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Microbial Degradation of Estrogens in Engineered and Natural Polluted Environments in Bahrain

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Doctor of Philosophy in Environment and Sustainable Development

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☐ Without making any changes to the thesis.

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Abstract

Microbial biodegradation is the main route for estrogen elimination from wastewater treatment plants (WWTPs) and polluted environments to avoid estrogens environmental hazard due to their endocrine-disruption and carcinogenic properties. However, information on the biodegradation of estriol (E3) and the synthetic estrogen ethinylestradiol (EE2) and involved microorganisms and their ecological relevance is very limited. In this study, raw sewage (inflow wastewater), activated sludge, and thickened sludge samples were collected from a municipal WWTP in Bahrain to isolate and characterize E3- and EE2-degrading microbial cultures. Three E3-degrading microbial consortia were obtained via enrichment culturing. The three consortia utilized E3 as a carbon source in minimal medium. On the contrary, pure bacterial cultures isolated from the consortia could not grow on E3 as the sole carbon source. Instead, they transformed E3 to 16 α -OH-E1 as a key product. Phylogenetic analysis revealed that the E3-transforming bacteria were affiliated with the genera *Hydrogenophaga*, *Microbacterium*, and *Gordonia*. The inflow consortium (enriched from raw sewage) grew on and utilized E1, E2, in addition to E3 individually and as a mixture in minimal medium, raw sewage, and activated sludge microcosms; albeit EE2 was not degraded. Degradation of E3 by the inflow consortium proceeds via the 4,5-*seco* pathway, in which E3 is initially transformed to E1. During E3 biodegradation in a 14-day experiment, the community structure of the inflow consortium underwent remarkable temporal changes. Up to day 6 of the biodegradation experiment, the inflow consortium was dominated mainly by the genera *Achromobacter*, *Hyphomicrobium*, *Microbacterium*, *Cupriavidus*, and *Aminobacter*. This community strongly shifted towards *Croceicoccus estronivorus* which dominated the community starting from day 8 till day 14. Growth of the inflow consortium on dimethylsulfoxide in the absence of estrogens eliminated E3-degrading members of the community, and the inflow consortium lost the E3 biodegradation phenotype. We conclude that natural estrogen-degrading microbial communities exist not only in activated sludge, but also in raw sewage. Complete mineralization of E3 requires concerted action of the several community members of the inflow consortium at different time intervals and depending on the substrate and product concentrations. However, functional resilience of the consortium is a crucial factor that needs to be carefully addressed. Time and

substrate/products concentrations are important determinants of the community composition.

Keywords: Estrogens, Estriol, Biodegradation, Activated sludge, 4,5-*seco* pathway, Consortia.

Dedication

To the soul of my father, whom I never met,

to my beloved mother,

Who first sparked my curiosity and taught me to love
science,

Your support, sacrifices, and belief in me have been my
greatest source of strength.

This work is a testament to your wisdom, resilience,
and the values you instilled in me.

Thank you for inspiring me to never give up on my
dreams.

With all my love and gratitude,

Jaleela

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List of Abbreviations

CDM	Chemically Defined Medium
DMSO	Dimethyl Sulfoxide
E1	Estrone
E2	17 β -Estradiol
E3	Estriol
E4	Estetrol
EDCs	Endocrine Disrupting chemicals
EE2	17 α - ethinylestradiol
GCC	Gulf Cooperation Countries
GC-MS	Gas Chromatography -Mass Spectrometry
HIP	3 α -H-4 α (3'-propanoate)-7 α β - methylhexahydro-1,5-indanedione
HPLC	High Performance Liquid Chromatography
HRMS	High-Resolution Mass Spectrometry
OecC	4-Hydroxy 4,5-dioxygenase
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PEA	Pyridinestrone Acid
PNECs	Predicted No Effect Concentrations
rpm	Revolution per minute
TCA	Tricarboxylic Acid Cycle
UPLC	Ultra-Performance Liquid Chromatography
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

1. Introduction

In modern times environmental pollution by hazardous chemicals is one of the most critical global challenges. Some of these chemical pollutants can interfere with the natural functions of hormones in humans and animals, and hence are termed endocrine-disrupting chemicals (EDCs) (Chiang et al., 2020; Zhang et al., 2016). EDCs have been defined by the World Health Organization (WHO) as "an exogenous substance or a mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (Bergman et al., 2012).

In the same context, scarcity of freshwater resources has become a global challenge that threatens sustainable development in many countries. This issue will escalate in the near future due to the increasing global human population, urbanization, and the negative impacts of climate change (Stefanakis, 2022; Stikker, 1998). Hence, reuse and recycling of wastewater from municipal, industrial, and agricultural sectors are being adopted as counter measures to increase the water resource base. However, wastewater streams require efficient treatment and remediation processes to remove hazardous pollutants, like EDCs, and ensure safe reuse (Stefanakis, 2018).

