.6 Sequencing

Recombinant plasmid containing about 1.5 kb insert was sequenced by Macrogen Inc. laboratory, Seoul, Korea. Sequences were analyzed by Basic Local Aliment Search Tool (BLAST), (National Center for Biotechnology Information, Bethesda, Maryland, USA).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Primary screening of estrogen-degrading bacteria

For screening of estrogen-degrading bacteria, the concentrations of estrogens used in the previous reports were usually in the range of 100-200 mg/L (167 mg/L, Fujii *et al*., 2002; 150 mg/L, Chao *et al*., 2004; and 100 mg/L, Yoshimoto *et al*., 2004). Yu *et al.* (2007) successfully screened 14 phylogenetically diversed E2-degrading bacteria at a very low concentration of 3 mg/L. In this study, the bacteria from each soil suspension were subjected to primary screening in IS medium containing 100 mg/L of E1, E2 or EE2 as described in section 3.3.2.1. Due to the low solubility of estrogens, the solid form of each estrogen was observed in each tube. Once the bacteria degraded the soluble estrogen, the solid part would be solubilized to its equilibrium. With 3 times subculturing, the viable cells in the media were expected to use estrogen as carbon source. There were 84, 83 and 62 colonies from E1, E2 and EE2 enrichment media, respectively. These colonies were then confirmed for their ability to utilize estrogens by replica plating to IS-agar plate with estrogen (estrogen plate) and without estrogen (control plate) as described in section 3.3.2.1. The positive colonies showing growth on estrogen plate but not on the control plate, had the tendency to utilize estrogens. Four and 10 colonies with positive growth on IS-agar plates with E1 and E2 were obtained, respectively. However, no bacteria exhibited positive growth on EE2 plate. In contraceptive plants that produced EE2, Haiyan *et al*. (2007) isolated *Sphingobacterium* sp. JCR5 that was capable of degrading EE2. This is not surprising as EE2 is a synthetic estrogen and it is not normally used in farming activities. Moreover, it was reported that ammonia-oxidizing bacteria from nitrifying activated sludge were capable of degrading EE2 via co-metabolism through ammonia monooxygenase (Shi *et al*., 2004 and Ren *et al*., 2007). These bacteria were screened from nitrifying activated sludge; therefore, the animal farm soils may not be a good source of EE2-degrading bacteria.

4.2 Secondary screening of estrogen-degrading bacteria

Single colonies of the bacteria obtained from primary screening were separately cultured in IS medium with two different initial concentrations of each estrogen, 20 mg/L, as a low initial concentration, and 100 mg/L, as a high initial concentration. Estrogens left in the tubes were slightly noticeable. To ensure the complete solubility of estrogens, an equivalent volume of methanol was added into 20 mg/L initial concentration set and two volumes of methanol were added into 100 mg/L initial concentration set. The methanol added is lethal to the bacterial cell resulting in termination of degrading activity. After that, the amount of estrogen left in the culture was determined with HPLC (Table 4.1). For E1, the isolated bacteria showed 22-54% degradation (23-32 μg) of the initial amount of 60 μg (20 mg/L), whereas the other concentration (100 mg/L) was slightly degraded by isolate S02 (2%, 7 μg). All 4 selected colonies could degrade E1 better at low initial concentration. For E2, 10 different colonies of bacteria showed 33-92% degradation (15-52 μg) of the initial amount of 60 μg, and 0-61% degradation (0-161 μg) of the initial amount of 300 μg. From Table 4.1, 9 out of 10 isolates could degrade E2 at 20 mg/L with higher percentage than at 100 mg/L. There were 8 isolates showing more than half of E2 degradation at the low initial concentration. For high initial concentration, only 2 colonies, P42 and S19, could degrade E2 at high levels, 61% and 56% respectively.

The results of secondary screening indicated that the bacteria isolated from animal farms degraded estrogens better at low concentration. At high estrogen concentration, estrogens might cause some cellular stresses and oxidative damages to DNA, protein and membrane in the same way as bisphenol A, an estrogen-like compound, as reported by Kim *et al*. (2002). Nevertheless, the concentrations of estrogen found in the environment were considerably low, in the range of μg/L to ng/L (Nichols *et al.*, 1998 and USEPA, 2000). Thus, time course of estrogen degradation in this study was conducted only at low concentration.

Table 4.1: Estrogen degradation by bacteria obtained in secondary screening

Estrogen	**Bacterial colony**	**Soil source**	**20 mg/L Estrogen**		**100 mg/L Estrogen**	
			% Degradation	Amount degraded (μg)	% Degradation	Amount degraded (μg)
E1	C07	cow farm	52	31	0	0
	C51	cow farm	54	32	0	0
	C60	cow farm	29	30	0	0
	S02	sheep farm	22	23	2	7
E2	C27	cow farm	33	20	34	90
	Cp36	pregnant cow farm	92	55	28	73
	P23	pig farm	51	31	7	12
	P42	pig farm	88	53	61	161
	R07	rabbit farm	55	33	3	7
	R08	rabbit farm	77	46	0	0
	R09	rabbit farm	70	42	18	48
	R10	rabbit farm	48	29	11	28
	R12	rabbit farm	65	39	10	27
	S19	sheep farm	86	52	56	147

4.3 Time course study of estrogen degradation

To study time course of degradation, the concentration of estrogen in culture media was periodically determined. Thus, the culture media was prepared to be homogenous as best possible. The maximum solubility of estrogen shown in Table 2.1 is around 13 mg/L. In the preliminary test, estrogen was homogeneously obtained at 15 mg/L. This was therefore, the starting concentration of estrogens used in the time course study. The time course of degradation was carried out for all isolates obtained from the secondary screening. The degradation patterns are shown in Figure 4.1 and Figure 4.2 (data in Appendix C).

The degradation of E1 by 4 different isolates is demonstrated in Figure 4.1. In 4 days bacterial isolates of C07, C51 and C60 from cow farm soil and S02 from sheep farm soil degraded E1 up to 37%, 48%, 27% and 28%, respectively (Table 4.2). The degradation of E1 by these 4 isolates did not show any detectable metabolite in the HPLC profile. However, these isolates might have degraded E1 to small carbon molecules, which could not be detected by HPLC. The isolate C07 degraded E1 with high performance in the first day after which E1 remained constant or slightly decreased in the last 3 days. The isolate C51 showed consistent rate of E1 degradation in the first 3 days; at day 4 the rate tended to decrease slightly. In Figure 4.1 (B), the E1 degradation of this isolate continued after 4 days. For the isolates C60 and S02, E1 was not degraded in the first 3 days but their degrading activities were found in day 4. In comparing with the percentage of degradation in primary screening, these two isolates might degrade E1 better if longer incubation time was allowed. Since the isolate C51 obtained from cow farm soil had the highest ability to degrade E1 in the given period, it was selected to use for further experimentation.

Figure 4.2 indicated that 9 out of 10 isolates exhibited strong E2 degrading activity of 43-81%, an equivalent of about 0.5-1.2 mg from the initial amount of 1.5 mg E2 (Table 4.3). These isolates are C27 from cow farm soil, Cp36 from pregnant cow farm soil, P42 from pig farm soil, R07, R08, R09, R10, and R12 from rabbit farm soil and S19 from sheep farm soil.

Two different patterns of E2 degradation were observed. Seven isolates: C27, P42, R07, R08, R09, R10 and R12 showed degrading activity of 43-77% (about 0.5-0.9 mg). The degradation patterns of these 7 isolates are categorized as pattern A,

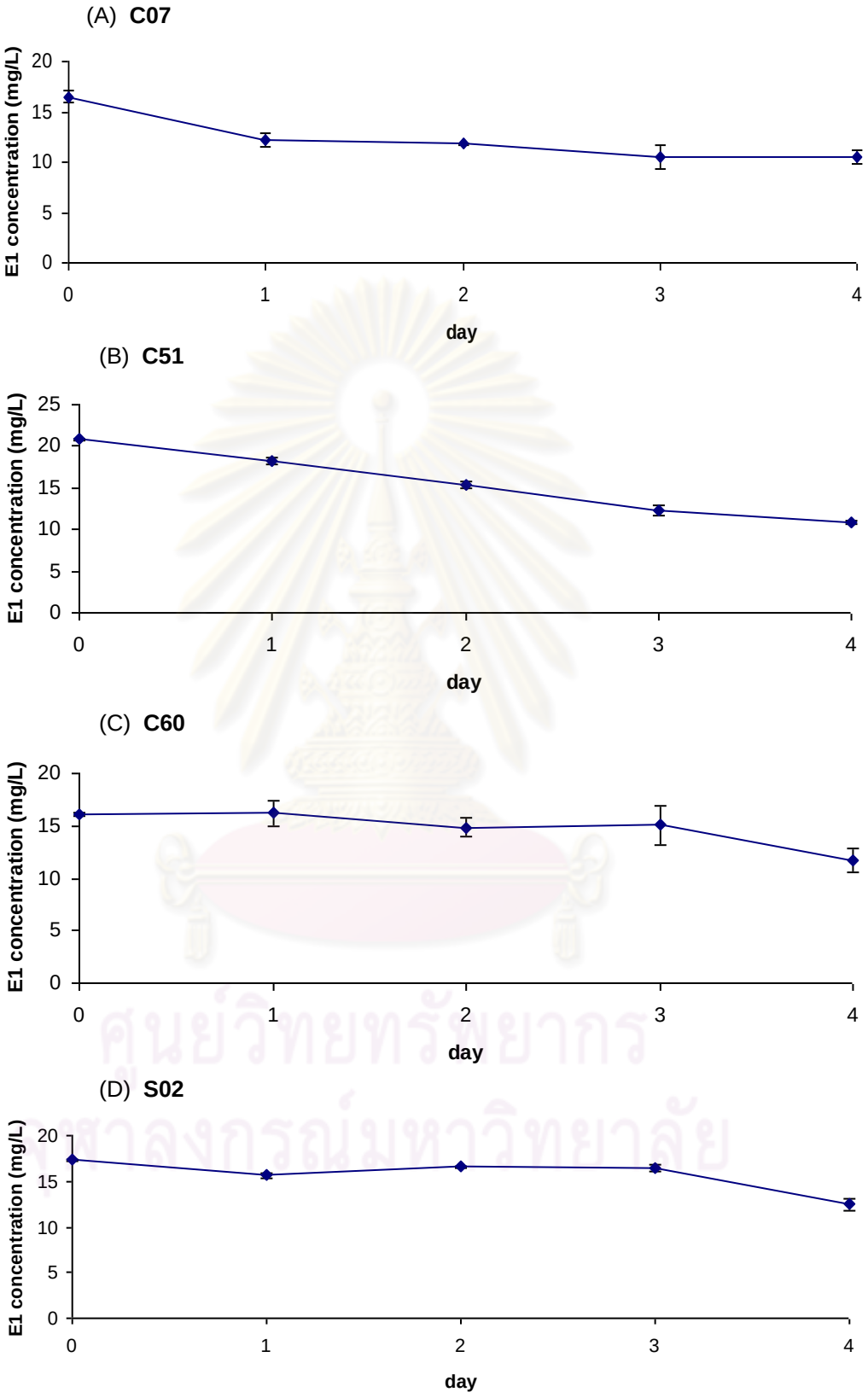


Figure 4.1: E1 degradation by bacteria isolated from animal farm soils
(A) isolate C07 (B) isolate C51 (C) isolate C60 and (D) isolate S02

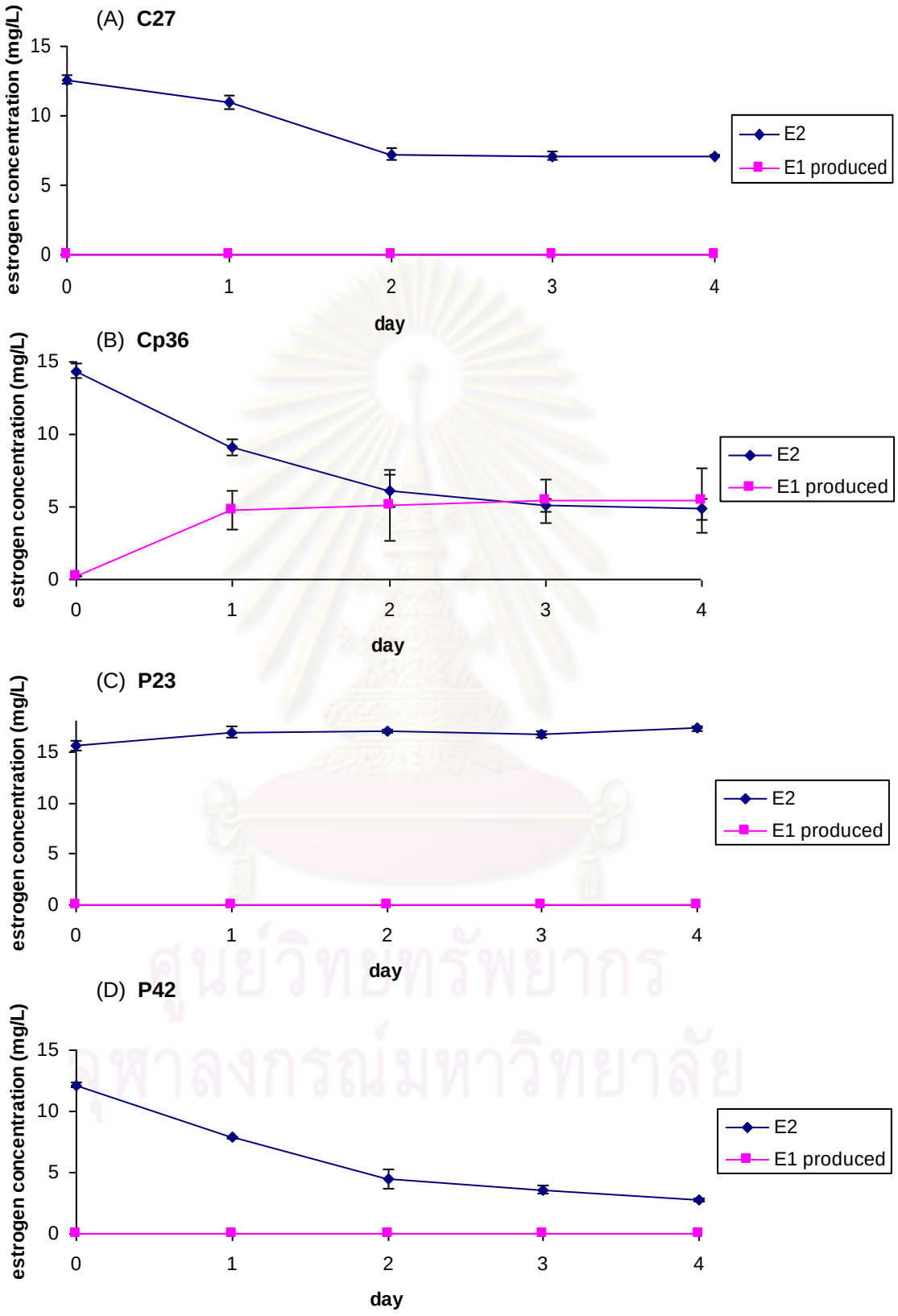


Figure 4.2: E2 degradation by bacteria isolated from animal farm soils
(A) isolate C27 (B) isolate Cp36 (C) isolate P23 and (D) isolate P42