Steroid hormones, like estrogens, are produced by humans and animals where they play key physiological roles in regulating reproductive systems and the development of secondary sex characteristics (Bilal et al., 2021). Although estrogens are necessary for humans and other vertebrates, chronic exposure to even trace amounts of estrogens (sub-nanomolar levels) can disrupt the endocrine system and sexual development (Kolodziej et al., 2003; Lambert et al., 2015; Lee et al., 2006; Regnault et al., 2018). Hence, estrogens are considered EDCs and were classified by the WHO as group-1 carcinogens, and some catechol metabolites of estrogens were reported as carcinogens (American Cancer Society, 2015). These

hazardous properties raised substantial concerns about environmental pollution with estrogens.

Steroid estrogens are represented by three naturally occurring hormones, namely estrone (E1), estradiol (E2), and estriol (E3), as well as the synthetic estrogen ethinylestradiol (EE2) (Pratish et al., 2020a). Estrogens are chemical pollutants in aqueous environments and raise significant concerns for water and wastewater quality (Roudbari & Rezakazemi, 2018). They enter the environment via different routes, but mainly through discharge of urine and feces reaching wastewater treatment plants (WWTPs) (Adeel et al., 2017). Estrogens are commonly present in wastewater in trace amounts, ranging from ng/L to µg/L, yet they are associated with adverse ecological impact and influence marine organisms in the impacted area, causing acute and chronic toxicity to organisms, accumulation in the ecosystem, as well as loss of habitat and biodiversity (Adeel et al., 2017; Chiang et al., 2020).

Estrogens accumulate in the tissues of fish and other marine organisms (Ros et al., 2016). These compounds have direct biological effects, causing increased liver size, increased plasma glucose concentration, smaller gonad size, and less mature gametes (Thomas et al., 2017). However, this may adversely disrupt the reproductive potential of marine organisms, reducing their numbers and thus causing decline in a sustainable food source for the exposed populations (Elliott et al., 2017). Likewise, the accumulation of estrogens seriously threatens the human food chain. Eventually, they build up in the human body and trigger health concerns, such as breast cancer in women and prostate cancer in men (Adeel et al., 2017). Consequently, the effective elimination of estrogens and other EDCs from the environment has become an important research area.

Efficient removal of estrogens during wastewater treatment is essential to avoid environmental pollution. Physical removal methods such as reverse osmosis

and adsorption on activated carbon do not eliminate the pollutants, albeit only transfer them from one phase to another. Moreover, chemical oxidation is not economically viable, and chemicals are reactive, and the process is associated with by-products with unknown effects (Bolong et al., 2009; Koh et al., 2008; Liu et al., 2009). Several techniques such as electrochemical oxidation adsorption, photocatalysis, and membrane filtration have been proposed for removal of EDCs from the aqueous environment, albeit can be associated with high cost, operational complexity, and environmental impact (Ben et al., 2017).

On the contrary, microbial biodegradation is a green and economical process which plays a key role in estrogen removal from WWTPs (de Lorenzo, 2017). Several estrogen-degrading bacteria have been isolated mainly from activated sludge collected from WWTPs. These bacteria were used in several studies with focus on biodegradation of E1 and E2 (Chiang et al., 2020). However, these studies mostly applied pure cultures and single estrogen substrates, which is not necessarily environmentally relevant where there is a mixture of estrogens and other carbon sources, and microbes exist as complex and heterogeneous communities. Moreover, there is a lack of knowledge on the mechanisms and microbes involved in E3 and EE2 biodegradation. This is particularly important for the GCC countries where estrogen biodegradation has been very rarely investigated, despite some reports on environmental pollution with estrogens and several other EDCs.

Research Problem

Estrogens are currently recognized as harmful environmental pollutants, and WWTPs are a key source of environmental pollution with estrogens. In addition, scarcity of freshwater resources in many countries around the globe, including the GCC countries, has rendered the reuse of treated wastewater a strategic direction. However, safe reuse of wastewater requires implementation of efficient treatment and remediation technologies to remove hazardous pollutants, like steroid estrogens. Microbial biodegradation is recognized as the principal mechanism by

which estrogens can be eliminated from the environment. However, there is still several knowledge gaps on the mechanisms of estrogen biodegradation that need to be sufficiently characterized in terms of the catabolic pathways (intermediates, genes, enzymes, gene regulation) and the key degrading microbes. This is particularly crucial for E3 and EE2 due to scarcity of knowledge. Thus, further systematic studies are needed for better understanding of the underlying biochemical and microbiological processes, which is crucial for exploiting the full potential of bioremediation for estrogens removal.

The climate change context exacerbates pressures to upgrade technologies capable of efficiently removing chemical micropollutants. Massive consumption coupled with unpredictable weather conditions, such as drought, leads not only to freshwater scarcity but also to the deterioration of water quality (Nunes, 2021). Furthermore, freshwater scarcity in Bahrain and other GCC countries imposes recycling wastewater, treated to a final effluent that can be safely and sustainably reused for different purposes, predominantly in agriculture.