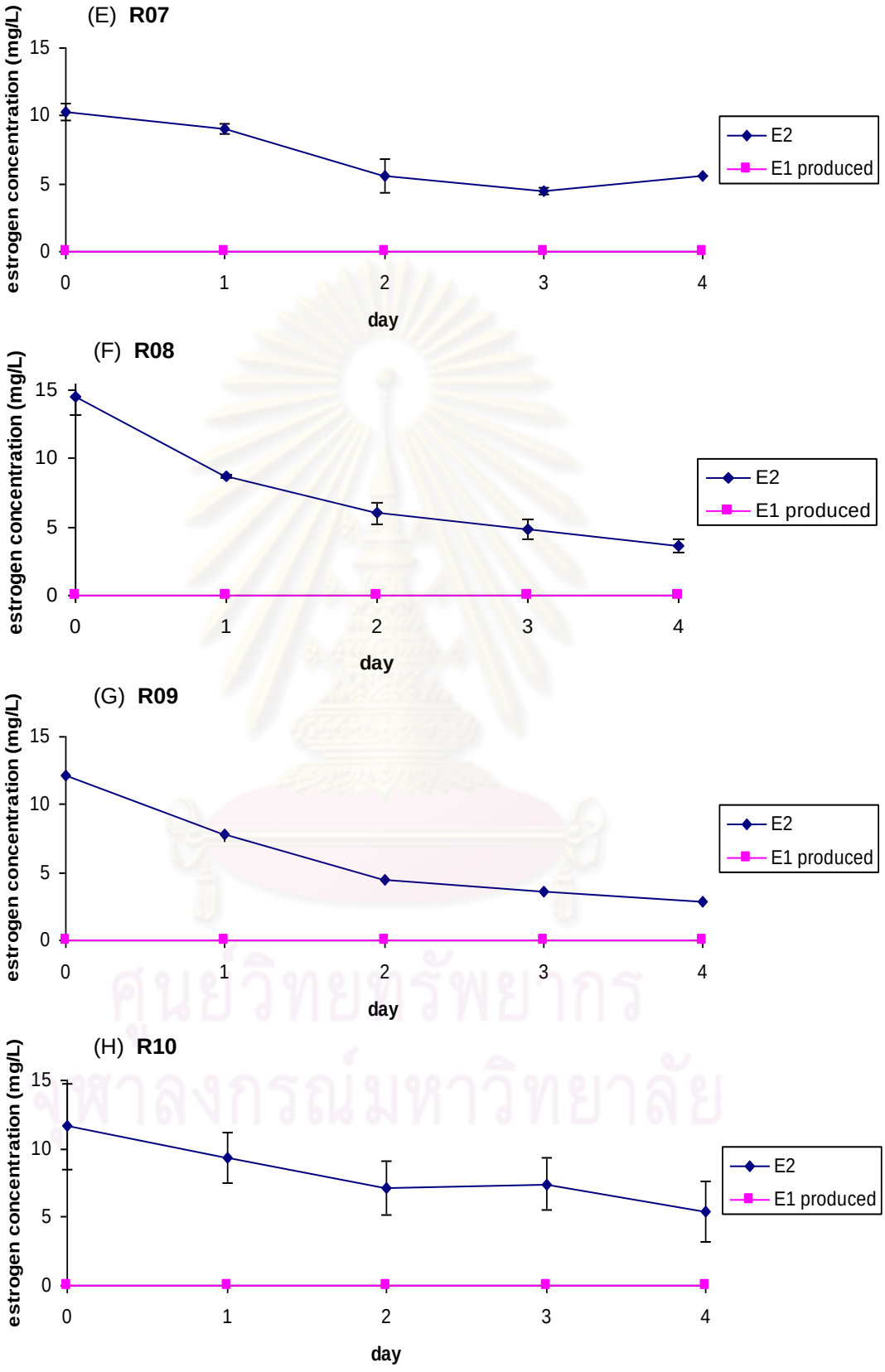


Figure 4.2 (Cont.): E2 degradation by bacteria isolated from animal farm soils (E) isolate R07 (F) isolate R08 (G) isolate R09 and (H) isolate R10

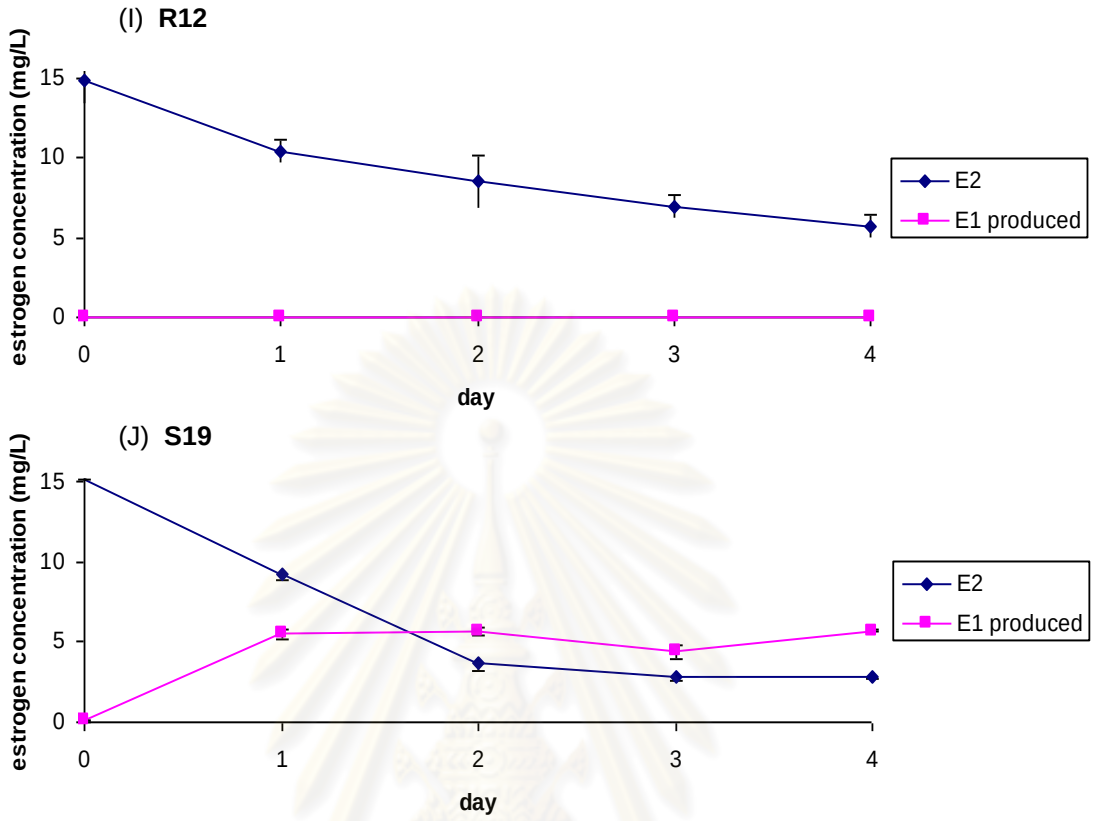


Figure 4.2 (Cont.): E2 degradation by bacteria isolated from animal farm soils (I) isolate R12 and (J) isolate S19

Table 4.2: Degradation of E1 by bacteria isolated from animal farm soils

Isolates	Amount of E1 degraded (mg)	% Degradation
C07	0.61	37
C51	1.00	48
C60	0.44	27
S02	0.48	28

Table 4.3: Degradation of E2 by bacteria isolated from animal farm soils

Isolates	Amount of E2 degraded (mg)	% Degradation	Amount of E1 produced (mg)
C27	0.5	43	-
Cp36	1.0	66	0.5
P23	0	0	-
P42	0.9	77	-
R07	0.5	46	-
R08	1.1	75	-
R09	0.9	66	-
R10	0.6	53	-
R12	0.9	62	-
S19	1.2	81	0.6

where E2 was degraded at a rapid rate in the first two to three days and remained constant or slightly decreased. No other compounds could be detected with absorbance at 210 nm. The absence of E1 in HPLC profiles indicates that these isolates may have the ability to degrade E2 to simple organic compounds as suggested by Fujii *et al*. (2002) was previously found in his study using *Novosphingobium tardaugens* ARI-1. Similar results were found with *Rhodococcus zopfii* and *Rhodococcus equi* (Yoshimoto *et al*., 2004).

The E2 degradation of the other two isolates, Cp36 and S19, are categorized as pattern B, with a metabolite detected by HPLC having the same retention time as E1 (Figure 4.3). In Figure 4.4, the absorption spectrum of E1 and the metabolite from E2 degradation by isolate S19 were shown to be similar. The same spectrum was observed for the metabolite from E2 degradation by isolate Cp36 (data not shown). This confirms that the metabolite from this degradation pattern was E1. The degradation of E2 by these two isolates happened rapidly in the first two days, and then slowed down. The amount of detected metabolite increased in the first day and became constant thereafter. At day 4, Cp36 and S19 could degrade E2 up to 66% (1.0 mg) and 81% (1.2 mg) and 0.5 and 0.6 mg of E1 were produced, respectively (Table 4.3). However, the amount of E1 produced was only half of the amount of E2 degraded. It is possible that E1 was degraded to small carbon compounds. Similar degradation patterns were also found in bacteria isolated from activated sludge, strains KC1-14 (Yu *et al*., 2007) and *Sphingomonas* sp. strain D12 (Chao *et al*., 2004).

For P23, the concentration of E2 remained constant in the first 4 days and slightly decreased by 17% after culturing for 6 days (data from preliminary study not shown). This may be due to its requirement for longer lag phase as compared to the other 9 isolates.

Previously, a number of E2-degrading bacteria were reported with high E2 degrading activity. Fujii *et al*. (2002) found that *N. tardaugens* ARI-1 isolated from activated sludge could degrade 83% of 5 mg of E2 in 30 mL of medium (167 mg/L) in 20 days. *Sphingomonas* sp. strain D12 isolated by Chao *et al*. (2004) from soil and activated sludge showed a trend of more rapid rate. It degraded E2 up to 97% of 600 μg of E2 in 4 mL medium (about 150 mg/L) in 4 days and degraded 95% of 600 μg of E1 in a day. Similarly, Yoshimoto *et al*. (2004) found *Rhodococcus zopfii* and

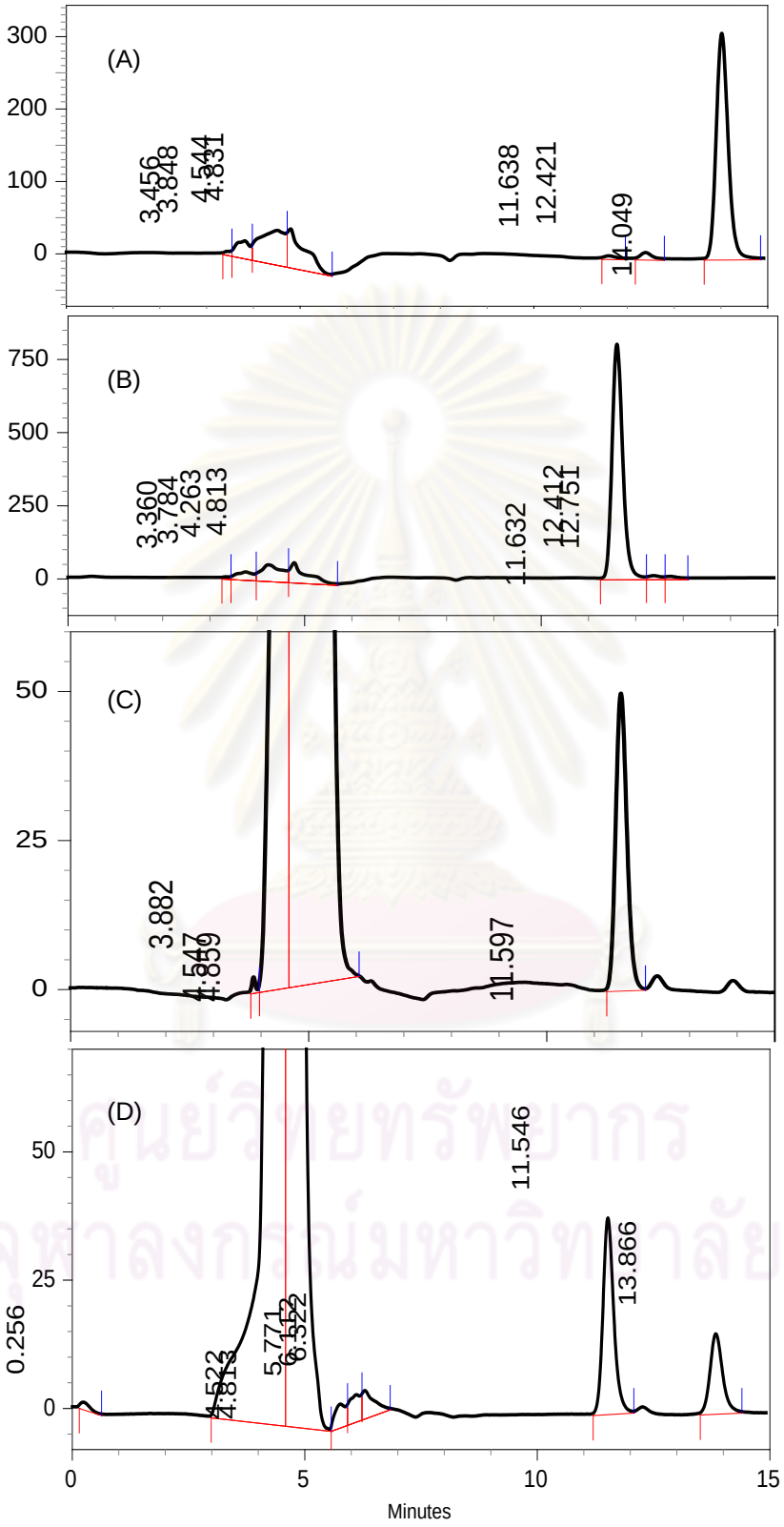


Figure 4.3: HPLC chromatograms of (A) standard E1, (B) standard E2, (C) S19 incubation sample at day 0 and (D) S19 incubation sample at day 4

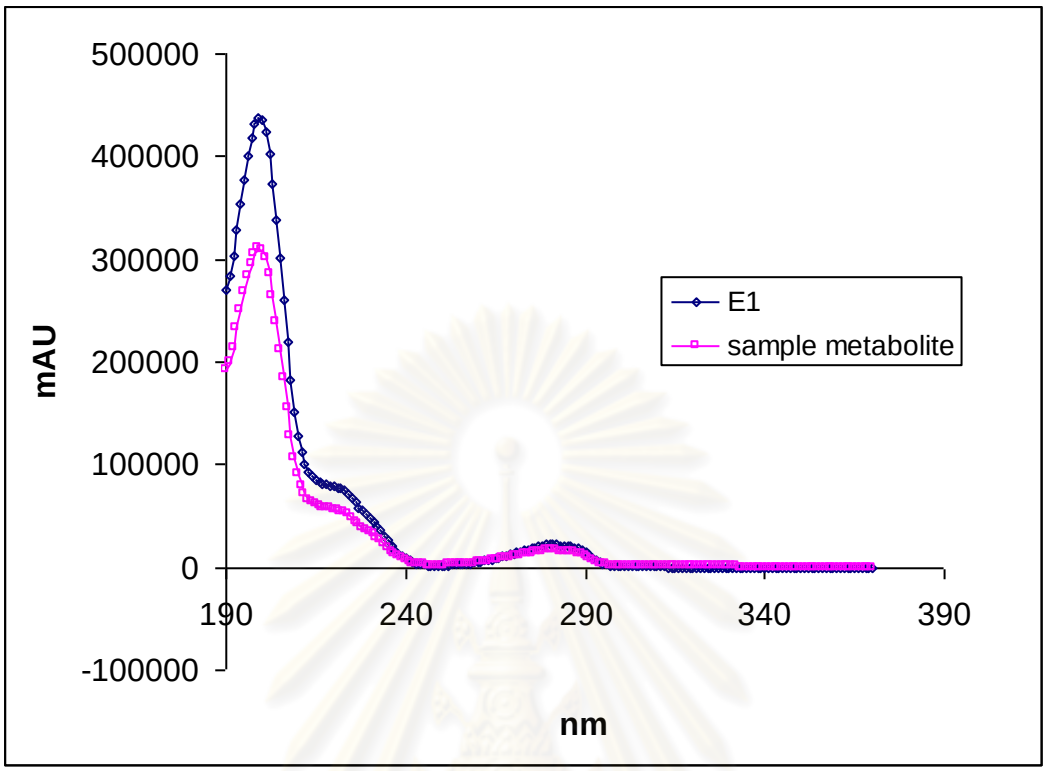


Figure 4.4: Absorption spectrum of E1 and metabolite from degradation of E2 by isolate S19 at day 4

Rhodococcus equi from activated sludge to be capable of degrade both E2 and E1 (100 mg/L, 10 mL) nearly complete (99%) in 24 hours. These studies used high concentration of E2 in the range of 100-200 mg/L. In contrast, Yu *et al.* (2007) used relatively low concentration (3 mg/L) to screen and isolate estrogen-degrading bacteria. Fourteen isolated bacteria could degrade more than 50% of E2 in 7 days.

It is difficult to compare the rate of estrogen degradation among different experiments due to the variation of cultivation conditions and initial amount of estrogens. This experiment use the low concentration of estrogens (15 mg/L) to investigate time course of estrogen degradation because the bacteria were expected to apply in ambient concentrations of estrogens in contaminated sites.

4.4 Degradation of related hormones

The estrogen-degrading bacteria isolated from animal farm soils were investigated for their ability to degrade other hormones of similar structure: E1, E2, EE2, and MT. For E1-degrading bacteria, the isolate C51 was selected due to its highest degradation ability. For E2-degrading bacteria, the bacteria showing the degradation pattern A was selected due to its ability to degrade E2 to simple organic compounds (non-estrogenic compounds). This biochemical property deems preferable for application in environmental treatment. The isolate P42 having the highest degradability in this group was used in the study. For control set, IS medium with 15 mg/L of each hormone was run without adding bacteria. Results and data are presented in Figure 4.5-4.8, Table 4.4 and Appendix C.

After 4 days, the bacterial isolates, C51 and P42 slightly degraded E1 by 38% and 30% (0.56 and 0.46 mg) from the initial amount of E1 added. The level of E1 was rapidly decreased in the first day, and remained constant thereafter. When compared with the control set, which decreased only 3% (0.04 mg) after 4 days, the percentage of degradation of C51 and P42 were 35% and 27%, respectively. It is concluded that the E2-degrading bacteria isolate P42 was able to degrade E1.

The degradation of E2 occurred in both control set and experimental set. The isolate C51 degraded E2 up to 79% (0.13 mg) and the isolate P42 degraded 80% (0.13 mg) of initial E2 added, while 66% (0.11 mg) was observed in control (Table 4.4). When subtracted with the value obtained in the control set, the degradation of E2 by

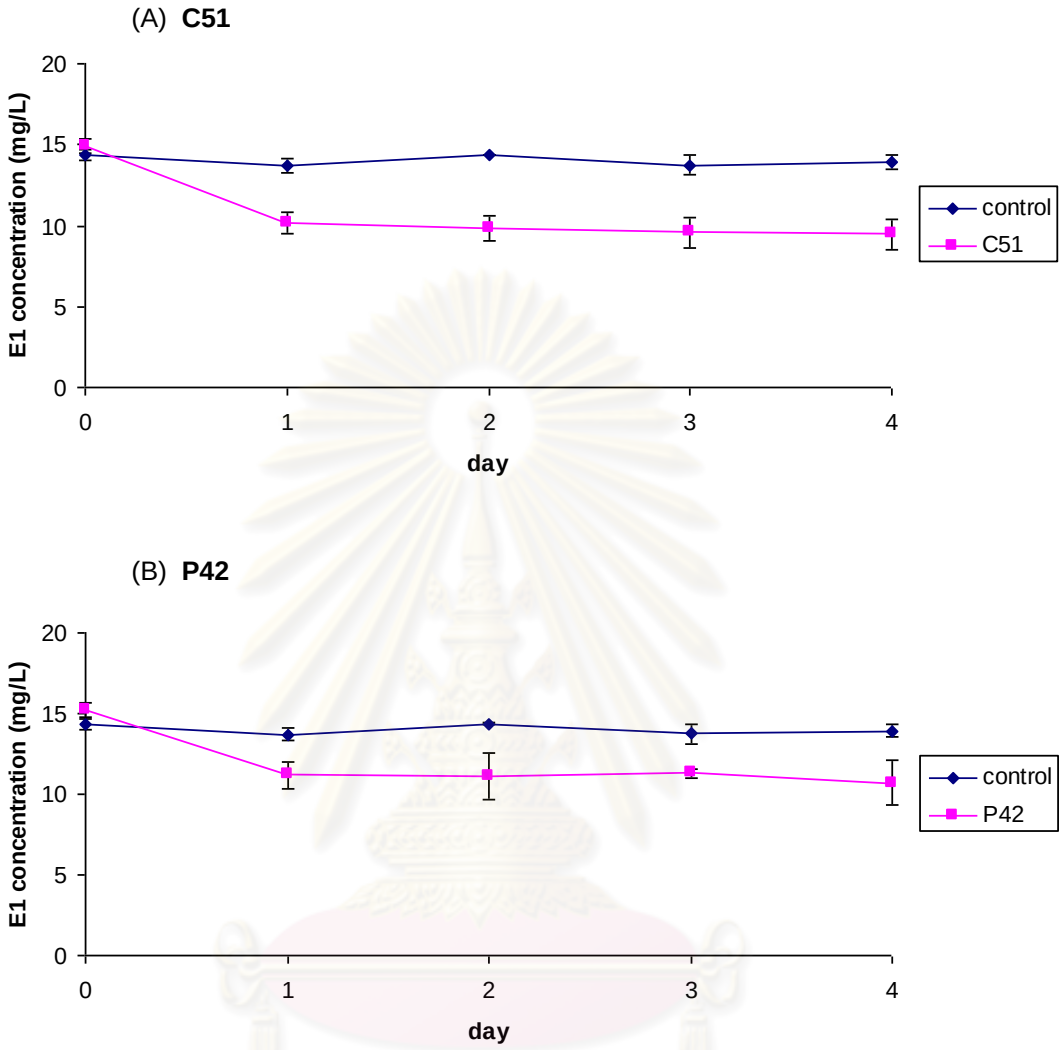


Figure 4.5: E1 degradation by bacteria (A) isolate C51 and (B) isolate P42. Control experiments were performed without bacterial inoculation.

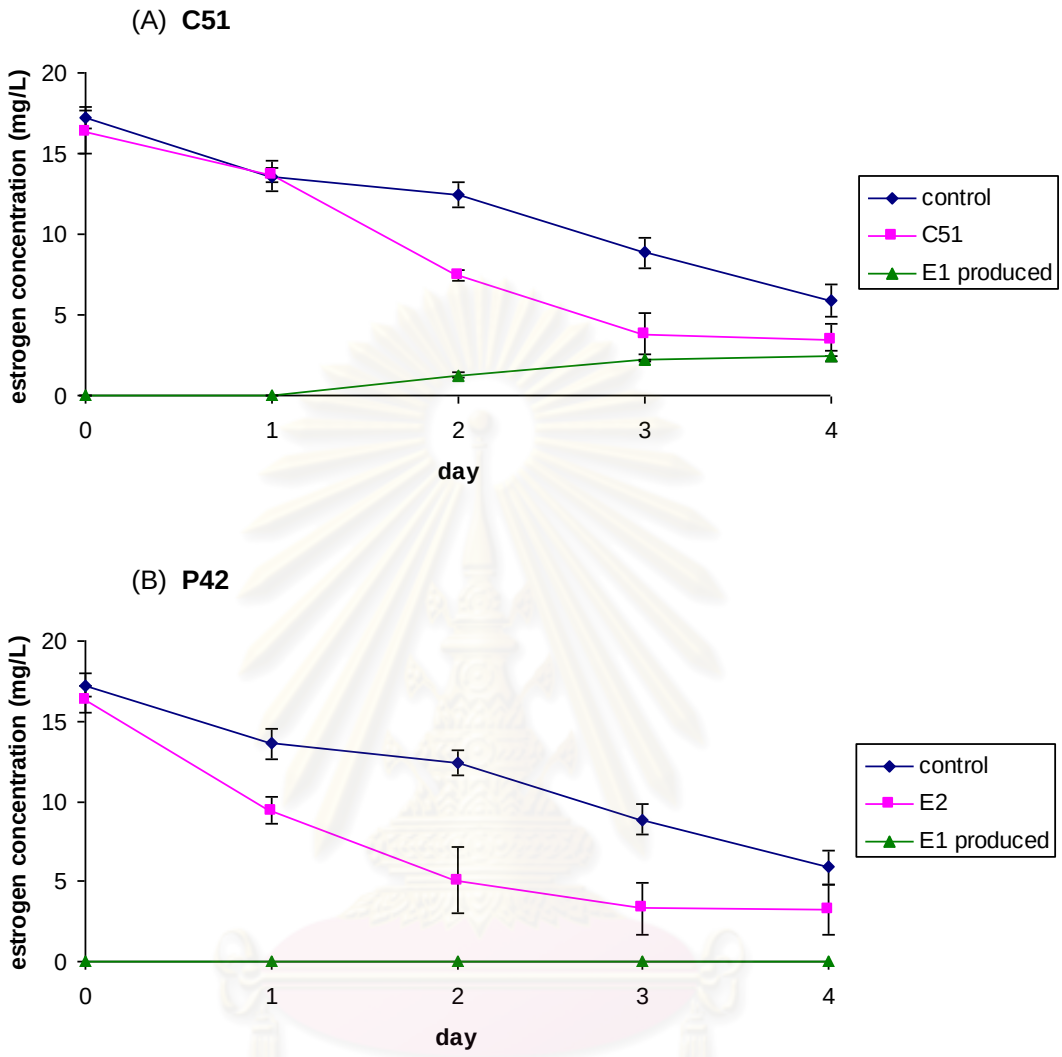


Figure 4.6: E2 degradation by bacteria (A) isolate C51 and (B) isolate P42. Control experiments were performed without bacterial inoculation.