Research Questions

This study is designed to address mechanisms and unravel bacterial communities involved in biodegradation of the steroid estrogens E3 and EE2. Hence the research questions are:

1. Which E3- and EE2-degrading bacteria can be isolated from wastewater?
2. Is the degradation of E3 and EE2 growth-associated or co-metabolic?
3. What metabolic pathway do the bacterial isolates employ to degrade E3?
4. How does the structure of E3-degrading bacterial communities change over time?
5. Are the estrogen-degrading microbial communities active in raw sewage and activated sludge?

Research Objectives

This study aims to identify and characterize E3- and EE2-degrading bacteria and elucidate the biodegradation pathway(s) in samples collected from a central wastewater treatment plant in Bahrain (Tubli plant), using culture-dependent and culture-independent techniques to address the following objectives:

- To isolate and characterize E3- and EE2-degrading bacteria and bacterial consortia from a wastewater treatment plant.
- To identify the isolated bacteria.
- To unravel key E3-degrading bacteria in bacterial communities isolated from wastewater.
- To elucidate the E3 biodegradation pathway by a mixed bacterial consortium.
- To test the potential of estrogen degradation in wastewater.

Hypotheses

This study is based on the hypothesis that if estrogens exist in municipal wastewater, it will be possible to isolate E3- and EE2-degrading bacteria from wastewater treatment plants in Bahrain, and the isolated bacteria will be able to degrade estrogens in wastewater and activated sludge microcosms.

Research Significance

This study will contribute to the growing field on estrogen biodegradation mechanisms and involved microbial communities. The contributed knowledge is fundamental for initiating further investigations to develop bioremediation as a major mechanism for estrogens removal from the environment. This will enable green and more economic elimination of these hazardous pollutants from wastewater, thus contributing to the reuse/recycle of wastewater initiatives. Achieving the 2030 vision and the Sustainable Development Goals (SDGs) necessitates monitoring water quality and applying the prevailing regulatory measures to develop new techniques and technologies to resolve environmental concerns.

2. Literature Review

2.1. Estrogens: Biosynthesis, Physiological roles, and Properties

Estrogens are steroid hormones that function as signaling molecules including natural estrogens such as estrone (E1), 17 β -estradiol (E2), estriol (E3), as well as the synthetic estrogen 17 α -ethinylestradiol (EE2) (Bilal et al., 2021). The precursor of biosynthesis of natural estrogens is dietary cholesterol (C₂₇H₄₆O), particularly the low-density lipoprotein (LDL) (Olivera & Luengo, 2019) (Figure 2.1).

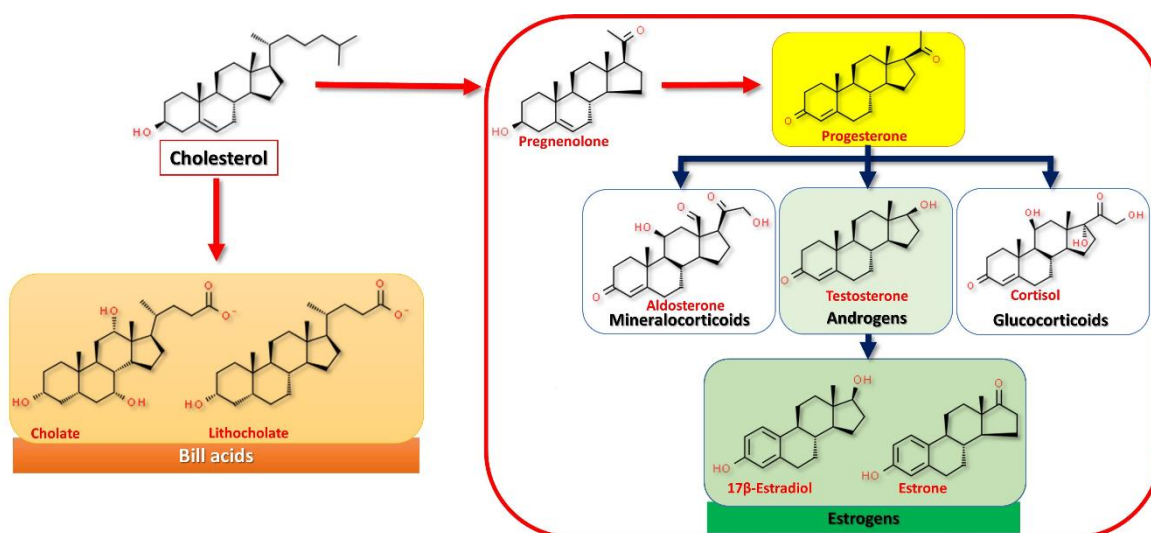


Fig. 2.1: Cholesterol as a precursor of steroids (Olivera & Luengo, 2019).