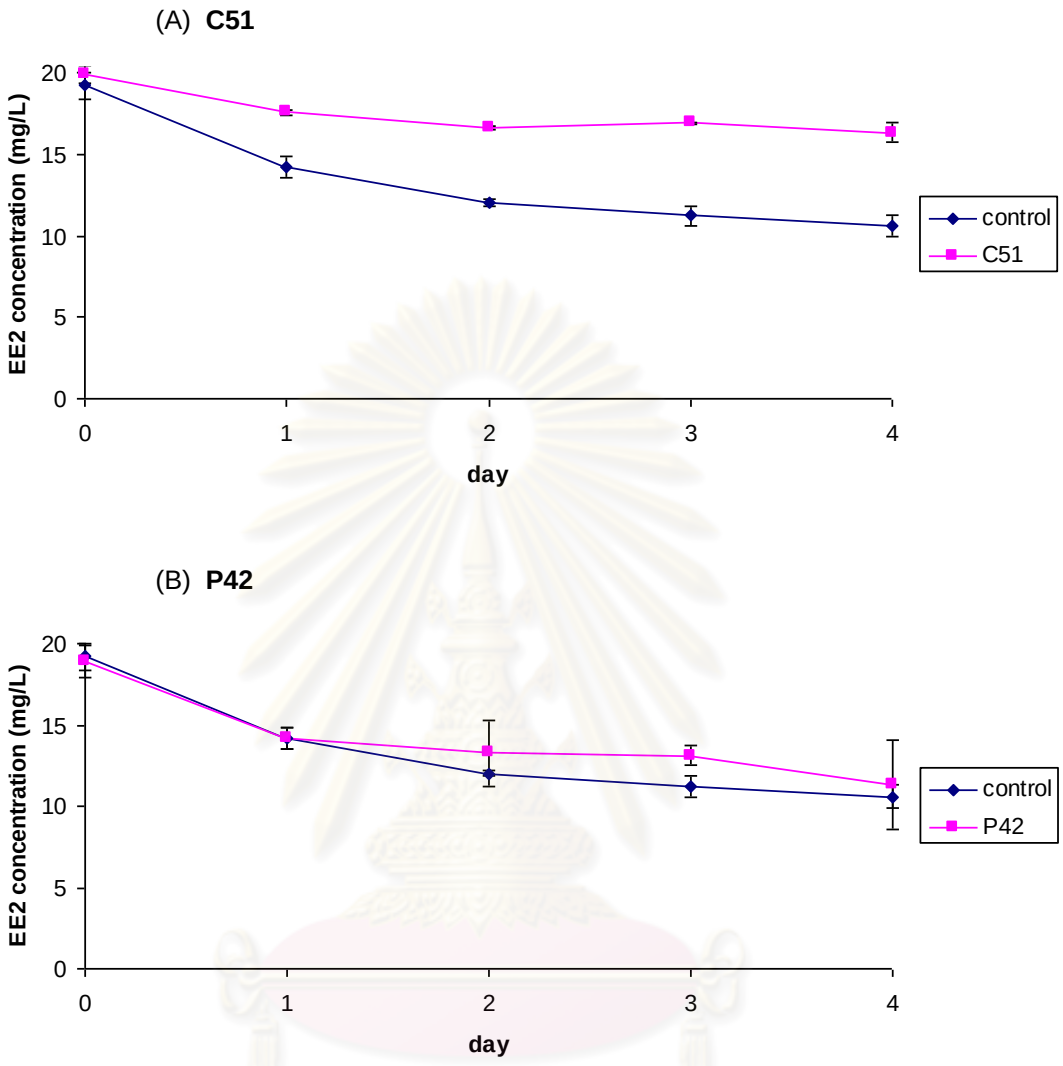


Figure 4.7: EE2 degradation by bacteria (A) isolate C51 and (B) isolate P42. Control experiments were performed without bacterial inoculation.

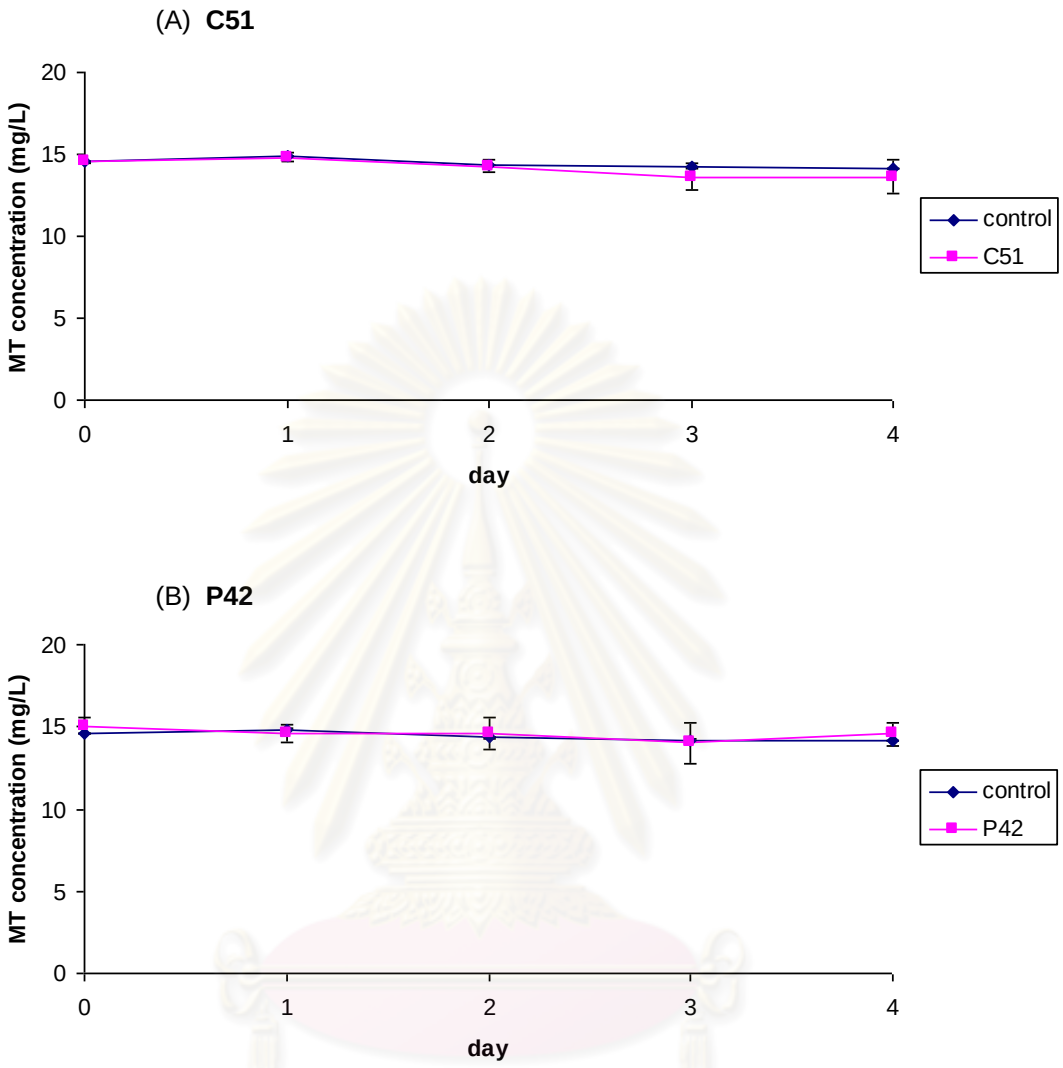


Figure 4.8: MT degradation by bacteria (A) isolate C51 and (B) isolate P42. Control experiments were performed without bacterial inoculation.

Table 4.4: Hormones degradation by bacteria isolate P42 and C51.

Δ% = Difference between sample and control.

Hormones		**E1**	**E2**	**EE2**	**MT**
Control	%	3	66	45	3
	mg	0.04	0.11	0.78	0.05
C51	%	38	79	18	6
	mg	0.56	0.13	0.36	0.09
	Δ%	35	13	-27	3
P42	%	30	80	40	3
	mg	0.46	0.13	0.76	0.05
	Δ%	27	14	-5	0

C51 and P42 were 13% and 14%, respectively. It is noted that the degradation by isolate C51, which is an E1-degrading bacterium, showed a metabolite peak after 2 days of inoculation (0.02 mg). The retention time of the metabolite peak was essentially the same as E1.

In the case of EE2, the results indicated higher degradation in the control set. Since photolysis of E2 and EE2 in aqueous solution was reported by Mazellier *et al* (2008), a test was performed to check if the decrease of E2 and EE2 in the control set were caused by photodegradation. Two control sets, under normal experimental condition (daylight and fluorescent light) and without any light irradiation (dark) were incubated. The amount of E2 and EE2 was measured every 24 hours for 4 days (Figure 4.9). The result showed that the reduction of E2 in both cases were nearly equal, while light seemed to have slightly more degrading effect on EE2. However, other factors may possibly cause reduction of E2 and EE2 levels in these experiments.

For MT degradation, slight decreases at day 4 were observed in all samples: 6% (0.09 mg) for C51 culture, 3% (0.05 mg) for P42 culture, and 3% (0.05 mg) in control set. Therefore, the degradation of MT did not seem to occur although the structure of MT is similar to E1 and E2 (Figure 3.2).

In conclusion, both P42 and C51 exhibited the ability to degrade E1 and E2, but could not degrade EE2 and MT. The ability of some E2-degrading bacteria to use other forms of estrogens have been reported in previous studies. *N. tardaugens* ARI-1 was found to degrade E1 and E3, but not EE2. *Sphingomonas* sp. strain D12 was able to degrade E1, while *R. zopfii* and *R. equi* could degrade E1, E3 and EE2. It is notable that all E2-degrading bacteria reported could degrade E1; some of them degraded E3; and only four strains of *R. zopfii* and *R. equi* degraded EE2.

4.5 Identification of estrogen-degrading bacteria

Seven isolates of E2-degrading bacteria, Cp36, P42, R07, R08, R09, R12 and S19, and an E1-degrading bacterium, C51, were selected to be identified using their biochemical properties together with 16S rRNA gene sequencing.

Single colony of each isolate is shown in Figure 4.10 and their colony morphologies were described in Table 4.5. The selected bacteria shared some similar morphology: round shape, entire margin, convex elevation and smooth surface. The

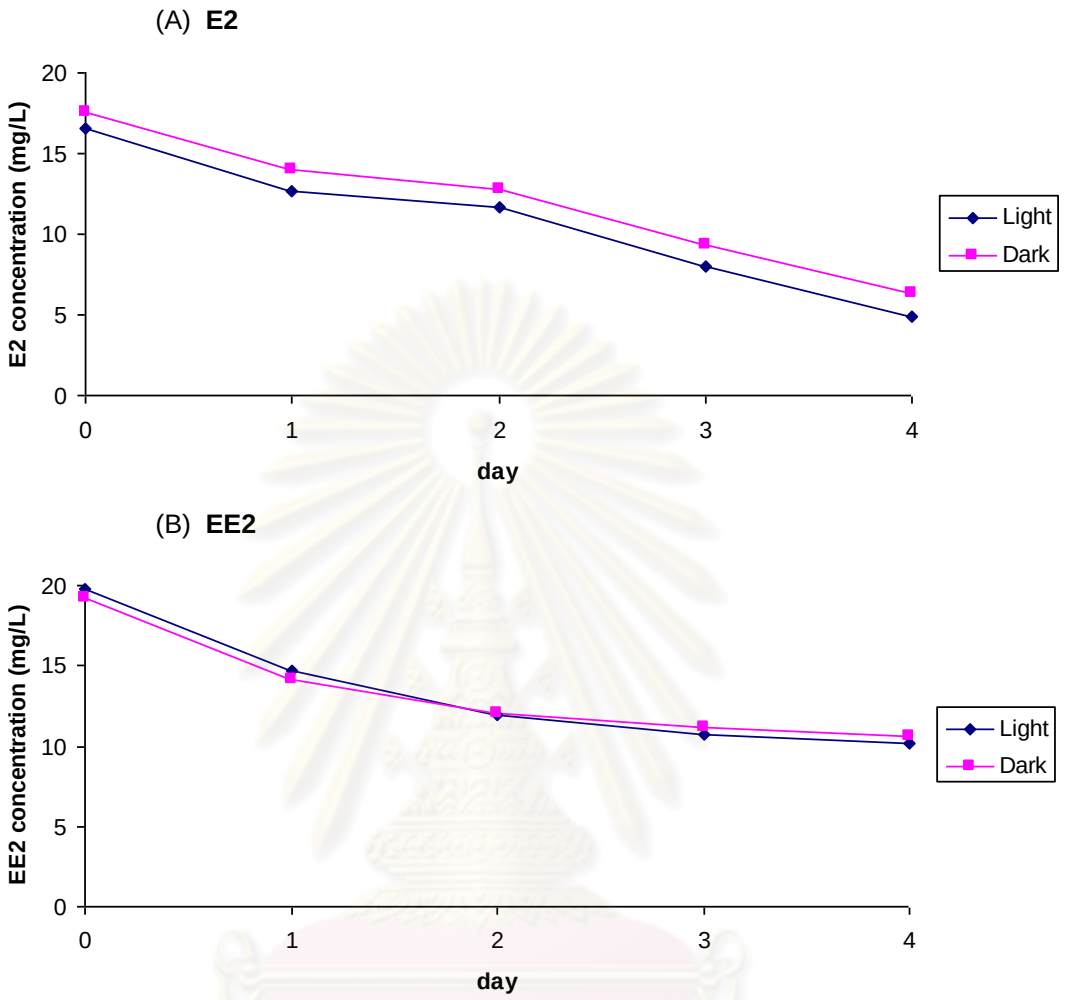


Figure 4.9: Degradation of E2 (A) and EE2 (B) in normal condition with exposure to daylight and fluorescent light and in the dark

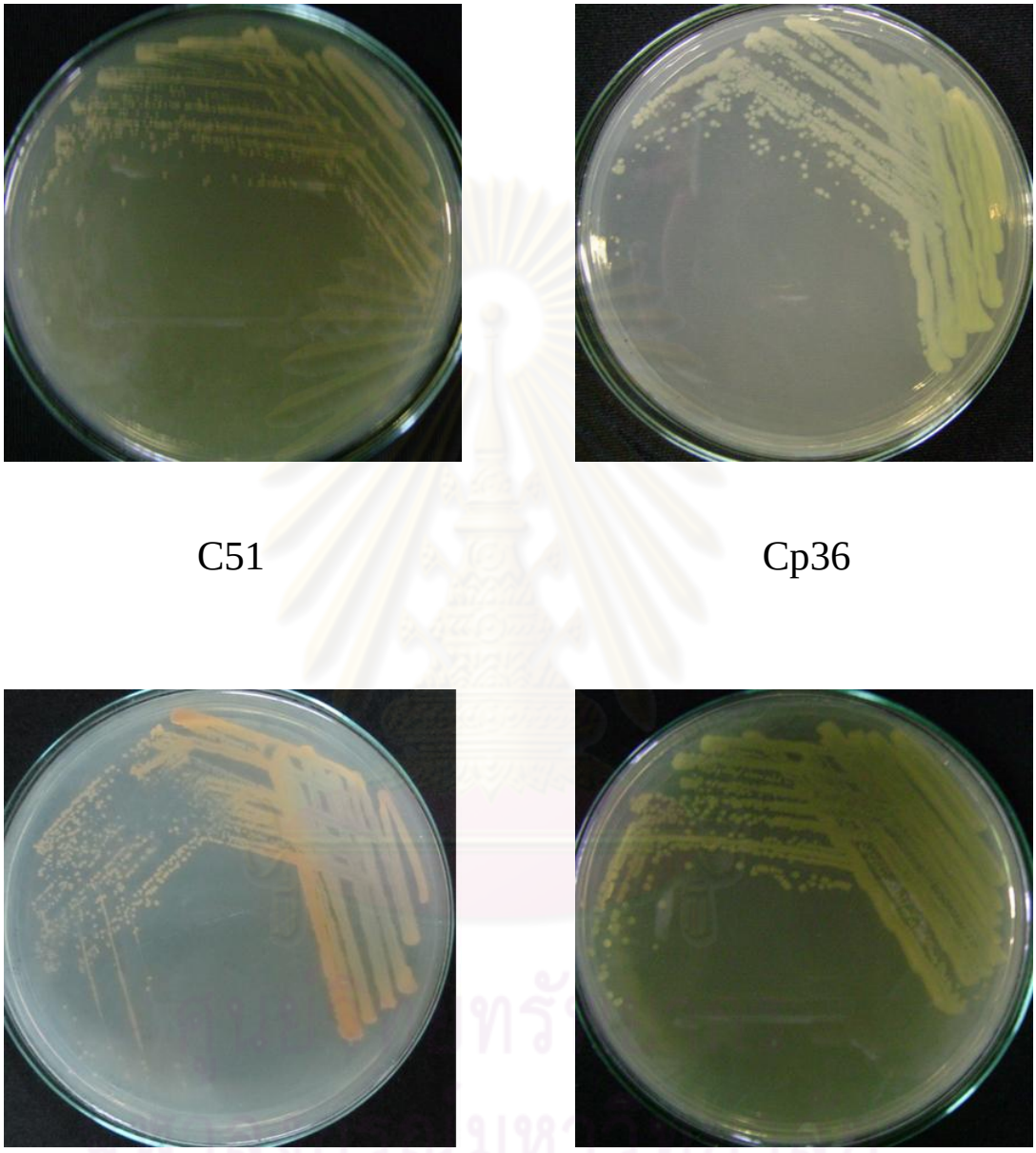
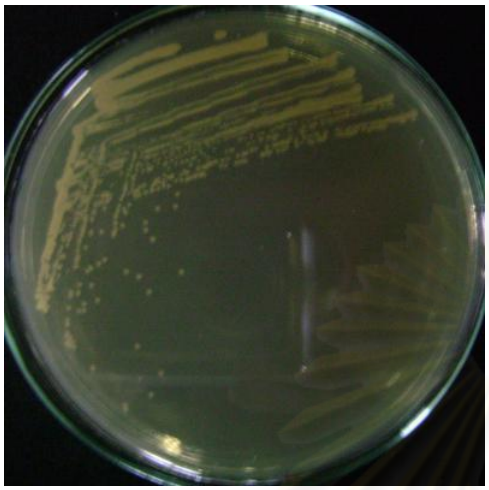
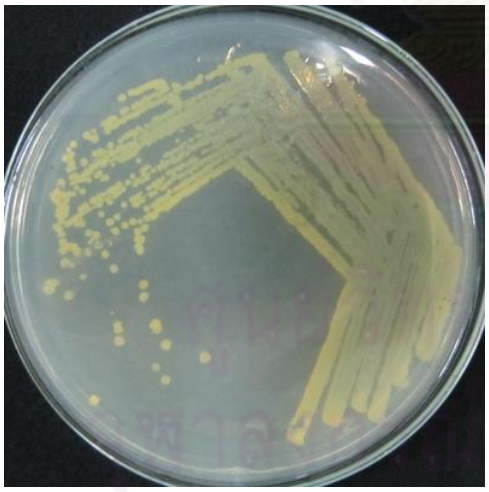


Figure 4.10: Single colonies of estrogen-degrading bacteria on LB-agar plates after incubation at 30°C for 2 days

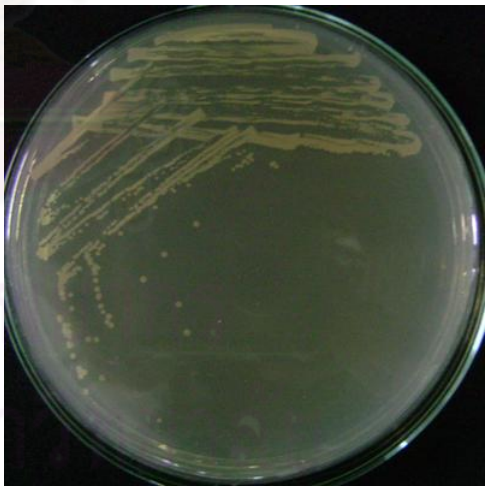


R08

R09



R12



S19

Figure 4.10 (cont.): Single colonies of estrogen-degrading bacteria on LB-agar plates after incubation at 30°C for 2 days

Table 4.5: Colony morphology of estrogen-degrading bacteria on LB-agar plates after 2 day incubation at 30°C

Isolate	Soil source	Morphology					
		Diameter (mm.)	Shape	Margin	Elevation	Surface	Color
C51	cow farm	1	round	entire	convex	smooth	yellow
Cp36	pregnant cow farm	1	round	entire	convex	smooth	yellow
P42	rabbit farm	1	round	entire	convex	smooth	orange
R07	rabbit farm	1	round	entire	convex	smooth	yellow
R08	rabbit farm	1	round	entire	convex	smooth	yellow
R09	rabbit farm	1.5	round	entire	convex	smooth	yellow
R12	rabbit farm	1.5	round	entire	convex	smooth	yellow
S19	Sheep farm	1	round	entire	convex	smooth	yellow

colony sizes of these isolates are around 1-1.5 mm. All isolates had yellow colonies in different tones except for P42 which showed orange colony. These 8 selected bacteria were sent to test their biochemical properties at National Institute of Health (NIH); the results are shown in Table 4.6.

The 8 isolates were identified to belong to 5 different bacterial species: C51, R09 and R12 were *Aureobacterium* sp.; Cp36 and S19 were *Arthrobacter* sp.; P42 was *Brevibacterium brevi*; R07 was *Cupriavidus pauculus*; and R08 was *Pseudomonas pseudoalcaligenes*.

In addition to identifying the bacterial species by their biochemical properties, 16S rRNA gene sequencing was also used. To prepare the DNA template for 16S rRNA gene sequencing, bacterial chromosomal DNA was extracted using the methods described in section 3.3.4.2.1. Their concentrations and molecular weights were estimated by comparison of the band intensity and relative mobility with standard DNA marker (λ/*Hin*dIII) on 1.5% agarose gel electrophoresis. Figure 4.11 showed the bands of extracted chromosomal DNA of selected isolates. It was found that extracted DNA had molecular weight over 23.1 kb and showed high purity which corresponded with their A_{260}/A_{280} ratio (1.8-2.0). The DNA concentration was about 0.3-0.5 μg/μL. Thus, the quality of DNA could be appropriately used for PCR amplification. Approximately 500 ng of chromosomal DNA was used as DNA template in the PCR amplification with the universal primer 27F and 1492R in a total volume of 50 μL. The annealing temperature of the reaction was varied from 42 to 58.6°C to determine the optimum temperature. The PCR products of all isolates were obtained at low annealing temperature: 42, 43.1 and 46.3°C (Figure 4.12) with a single band of estimated molecular weight of 1.5 kb. The annealing temperature of 43°C was finally used in order to achieve the highest intensity of PCR amplification. The PCR products were cloned into pGEM®-T Easy Vectors using T4 DNA ligase. The ligation products were transformed into competent cells of *E. coli* JM109 by electroporation. The transformant cells were spread onto the selective media, the LB plate with ampicillin/IPTG/X-gal. White colonies growing on the plate were picked and their plasmids were extracted. The EcoRI digestion was performed to confirm size of inserted DNAs. Figure 4.13 showed 3 product bands of approximately 3, 1 and

Table 4.6: Biochemical properties of estrogen-degrading bacteria

Characteristics	**C51**	**Cp36**	**P42**	**R07**	**R08.**	**R09**	**R12**	**S19**
Gram stain	+	+	+	-	-	+	+	+
Hemolysis	γ	γ	γ	-	-	γ	γ	γ
H_2S production	ND	ND	ND	-	-	ND	ND	ND
Indole production	ND	ND	ND	-	-	ND	ND	ND
N_2 gas production	ND	ND	ND	-	-	ND	ND	ND
Enzyme activity:								
Catalase	+	+	+	ND	ND	+	+	+
Oxidase	-	-	+	+	+	-	-	-
Urease	-	-	-	+	-	-	-	-
Esculin hydroxylase	+	+	-	-	+w	+	+	+
Gelatinase	-	-	-	-	-	-	-	-
Alkaline phosphatase	+	+	ND	ND	ND	+	+	+
Substrate utilization:								
Glucose	+	+	+w	-	+	+	+	+
Lactose	-	-	ND	-	-	-	-	-
Maltose	+	+	ND	-	-	+	+	+
Mannitol	+	-	-	-	-	+	+	-
D-xylose	-	-	-	-	-	-	-	-
Fructose	+	-	ND	-	+	+	+	-
Citrate	+	+	+	+	-	+	+	+
Nitrate	-	-	-	-	+	-	-	-
Malonate	ND	ND	ND	-	ND	ND	ND	ND
Acetate	ND	ND	ND	+	-	ND	ND	ND
Lysine decarboxylation	ND	ND	ND	-	-	ND	ND	ND
Arginine decarboxylation	ND	ND	ND	-	-	ND	ND	ND
Ornithine decarboxylation	ND	ND	ND	-	-	ND	ND	ND
Growth at 42°C	ND	ND	ND	+	+	ND	ND	ND
Voges-Proskauer test	+	-	-	ND	ND	+	+	-
Motility	+	+	+	+	+	+	+	+
CAMP test	-	-	ND	ND	ND	-	-	-

ND = no data available, +w = with gas production, CAMP = Christie Atkins Munch-Petersen