In steroidogenesis, cholesterol is converted into sterane, a C₁₈ steroid derivative. The leading site of estrogen biosynthesis is the granulosa cells of the ovaries (Vazakidou et al., 2024). Yet, the translocation of cholesterol occurs in the inner membrane of the mitochondria and is regulated by a steroidogenic acute regulatory protein (StAR) (Lennartz et al., 2024).

In the mitochondrial membrane, cholesterol is transformed to pregnenolone by the enzyme P450_{scc}, which is further converted to androstenedione by the enzymes

CYP17A1 and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) using dehydroepiandrosterone (DHEA) (Cui et al., 2013). Androstenedione disperses to the granulosa cells across the basal lamina, where it produces E1 and E2 by the enzymes CYP19A1 (aromatase) and 17 β hydroxysteroid dehydrogenase (17 β -HSD), respectively (Cui et al., 2013).

On the other hand, E3 biosynthesis occurs primarily during pregnancy and almost entirely by the placenta from the dietary cholesterol as well. Cholesterol is converted into pregnenolone and progesterone in the placenta, where they are then metabolized in the fetal adrenal gland and hydroxylated in the fetal liver by the enzyme CYP3A7 and transmitted back to the placenta (Leeder et al., 2005). They are then converted into 16 α -OH-E1, which is transformed to estriol by the action of the enzyme 17 β -HSD and released into the maternal circulation. In non-pregnant women, estriol is formed predominantly in the liver by hydroxylation of estrone and estradiol by the CYP enzymes. Estrone is produced chiefly during menopause from the aromatization of androstenedione within the extra-granular tissues. It can also be transformed to estriol by the enzyme 17 β -HSD in peripheral tissues such as breast and adipose tissues, smooth muscle cells, brain tissue, and bone cells, where it enters the circulation, however in lesser quantities (Fuentes & Silveyra, 2019).

Estrogens are endogenous chemical messengers produced by the endocrine system of humans and animals and carried out by the blood to the target tissues. Natural estrogens are essential sex hormones that play important physiological roles in the development of female reproductive system and secondary sexual characteristics (Fuentes & Silveyra, 2019) in addition to male external normal genitalia (Baskin et al., 2022). However, the classical role of endogenous estrogens in the human body has changed. By now, it is known that estrogens have roles in regulating immunity through their effect on the immune cells to prevent atherosclerosis and endothelial dysfunction by maintaining the endothelial cells and promoting angiogenesis (Trenti et al., 2018). Furthermore, estrogens regulate

critical psychological pathways, such as in the pathophysiology of schizophrenia, particularly in men (Brand et al., 2021). The synthetic estrogen EE2 is commonly used in contraceptives. It is one of the most common medications for humans and animals and has raised various health issues and ecological concerns (Laurenson et al., 2014). EE2 is a highly potent oral contraceptive and ranked among the top 15 US active ingredients in terms of its daily use (Kostich & Lazorchak, 2007).

The structure of estrogens comprises 18 carbon atoms and shares the same core molecular structure of sterane, tetracyclic framework, consisting of four fused rings (A, B, C, and D) (Figure 2.2, Table 1) (Bilal et al., 2021). The A ring is phenolic, B and C rings are cyclohexane, whereas D is a cyclopentane ring. The structural differences among the different estrogens lie in the configuration of the D-ring, specifically, carbons at positions 16 and 17.