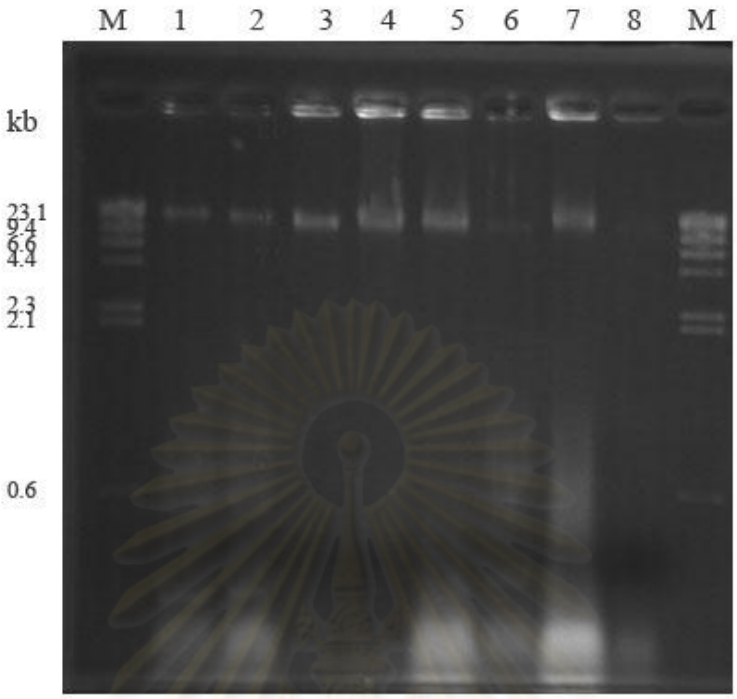


Figure 4.11: Chromosomal DNA of selected isolates

Lane 1 = Isolate R07 Lane 2 = Isolate R08

Lane 3 = Isolate R09 Lane 4 = Isolate R12

Lane 5 = Isolate S19 Lane 6 = Isolate Cp36

Lane 7 = Isolate P42 Lane 8 = Isolate C51

Lane M = λ/*Hin*dIII DNA marker

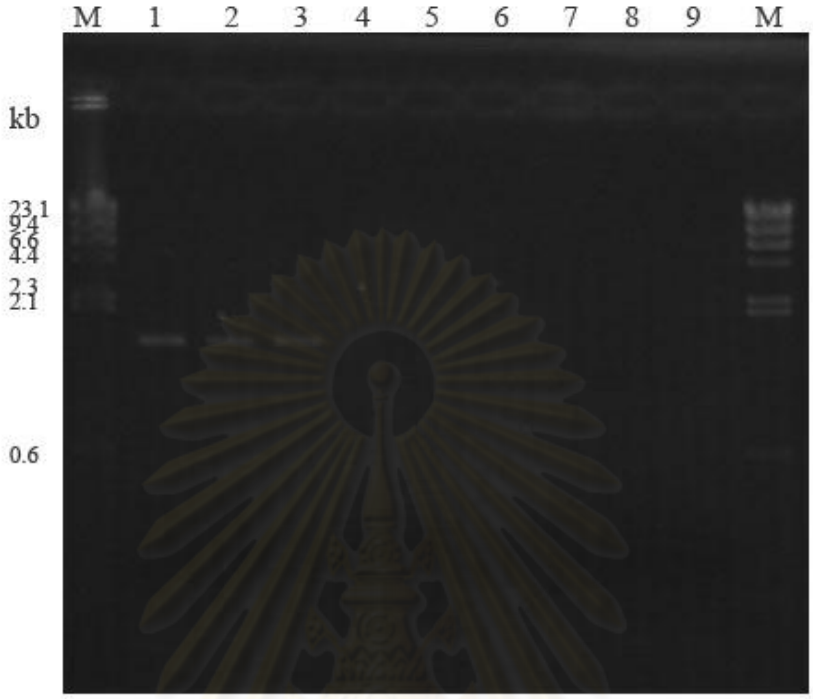


Figure 4.12: PCR products of 16S rRNA gene of isolate R09 at various annealing temperatures

Lane 1 = 42°C Lane 2 = 43.1°C
Lane 3 = 46.3°C Lane 4 = 48.4°C
Lane 5 = 50.6°C Lane 6 = 52.8°C
Lane 7 = 54.8°C Lane 8 = 56.5°C
Lane 9 = 58.6°C
Lane M = λ/*Hin*dIII DNA marker

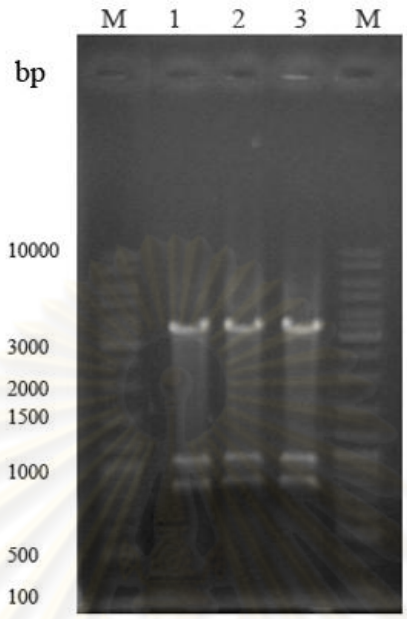


Figure 4.13: Digestion of recombinant plasmid with *Eco*RI

Lane 1 = Isolate R07 Lane 2 = Isolate R12

Lane 3 = Isolate R08

Lane M = 100 bp DNA marker

0.8 kb. The upper bands of 3 kb corresponded with the linear form of pGEM®-T Easy Vectors, while the two lower bands were inserted DNAs of which the molecular weight summation was equal to that of the PCR products. The recombinant plasmids were sequenced by Macrogen Inc. laboratory (Appendix D). Closely related sequences were identified by comparing the partial 16S rRNA gene sequences with those in the GenBank using the Basic Local Aliment Search Tool (BLAST).

Based on the results of 16S rRNA sequencing, the bacterial isolates C51, R09, R12 and S19 are identified to be *Microbacterium* spp. considering their high degree of identities (99%) as shown in Table 4.7, 4.12, 4.13 and 4.14.

For isolate Cp36, 98% identity to *Alcaligenes faecalis* was found together with 96-99% identity to the other 4 unidentified bacteria (Table 4.8). In addition, almost 50 strains in lower ranks range from 94-95% are mostly *Alcaligenes* spp. From these data, Cp36 was likely to be a member of genus *Alcaligenes*.

The sequence of isolate P42 had 98% sequence identity to 2 strains of bacteria in genus *Planococcus* (Table 4.9) indicating the possibility of P42 being a member of genus *Planococcus*.

In Tables 4.10 and 4.11, the 5 top most related strains to isolates R07 and R08 were similar with 97% identity and belonged to *Pusillimonas* sp. and *Alcaligenes* sp. It is most likely that the isolates R07 and R08 were members of family Alcaligenaceae.

The results obtained from biochemical property study and 16S rRNA gene sequence analysis are summarized in Table 4.15. C51, R09 and R12 had identical biochemical properties which suggested that they belonged to *Aureobacterium* sp. while the 16S rRNA gene analysis indicated that they were *Microbacterium* sp. However, *Aureobacterium* sp. and *Microbacterium* sp. are synonyms as classified in the NCBI database. It was concluded that C51, R09 and R12 were members in the same genus, *Microbacterium*.

Cp36 and S19 had the same biochemical properties as the *Arthrobacter* sp. but their 16S rRNA gene sequence analysis show different results. For Cp36, the biochemical properties and the sequencing result did not correspond. With numerous sequences in the GenBank, it is generally accepted that the 16S rRNA gene sequence analysis provides more reliable result than the biochemical test. The isolate Cp36 is

Table 4.7: Five most related strains to C51, as sorted by max score (1,485 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Microbacterium sp.* Atl-19, 16S rRNA gene, partial sequence	2704	2704	100%	99%
2.	*Microbacterium schleiferi* strain 2PR54-18, 16S rRNA gene, partial sequence	2686	2686	100%	99%
3.	*Microbacterium lacticum* strain 3388, 16S rRNA gene, partial sequence	2662	2662	98%	99%
4.	*Microbacterium aurum* strain TPL18, 16S rRNA gene, partial sequence	2662	2662	100%	98%
5.	Uncultured bacterium, clone SSmCB08-6, 16S rRNA, partial sequence,	2660	2660	98%	99%

Table 4.8: Five most related strains to Cp36, as sorted by max score (1,500 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Alcaligenes faecalis* strain EBD, 16S rRNA gene, partial sequence	2501	2501	93%	98%
2.	Uncultured bacterium, clone PB2, 16S rRNA gene, partial sequence	2459	2459	90%	99%
3.	Uncultured bacterium partial, clone HAW-R60-B-745d-BE, 16S rRNA gene	2451	2451	99%	96%
4.	Uncultured bacterium partial, clone HAW-R60-B-745d-J, 16S rRNA gene	2451	2451	99%	96%
5.	Uncultured bacterium partial, clone SMG125, 16S rRNA gene	2451	2451	99%	96%

Table 4.9: Five most related strains to P42, as sorted by max score (1,511 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Planococcus sp.* Tibet-IX21, 16S rRNA gene, partial sequence	2671	2671	99%	98%
2.	Uncultured bacterium, clone FB04C09, partial 16S rRNA gene	2647	2647	98%	98%
3.	*Planococcus sp.* Smarlab 3302355, 16S rRNA gene, partial sequence	2612	2612	97%	98%
4.	Uncultured bacterium, clone 1103200820440, 16S rRNA gene, partial sequence	2569	2569	99%	97%
5.	Uncultured bacterium, clone 1103200820276, 16S rRNA gene, partial sequence	2569	2569	99%	97%

Table 4.10: Five most related strains to R07, as sorted by max score (1,102 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Pusillimonas sp.* ES-QY-3, 16S rRNA gene, partial sequence	1906	1906	99%	97%
2.	Uncultured bacterium, clone DR550SWSAEE14, 16S rRNA gene, partial sequence	1903	1903	99%	97%
3.	*Alcaligenaceae bacterium* BZ45, 16S rRNA gene, partial sequence	1879	1879	99%	97%
4.	*Pusillimonas terrae*, 16S rRNA gene, partial sequence	1868	1868	98%	97%
5.	*Alcaligenes sp.* H, 16S rRNA gene, partial sequence	1860	1860	99%	97%

Table 4.11: Five most related strains to R08, as sorted by max score (1,197 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Pusillimonas sp.* ES-QY-3, 16S rRNA gene, partial sequence	1901	1901	91%	97%
2.	Uncultured bacterium, clone DR550SWSAEE14, 16S rRNA gene, partial sequence	1897	1897	91%	97%
3.	*Alcaligenaceae bacterium* BZ45, 16S rRNA gene, partial sequence	1873	1873	91%	97%
4.	*Pusillimonas terrae*, 16S rRNA gene, partial sequence	1862	1862	90%	97%
5.	*Alcaligenes sp.* H, 16S rRNA gene, partial sequence	1855	1855	91%	97%

Table 4.12: Five most related strains to R09, as sorted by max score (1,477 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Microbacterium oxydans* strain B5, 16S rRNA gene, complete sequence	2671	2671	99%	99%
2.	*Microbacterium sp.* TS-YF-2, 16S rRNA, partial sequence	2665	2665	99%	99%
3.	*Microbacterium sp.* PHD-5, 16S rRNA gene, partial sequence	2649	2649	99%	98%
4.	*Microbacterium sp.* CME1, 16S rRNA gene, complete sequence	2647	2647	99%	98%
5.	*Microbacteriaceae bacterium* KVD-1982-06, 16S rRNA gene, partial sequence	2645	2645	99%	98%

Table 4.13: Five most related strains to R12, as sorted by max score (1,487 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Microbacterium oxydans* strain B5, 16S rRNA gene, complete sequence	2739	2739	99%	99%
2.	*Microbacterium sp.* TS-YF-2, 16S rRNA, partial sequence	2734	2734	99%	99%
3.	*Microbacterium sp.* PHD-5, 16S rRNA gene, partial sequence	2717	2717	99%	99%
4.	*Microbacterium sp.* CME1, 16S rRNA gene, complete sequence	2715	2715	99%	99%
5.	*Microbacteriaceae bacterium* KVD-1982-06, 16S rRNA gene, partial sequence	2713	2713	99%	99%

Table 4.14: Five most related strains to S19, as sorted by max score (1,484 bp)

Rank	Strain	Max score	Total score	Query coverage	Max identity
1.	*Microbacterium resistens*, 16S rRNA gene, partial sequence	2680	2680	99%	99%
2.	*Microbacterium sp.* 35N43-1, 16S rRNA gene, partial sequence	2669	2669	99%	99%
3.	*Microbacterium resistens* strain 3352, 16S rRNA gene, partial sequence	2660	2660	98%	99%
4.	*Microbacterium resistens* strain DMMZ 1710, 16S rRNA, partial sequence	2645	2645	97%	99%
5.	*Microbacterium oxydans* strain B5, 16S rRNA gene, complete sequence	2603	2603	99%	98%

Table 4.15: Summary of estrogen-degrading bacteria identification by their biochemical properties and 16S rRNA gene sequences

Isolate	**Soil source**	**Biochemical properties**	**16S rRNA sequences**
C51	cow farm	*Aureobacterium* sp.*	*Microbacterium* sp.
Cp36	pregnant cow farm	*Arthrobacter* sp.	*Alcaligenes* sp.
P42	pig farm	*Brevibacterium brevi*	*Planococcus* sp.
R07	rabbit farm	*Cupriavidus pauculus*	Alcaligeneae bacteria
R08	rabbit farm	*Pseudomonas pseudoalcaligenes*	Alcaligeneae bacteria
R09	rabbit farm	*Aureobacterium* sp.*	*Microbacterium* sp.
R12	rabbit farm	*Aureobacterium* sp.*	*Microbacterium* sp.
S19	sheep farm	*Arthrobacter* sp.	*Microbacterium* sp.