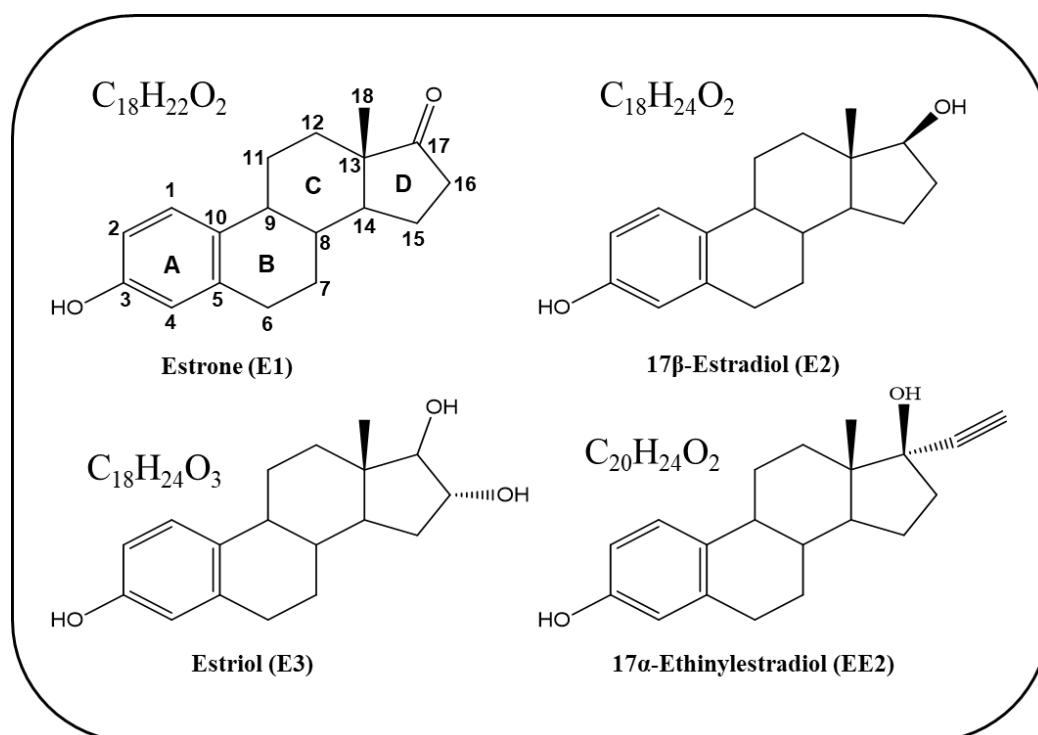


Fig. 2.2: Chemical structure of natural and synthetic estrogens.

Table (2.1): Properties of natural and synthetic estrogens (Zhang et al., 2016).

Endocrine disrupting compound	Molecular weight (g/mol)	Water solubility (mg/L)	Log K _{ow}	pK _a	Relative estrogenic potency (E-Screen)
Estrone (E1)	270.4	30	3.13	10.5 ^a	0.01
17 β -Estradiol (E2)	272.4	3.6	4.01	10.7 ^a	1
Estriol (E3)	288.4	441	2.45	10.4 ^a	0.3
17 α -Ethinylestradiol (EE2)	296.4	116	3.67	10.4 ^a	1.25

Based on that, there are three well-known natural estrogens: Estrone (E1), which has a carbonyl group at C17 and one hydroxyl group at C3; 17 β -estradiol (E2), that has two hydroxyl groups, one at C3 and another at C17; Estriol (E3) has two hydroxyl groups at C16 and C17 in addition to the C3 hydroxyl group. The synthetic estrogenic hormone 17 α -ethinylestradiol (EE2) is a derivative of the natural E2 by adding an ethinyl group to C17 of estradiol (Bilal et al., 2021).

Among the three major estrogens, estrone (molecular weight 270.4 g/mol) is the first known steroidal hormone discovered in 1929. It is a weak estrogen, 1/3 that of estradiol, and a precursor or intermediate metabolite of E2, but it is the most predominant circulating estrogen in postmenopausal women. As mentioned earlier, E1, as well as the other two estrogens, is synthesized from the organic sterol molecule, cholesterol, through a series of reactions and produced mainly from the ovaries, though it is also produced from the adipose tissues and placenta (Cui et al., 2013).

The general pathway of estrone synthesis is via the conversion of androstenedione to estrone by the enzyme aromatase; estrone, on the other hand, produces estradiol by the enzyme 17 β -hydroxysteroid dehydrogenase (17 β -HSD).

Thus, the most important physiological functions of E1 seem to be a repository for the other estrogens, besides maintaining female characteristics and preventing osteoporosis in postmenopausal women. It is excreted in urine and feces as estrone sulfate (Hamad & Elbadri, 2018). The reference level of E1 in the plasma of premenopausal women is 17-200 pg/mL, and it reduces considerably to 7-40 pg/mL in postmenopausal women (Denver et al., 2019).

17 β -Estradiol is the primary female sex hormone, the most potent and foremost prevalent estrogen during the female reproductive years. E2 is twelvefold and eightyfold more potent than E1 and E3, respectively. E2 is produced mainly within the follicular cells of the female ovaries but also by other endocrine and nonendocrine tissues such as testes in the male, adrenal cortex, liver, fat, breasts, and brain (Cui et al., 2013). In females, E2 plays critical physiological functions, particularly initiating and maintaining the menstrual cycle and reproductive stages, during which it also supports the oocysts, and together with the luteinizing hormone, it triggers ovulation (Kauffman, 2022).

Estradiol is synthesized from cholesterol via a series of chemical reactions that involve metabolites and are catalyzed by enzymes. The principal pathway of E2 synthesis consists of generating androstenedione, which is converted to E1 by the enzyme aromatase and then to E2 by the enzyme 17 β -HSD. Alternatively, in another similar pathway, androstenedione is converted to testosterone, a primary male sex hormone that is aromatized to estradiol (Thomas & Potter, 2013).

While the E2 level in men is low compared to that of women, it also has critical sexual functions in men, as it alters libido, spermatogenesis, and erectile function. E2 receptors and the enzyme aromatase, which generate estradiol from testosterone, are abundant in the testes, penis and brain, the organs that are important for sexual functions (Schulster et al., 2016). The reference levels of E2 in human plasma are

15-350 pg/ml, 188-7192 pg/ml, and < 10 pg/ml in premenopausal, pregnant, and postmenopausal women, respectively (Denver et al., 2019).