* *Aureobacterium* sp. and *Microbacterium* sp. are synonymous.

therefore regarded as a member of genus *Alcaligenes*. On the other hand, the 16S rRNA gene analysis of S19 agreed with its biochemical test in suborder level, since *Arthrobacter* sp. and *Microbacterium* sp. are in the suborder Micrococcineae. Hence, S19 was considered a member of genus *Microbacterium.*

The isolate P42 was biochemically identified to be *Brevibacterium brevi* while the sequence analysis referred to *Planococcus* sp. These 2 bacteria are in the same family of Micrococcaceae**.** Based on the recognition of 16S rRNA gene analysis, it is suggested that P42 had the tendency to be *Planococcus* sp. or at least a member of *Micrococcaceae*.

The isolates R07 and R08 were similarly identified by sequence analysis to be members of the family Alcaligenaceae. In addition, R07 was biochemically identified to be *Cupriavidus pauculus*, which belongs to the family Alcaligenaceae. It is, without doubt to state that R07 was a member in family Alcaligenaceae. Nevertheless, R08 was identified by its biochemical properties to be *Pseudomonas pseudoalcaligenes* which was quite different from family Alcaligenaceae. When considering further, *Pseudomonas pseudoalcaligenes* is a gram-negative bacterium, whereas bacteria in the family Alcaligenaceae are gram-positive. In this case, biochemical properties seemed to be a better choice because the isolate R08 was tested to be a gram-negative bacterium. Hence, R08 was tentatively identified to be *Pseudomonas pseudoalcaligenes* or at least in the genus *Pseudomonas*.

In summary, the estrogen-degrading bacteria isolated from the animal farm soils in the current study are in family *Alcaligenaceae* (Cp36 and R07), genus *Microbacterium* (C51, R09, R12 and S19), genus *Planococcus* (P42) and genus *Pseudomonas* (R08). Previously, Yu *et al.* (2007) reported the finding of a *Microbacterium* sp. strain KC5 from activated sludge that converted E2 to E1. In comparison, the *Microbacterium* spp. investigated in this study exhibited three different types of estrogen degradation: E1 degradation - C51, E2 degradation - R09 and R12, and E2 degradation with E1 as a metabolite - S19. The additional experiment indicated that C51 could also use E2 as carbon source and E1 was released as a metabolite. It is noted that bacteria of the same genus can perform different metabolic functions in different environment. Further work is required to examine the similarity between strain KC5 and the isolates C51 and S19.

In essence, bacteria in the family Alcaligenaceae, genus *Planococcus* and genus *Pseudomonas* have never been previously reported on their estrogen-degrading activities. Although the activities of E1 and/or E2-degrading bacteria isolated in this study were not as high as those in the previous reports, they will be suitable for application in the areas contaminated with low level of estrogen. It is challenging to study further if these isolates can be cultured with other bacteria to improve the overall biodegradation.

CHAPTER V

CONCLUSION

5.1 Conclusion

This study successfully screened the estrogen-degrading bacteria from soil samples collected from different animal farms in Nakhonpathom Province. Using HPLC to investigate estrogen transformation together with identification of the isolated bacteria by 16S rRNA gene sequencing and biochemical properties, the following findings are resulted.

1. One isolate, C51 from cow farm soil, a *Microbacterium sp.*, degraded 48% of 15 mg/L initial concentration of E1 in 4 days.

2. Seven isolates were found to possess high E2 degrading ability, 53-82% of 15 mg/L E2 in 4 days: Cp36 from pregnant cow farm, P42 from pig farm, R07, R08, R09 and R12 from rabbit farm, and S19 from sheep farm.

3. Two different patterns of E2 degradation were observed. No detectable metabolites were generated from P42, R07, R08, R09 and R12, while E1 was detected as a metabolite of E2 degradation for Cp36 and S19.

4. Four novel isolates of E2-degrading bacteria were identified: 2 isolates in family Alcaligenaceae (Cp36 and R07), 1 isolate in genus *Planococcus* (P42) and 1 isolate in genus *Pseudomonas* (R08).

5. The isolates C51 and P42, as representatives of E1- and E2-degrading bacteria were studied for the ability to use other estrogenic compounds. Both bacterial isolates could degrade E1 and E2 but not EE2 and MT.

5.2 Suggestions for future work

The bacteria which did not give any metabolite from estrogen degradation such as isolates P42, R07, R08, R09 and R12 from this preliminary study are possible candidates for application in the treatment of estrogens contaminated area. However, there are several practical steps worth pursuing further. Degrading activity of each isolate can be examined in more details so as to find a suitable methodology to improve the enzymatic efficiency. Optimization of incubating condition, nutrients and

oxygen level for bacterial growth and estrogen degradation, and/or genetic engineering technique are suggested. In addition, the bacterial isolates in a mix-and match culture, either between E1- and E2-degrading bacterial groups or within each group may be an interesting option but would require good understanding in managing bacterial coexistence. Subsequently, cell immobilization may be an appropriate technique to stabilized and prolong the bacterial metabolic activity as well as to increase the estrogen-degrading efficiency. Immobilized cells can be subsequently studied for the application in the stabilization ponds and in the biological reactors in wastewater treatment plants in order to test the estrogen elimination from animal farms.

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APPENDICES

APPENDIX A

Standard curve for calculation

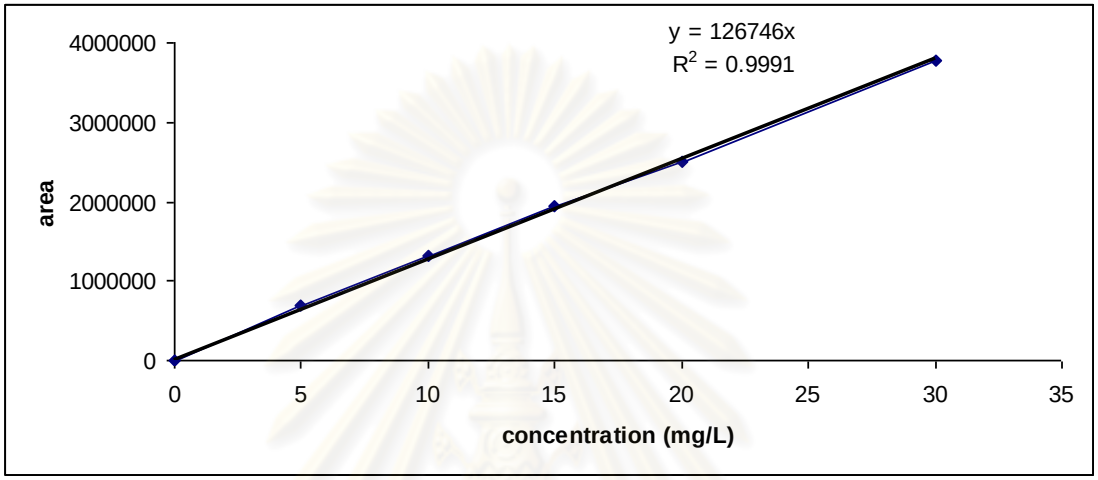

Figure A-1: Standard curve of E1

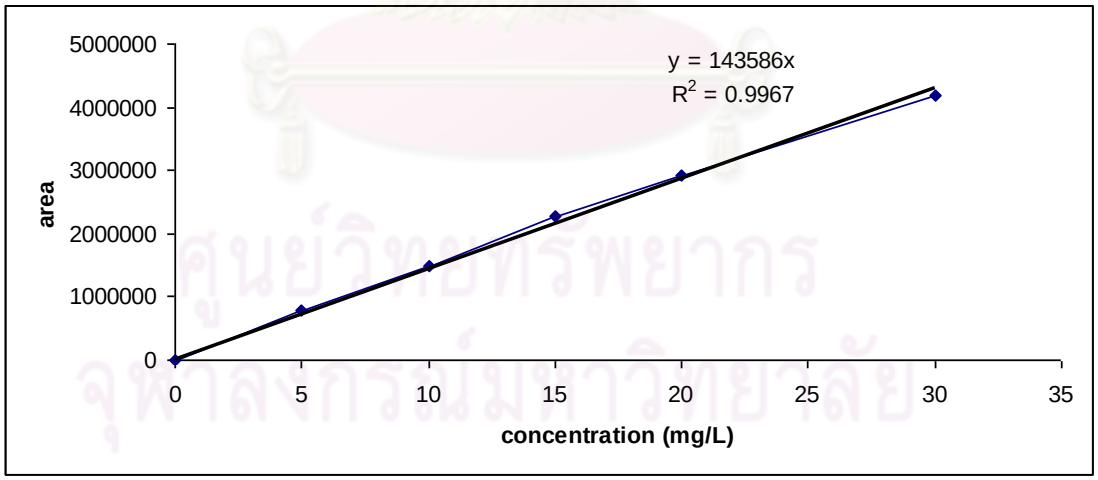

Figure A-2: Standard curve of E2

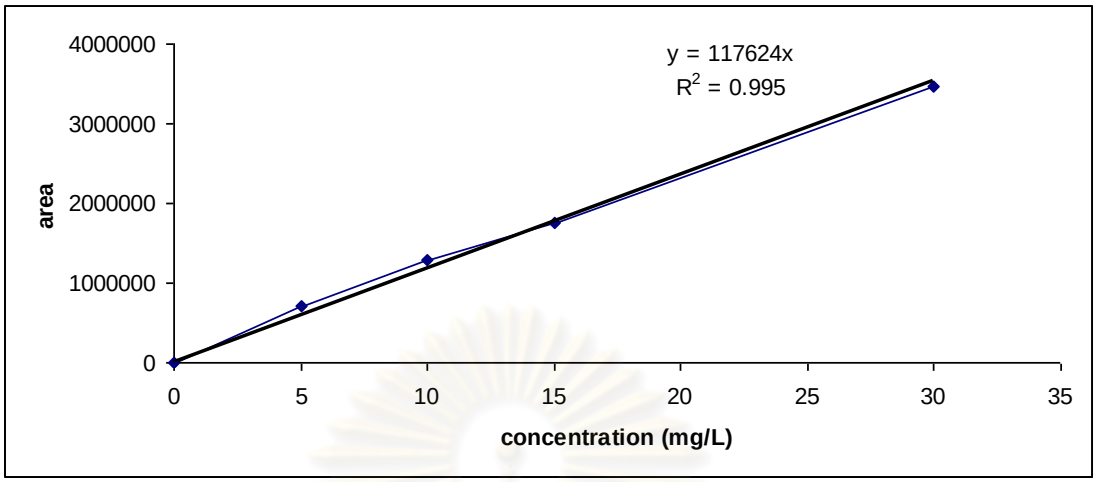

Figure A-3: Standard curve of EE2

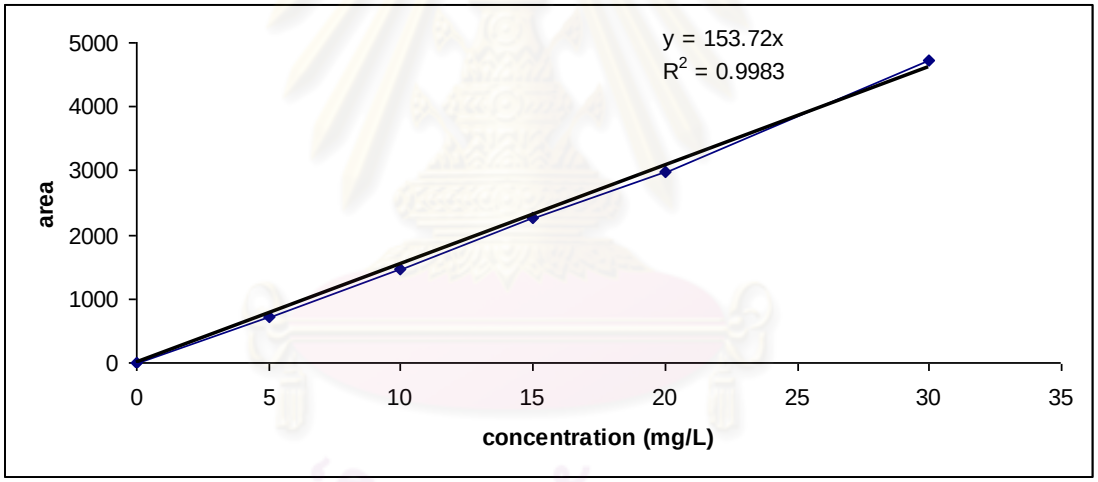

Figure A-4: Standard curve of MT

APPENDIX B

Preparation of reagents for DNA extraction

1. 1 M Tris-HCl (pH 8.0)

Tris (hydroxymethyl)-aminomethane	12.10	g

Adjust pH to 8.0 with 1 N HCl and make up the volume to 100 mL with distilled water.

2. 0.5 M EDTA (pH 8.0)

di-Sodium ethylene diamine tetra acetic acid	18.61	g

Adjust pH to 8.0 with 1 N NaOH and make up the volume to 100 mL with distilled water.

3. 10X Electrophoresis buffer (TBE)

Tris (hydroxymethyl)-aminomethane	107.90	g
Boric acid	55.03	g
Ethylene diamine tetra acetic acid	7.44	g

Adjust volume to 1 L with distilled water

APPENDIX C

Data of the time course study of estrogen degradation

Table C-1 to C-16 show the data of time course study of estrogen degradation as illustrated in Figures 4.1 and 4.2. Each table contains data of duplicate samples. The values of estrogen concentration (conc.) are in the unit of mg/L.

Table C-1: Time course study of E1 degradation by isolate C07

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	1508484	1438416	16.89232	16.10768	16.50089	0.554824
1	1135758	1047459	12.71845	11.72966	12.22406	0.699181
2	1046154	1061419	11.71505	11.88599	11.80052	0.120873
3	1011469	864608	11.32664	9.68206	10.50435	1.162894
4	979908	899063	10.97322	10.06789	10.52056	0.640165

Table C-2: Time course study of E1 degradation by isolate C51

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	1854442	1868559	20.76643	20.92451	20.84547	0.111779
1	1608516	1657035	18.01250	18.55582	18.28416	0.384185
2	1351815	1396127	15.13791	15.63412	15.38601	0.350877
3	1129016	1053303	12.64296	11.79510	12.21903	0.599524
4	983199	963849	11.01007	10.79338	10.90172	0.153220

Table C-3: Time course study of E1 degradation by isolate C60

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	1424081	1446031	15.94716	16.19296	16.07006	0.173807
1	1367846	1523682	15.31742	17.06251	16.18997	1.233961
2	1378392	1268172	15.43552	14.20125	14.81839	0.872758
3	1467832	1231500	16.43709	13.79059	15.11384	1.871355
4	1104803	963941	12.37181	10.79441	11.58311	1.115392

Table C-4: Time course study of E1 degradation by isolate S02

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1539340	1555198	17.23785	17.41543	17.32664	0.125569
1	1412099	1379395	15.81297	15.44675	15.62986	0.258957
2	1483490	1471060	16.61242	16.47324	16.54283	0.098421
3	1439920	1489752	16.12452	16.68255	16.40354	0.394590
4	1155425	1076195	12.93868	12.05146	12.49507	0.627365

Table C-5: Time course study of E2 degradation by isolate C27

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1314283	1360846	12.35693	12.79472	12.57582	0.309564
1	1198527	1126888	11.26859	10.59504	10.93181	0.476273
2	806685	735945	7.58448	6.91938	7.25193	0.470296
3	778409	731550	7.31863	6.87806	7.09834	0.311530
4	750181	763963	7.05322	7.18281	7.11802	0.091630

Table C-6: Time course study of E2 degradation by isolate Cp36

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1568392	1489615	14.74607	14.00541	14.37574	0.523728
1	1015301	930852	9.54589	8.75190	9.14890	0.561437
2	565449	731881	5.31637	6.88117	6.09877	1.106480
3	576460	508596	5.41990	4.78184	5.10087	0.451176
4	460842	568487	4.33285	5.34493	4.83889	0.715650

Table C-7: E1 production by isolate Cp36 during degradation of E2

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	16754	1489615	0.187615	0.233415	0.210515	0.032386
1	343886	930852	3.850907	5.754927	4.802917	1.346346
2	299715	731881	3.356271	6.791445	5.073858	2.429035
3	385965	508596	4.322116	6.485901	5.404009	1.530027
4	343379	568487	3.845230	6.994950	5.420090	2.227188

Table C-8: Time course study of E2 degradation by isolate P23

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1505891	1575712	15.24937	15.95642	15.60290	0.499953
1	1639095	1711854	16.59826	17.33505	16.96666	0.520991
2	1676225	1693925	16.97426	17.15350	17.06388	0.126741
3	1678070	1636518	16.99294	16.57217	16.78255	0.297533
4	1692234	1728854	17.13637	17.50720	17.32179	0.262218

Table C-9: Time course study of E2 degradation by isolate P42

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1212979	1190505	12.28321	12.05562	12.16942	0.160925
1	782621	767304	7.92520	7.77009	7.84764	0.109677
2	498752	386398	5.05060	3.91285	4.48173	0.804511
3	378645	333313	3.83434	3.37529	3.60481	0.324600
4	266131	287468	2.69500	2.91104	2.80301	0.152784

Table C-10: Time course study of E2 degradation by isolate R07

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1160840	1025983	10.91425	9.64632	10.28029	0.633962
1	927887	998324	8.72402	9.38627	9.05515	0.331125
2	466480	731006	4.38586	6.87294	5.62940	1.243541
3	447914	501870	4.21130	4.71860	4.46495	0.253649
4	587031	598046	5.51929	5.62285	5.57107	0.051781

Table C-11: Time course study of E2 degradation by isolate R08

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1440157	1648115	13.5404	15.49563	14.51801	1.382555
1	940176	922605	8.839564	8.674361	8.756962	0.116816
2	583233	698998	5.483575	6.572001	6.027788	0.769633
3	461300	565790	4.337157	5.319575	4.828366	0.694675
4	352048	426417	3.309966	4.009186	3.659576	0.494423

Table C-12: Time course study of E2 degradation by isolate R09

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1419938	1425416	13.35030	13.40181	13.37605	0.036419
1	1144281	1048405	10.75857	9.85714	10.30785	0.637407
2	715629	649158	6.72836	6.10340	6.41588	0.441912
3	561670	523714	5.28083	4.92398	5.10241	0.252337
4	502944	453371	4.72870	4.26261	4.49565	0.329573

Table C-13: Time course study of E2 degradation by isolate R10

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	1477035	1005939	13.89418	9.45787	11.67603	3.136947
1	1134928	859944	10.67063	8.08522	9.37792	1.828160
2	613878	902429	5.77170	8.48467	7.12818	1.918356
3	644031	929626	6.05520	8.74037	7.39779	1.898704
4	413176	745764	3.88469	7.01170	5.44820	2.211125

Table C-14: Time course study of E2 degradation by isolate R12

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	1686755	1470498	15.85892	13.82567	14.84229	1.437724
1	1160972	1054521	10.91549	9.91464	10.41507	0.707712
2	1033346	776826	9.71555	7.30374	8.50964	1.705406
3	791045	680484	7.43743	6.39793	6.91768	0.73504
4	555999	665203	5.22752	6.25426	5.74089	0.726017

Table C-15: Time course study of E2 degradation by isolate S19

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	1608305	1617313	15.12133	15.20603	15.16368	0.059887
1	949205	1004284	8.92446	9.44231	9.18338	0.366178
2	421657	358285	3.96443	3.36861	3.66652	0.421312
3	285300	317080	2.68240	2.98120	2.83180	0.211281
4	310552	295481	2.91982	2.77812	2.84897	0.100196

Table C-16: E1 production by isolate S19 during degradation of E2

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	0	13211	0	0.14794	0.07397	0.104609
1	505158	473131	5.65687	5.29822	5.47754	0.253600
2	486609	522646	5.44915	5.85270	5.65092	0.285353
3	421904	366994	4.72457	4.10968	4.41712	0.434795
4	507636	510470	5.68461	5.71635	5.70048	0.022441

Data of degradation of other related hormones

Table C-17 to C-29 show the data of time course study of related hormones as illustrated in Figures 4.5 to 4.8. Each table contains data of duplicate samples.

Table C-17: Control studies of E1 without any bacterial inoculation

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	769375	794449	14.13591	14.59660	14.36625	0.325759
1	730593	762653	13.42336	14.01240	13.71788	0.416517
2	781504	783562	14.35876	14.39657	14.37766	0.026739
3	724662	771699	13.31438	14.17860	13.74649	0.611093
4	742845	774338	13.64846	14.22710	13.93778	0.409157

Table C-18: Degradation of E1 by isolate C51

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	830440	792850	15.25787	14.56722	14.91254	0.488363
1	578396	527743	10.62700	9.69634	10.16167	0.658076
2	566520	505201	10.40880	9.28218	9.84549	0.796647
3	558265	485865	10.25713	8.92681	9.59197	0.940679
4	550014	478573	10.10554	8.79294	9.44924	0.928149

Table C-19: Degradation of E1 by isolate P42

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	813362	847212	14.94409	15.56602	15.25506	0.439774
1	577928	639647	10.61841	11.75238	11.18540	0.801843
2	550980	659969	10.12328	12.12576	11.12452	1.415967
3	625182	604176	11.48662	11.10067	11.29364	0.272907
4	528028	635914	9.70158	11.68380	10.69269	1.401637

Table C-20: Control studies of E2 without any bacterial inoculation

day	$area_1$	$area_2$	$conc._1$	$conc._2$	average conc.	SD
0	799442	846086	16.60557	17.57443	17.25148	0.68509
1	611758	675819	12.70710	14.03773	13.59419	0.940897
2	563182	616956	11.69810	12.81506	12.44274	0.789813
3	382928	448342	7.95397	9.31272	8.85980	0.960778
4	236675	307359	4.91608	6.38428	5.89488	1.038179

Table C-21: Degradation of E2 by isolate C51

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	740046	830078	15.37183	17.24193	16.30688	1.322357
1	641661	671250	13.32823	13.94284	13.63553	0.434592
2	345830	369809	7.18339	7.68147	7.43243	0.352195
3	138934	226832	2.88585	4.71164	3.79875	1.291028
4	133030	200227	2.76323	4.15901	3.46112	0.986965

Table C-22: E1 production by isolate C51 during degradation of E2

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	75947	63075	1.3954	1.1589	1.2772	0.167231
3	119843	117888	2.2019	2.1660	2.1839	0.025399
4	145728	120082	2.6775	2.2063	2.4419	0.333189

Table C-23: Degradation of E2 by isolate P42

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	759723	816238	15.78055	16.95445	16.36750	0.830072
1	480825	424034	9.98743	8.80780	9.39762	0.834125
2	313307	174578	6.50784	3.62624	5.06701	2.037601
3	214672	104607	4.45905	2.17284	3.31594	1.616594
4	209300	102908	4.34747	2.13755	3.24251	1.562647

Table C-24: Control studies of EE2 without any bacterial inoculation

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	938134	738317	19.79603	18.57959	19.18781	0.860152
1	694092	554358	14.64638	13.69778	14.17208	0.670759
2	563536	576877	11.89145	12.17297	12.03221	0.199061
3	509516	576580	10.75155	11.66670	11.20913	0.647109
4	480160	600529	10.13210	11.07206	10.60208	0.664657

Table C-25: Degradation of EE2 by isolate C51

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	924754	963127	19.51369	20.32342	19.91856	0.572564
1	838573	826052	17.69515	17.43093	17.56304	0.186826
2	783320	789231	16.52923	16.65396	16.59159	0.088198
3	803344	799034	16.95176	16.86081	16.90629	0.064310
4	793007	754179	16.73364	15.91431	16.32397	0.579353

Table C-26: Degradation of EE2 by isolate P42

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	929290	864128	19.60941	18.23440	18.92190	0.972283
1	694104	650582	14.64663	13.72825	14.18744	0.649392
2	695460	560327	14.67525	11.82374	13.24949	2.016321
3	641334	600486	13.53311	12.67115	13.10213	0.609494
4	628109	444487	13.25404	9.37934	11.31669	2.739826

Table C-27: Control studies of MT without any bacterial inoculation

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	2244.5	2245.8	14.60122	14.6113	14.60626	0.003563
1	2291.7	2276.8	14.90827	14.8134	14.86084	0.033543
2	2199.9	2213.2	14.31109	14.3992	14.35514	0.031153
3	2172.5	2197.6	14.13284	14.2981	14.21547	0.058429
4	2169.0	2171.6	14.11007	14.1287	14.11939	0.006587

Table C-28: Degradation of MT by isolate C51

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	2230.7	2242.4	14.51145	14.58756	14.54951	0.053820
1	2313.6	2246.7	15.05074	14.61553	14.83314	0.307738
2	2159.4	2232.3	14.04762	14.52186	14.28474	0.335338
3	2012.8	2179.5	13.09394	14.17838	13.63616	0.766814
4	1810.2	2034.5	11.77596	13.23510	13.60005	1.031772

Table C-29: Degradation of MT by isolate P42

day	area$_1$	area$_2$	conc.$_1$	conc.$_2$	average conc.	SD
0	2263.9	2368.9	14.72743	15.41049	15.06896	0.482996
1	2303.0	2178.9	14.98179	14.17447	14.57813	0.570856
2	2346.0	2132.8	15.26151	13.87458	14.56805	0.980713
3	2283.7	2023.0	14.85623	13.16029	14.00826	1.199211
4	2309.3	2162.8	15.02277	14.06974	14.54625	0.673895

APPENDIX D

16S rRNA gene sequences of isolated estrogen-degrading bacteria

>C51

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>Cp36

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CA

BIOGRAPHY

Miss Chanoknad Pornsamrit was born on August 23, 1984 in Ayutthaya Province, Thailand. She obtained her Bachelor Degree in Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2007. In April of the same year, she was accepted with full scholarship into the International Postgraduate Programs in Environmental Management, an interdisciplinary program operated by the National Center of Excellence for Environmental and Hazardous Waste Management through the Graduate School, Chulalongkorn University, Bangkok, Thailand. In her second year of research work, she presented a poster entitled "17β-estradiol degrading bacteria isolated from animal farm soils" at the 2nd Biochemistry and Molecular Biology Conference, organized by Khon Kaen University on May 7-8, 2009.