Estriol, also called trihydroxy estrin and 16α -hydroxy estradiol, was discovered in the urine of a pregnant woman in 1930. It is a weak estrogen; nevertheless, it is the predominant circulating estrogen during pregnancy but is not detectable in non-pregnant women. During pregnancy, E3 is synthesized in high quantities by the placenta (Chatuphonprasert et al., 2018). Unlike E1 and E2, E3 is not synthesized or secreted by the ovaries; instead, it is derived from 16α -hydroxylation of E1 and E2 by the enzyme cytochrome P450, CYP3A4, for example, primarily in the liver. Similarly, E1 and E2 are produced by the placenta; however, the E3 level increases by 1000 folds, whereas E1 and E2 increase by 100 folds only (Vazakidou et al., 2024).

The level of E3 in maternity circulation is approximately 8 to 13 ng/dl (Hamad & Elbadri, 2018). E3 is readily available for medication such as menopausal hormone therapy and other therapies, particularly for multiple sclerosis (MS), where a reduced risk of MS relapses in women was found during pregnancy when the hormone E3 increased (Varytė et al., 2020). The reference level of E3 in the plasma of premenopausal women is < 80 pg/ml and increases significantly to > 2100 in pregnant women; nevertheless, it is not detectable in postmenopausal women (Denver et al., 2019).

17α -Ethinylestradiol (EE2), is a synthetic steroidal hormone derived from E2 by the addition of ethinyl group on C17. This difference in structure at C17 leads to a substantial surge in the estrogenic action of the drug. When taken orally, it has an increased bioavailability and is more metabolism resistant. It is almost found in a combined form in all birth control pills and is nearly exclusively used for this purpose. It is also used in hormone replacement therapy (Aris et al., 2014). EE2 is released into the environment via urine and feces of animals and people consuming

it as a medication. It is one of the most potent estrogenic contaminants, reaching the water bodies mainly through the wastewater treatment plants (WWTPs).

2.2. Environmental pollution with estrogens: sources and impact

Natural and synthetic estrogens are among the most significant pollutants in different environments. Wastewater treatment plants (WWTPs), hospitals, the pharmaceutical industry, agricultural manure, and effluents from livestock feedlots are the main routes or sources of estrogen hormones pollution in the environment (Figures 2.3 and 2.4). All humans and animals excrete steroid hormones in different amounts depending on age, state of health, diet, or pregnancy. The estimated annual excretion of natural estrogens by humans and animals during anthropogenic activities was far higher in farm animals than in humans (Table 2.2.) (Yu et al., 2019).

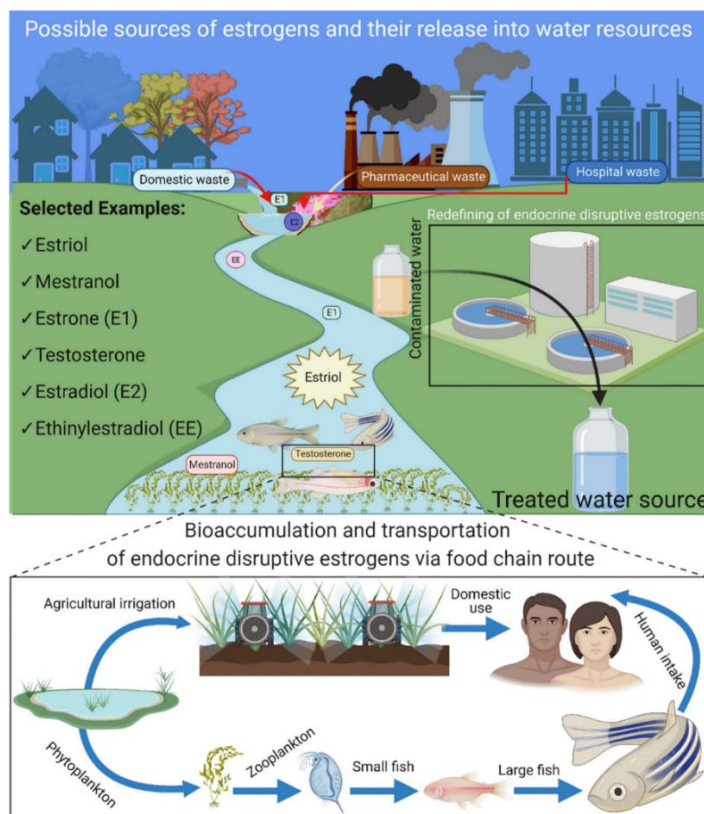


Fig. 2.3: Possible sources of estrogens and their release into water resources. The lower part shows a food-chain based bioaccumulation of estrogens (Reproduced from Bilal et al., 2021, with permission from ELSEVIER, License number 5711790544909).

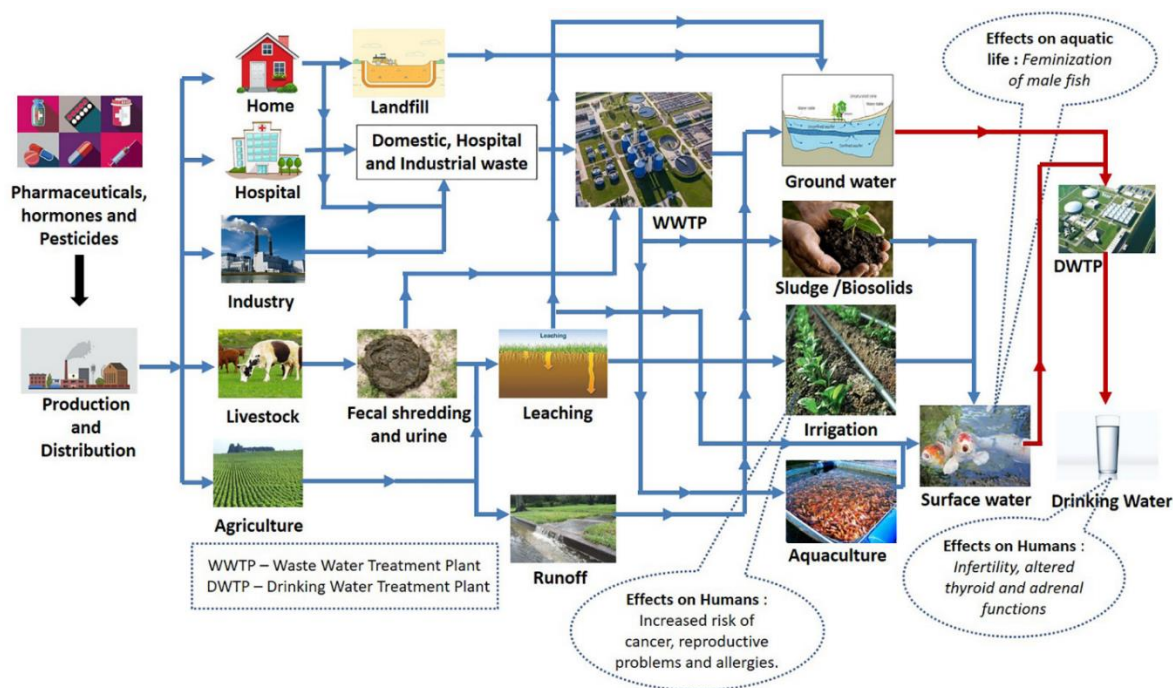


Fig. 2.4: Routes of steroidal estrogens contamination to ground and surface water bodies. (Reproduced from Syed et al., 2021, with permission from ELSEVIER, License number 5711791162777).

Table (2.2): Annual excretion of natural estrogens by humans and animals (Yu et al., 2019).

Countries/ regions	Population (million)	Annual estimated excretion of estrogens (t/year)			
		E1	17B-E2	E3	Total excretions
Global	Residents ^a , 7.7 x 10 ³	n.a	n.a	-	33.8 ^b
European Union	Animals, 1318	n.a	n.a	n.a	33
United Kingdom	Residents, 59	0.219	0.146	-	0.365
	Animals, 157.5	1.096	0.424	-	1.52
United States	Animals, 1981	n.a	n.a	n.a	49
	Residents, 277.048	1.802	0.78	8.206	10.788
China	Residents, 1370	5.11	1.387	27.375	33.872
	Animals, n.a	66.795	21.9	1160.7	1249.395
	Residents ^c , 1370	5.476	2.749	20.1	28.325

^aGlobal population in 2019 was predicted by United Nations Department of Economic and Social Affairs

^bThe total estrogen excretions is estimated by 4.4 kg/year per million residents of human urine excretion

^cEstimations are based on a high-resolution contaminant fate model

n.a., data was not available; –, no estimation

Natural estrogens are free or conjugated with sulfate and/or glucuronide groups. The free estrogens are more hydrophobic with lower solubility than the conjugated estrogens, even though they pose an estrogenic potential as considerable hazardous contaminants to the ecosystems (Yu et al., 2019). After being metabolized in the body via conjugation with sulfate and hydroxylation reactions, the inactive forms of estrogens are excreted into the environment in urine and feces, which end up in the WWTPs (Tables 2.3-2.5).

Table (2.3): Steroid estrogen concentrations (ng/L) in influent and effluent of WWTPs and their removal percentage (Liu et al., 2009b; 2009c; Pratush et al., 2020a).

Name of compound	Country	Influent concentration	Effluent concentration	Removal (%)
Estrone	Japan	259-326	n.d.–17	93–100
	Italy	25-132	2.5–82	22–95
	Australia	29-670	n.d.–72	111–100
	USA	57.8-83.3	6.3–49.1	41–89
	Canada	n.d.-33	n.d.–147	n.a.
Estradiol	Japan	n.d.-57	4.6–14 b	100–92
	Italy	4-25	0.35–3.5	59–98
	Australia	35-125	n.d.–30	44–100
	USA	11.2-161.6	1.5–5.4	52–99
	Canada	2.4-26	0.2–14.7	18.5–98.8
Estriol	Japan	n.d.	n.d.–151	100–0
	Italy	24-188	0.43–18	77–99
	Australia	23-660	n.d.–275	18–100
	USA	79.7-259.2	2.2–3.9	95–98
	Canada	n.d.-22	n.d.–29	n.a.

n.d.: not detected; n.a.: data not available

Table (2.4): Estrogen conjugates of E1, E2, and E3 found in WWTPs (Yu et al., 2019; Pratush et al., 2020a).

Compound name	Conjugate name	Conc. in WWTPs influent (ng/L)	Conc. in WWTPs effluent (ng/L)	Removal percentage (average)
Estrone	Estrone-3-sulfate	25	9	54.48%
		42	13	
		34.1	0.3	
		18	11.8	
		16.5	1.9	
		76	27	
		4.8	0.8	
		4.4	0.7	
	Estrone-3-glucuronide	4.3	0.7	35.7%
		11	7.4	
		0.4	n.d.–0.05	
		0.6	n.d.–0.6	
		3.6	n.d.	
Estradiol	Estradiol-3-glucuronide	5.2	n.d.	99.9%
		0.3	n.d.–0.07	
		13.3	0.5	
		2.0	0.4	
		2.5	0.7	
	Estradiol-17-glucuronide	18	91	40%
		n.d.–0.25	n.d.–0.25	
		10.5	n.d.–1.4	
		51	21	
		3	0.7	
		3.6	0.9	
	Estradiol-3-sulfate	3.3	n.d.	48%
		110	52	
		32	n.d.–0.07	
		3.6	0.4	
		2.7	1.8	
		13	5.3	
		5.5	0.7	
		4.9	0.7	
	Estradiol-3,17-disulfate	n.d.–0.28	n.d.–0.28	78%
		28	3.3	
Estriol	Estriol-3-glucuronide	22	72	n.d.
		n.d.–0.28	n.d.–0.8	
	Estriol-3-sulfate	14	2.2	84
	Estriol-17-glucuronide	30	n.d.	100

n.d.: not detected

Table 2.5: Example concentrations of estrogens detected in WWTP influent and effluent (Hamid & Eskicioglu, 2012; Huang et al., 2013).

Compound	Influent concentrations		Effluent concentrations	
	Lower (ng/L)	Higher (ng/L)	Lower (ng/L)	Higher (ng/L)
Estrone (E1)	2.4	670.0	<0.3	96.0
17 β -estradiol (E2)	2.4	150.0	0.2	30.0
Estriol (E3)	23.0	660.0	<0.5	75
17 α Ethinylestradiol (EE2)	<0.3	70.0	<0.3	26

Age and gender are two determinants of estrogens amounts in urinary excretion. The excretion rate of the natural estrogens E1, E2, and E3 in urine was reported to be 10.73, 4.71, and 8.12, 1194, 347, and 24,078, $\mu\text{g/day}$ for premenopausal, pregnant women, respectively; while postmenopausal women excrete 5, 2.78, and 2.78, respectively, whereas men excrete 3.9, 1.5, and 1.5 $\mu\text{g/day}$, of E1, E2, and E3, respectively (Liu et al., 2009). Consequently, the total concentration of steroid estrogens in sewage wastewater in a certain area depends on the gender ratio of the population. In WWTPs, the estrogens' conjugates revert to free estrogens (active forms) through enzymatic reactions performed by microbes existing in the WWTPs (Liu et al., 2009; Palme et al., 1996). The presence of estrogens in aquatic ecosystems is hazardous to aquatic life due to their endocrine-disrupting activity, and the hazards may include synthesis and secretion of vitellogenin (a female specific protein) in male fish, development of intersex characteristics and/or failure in the development of normal secondary characteristics (Racz & Goel, 2010; Ying et al., 2002) which will lead to changes in reproduction. In addition to their endocrine-disrupting activity, estrogens were listed by the WHO as “group 1” carcinogens

(http://monographs.iarc.fr/ENG/Classification/latest_classif.php).

Moreover, some catechol metabolites of estrogens such as 4-hydroxyestrone, were reported to exhibit carcinogenic activity (Fernandez et al., 2006; Liehr et al., 1986). Due to their biological activity at very low concentrations, EDCs can threaten the polluted environment with acute and chronic toxicity to organisms, accumulation in the ecosystem, loss of habitat and biodiversity, and a range of potential adverse effects on environmental and ecological health (Torres et al., 2021; Zheng et al., 2023).

Natural estrogens E1, E2, E3, and the synthetic estrogen EE2 are the major components of environmental estrogens. They have been identified in surface water at a concentration ranging from sub-nanogram to tens of nanograms per liter (Ojoghoru et al., 2021), and they are among the chemicals that contaminate the environment and disrupt the body's endocrine system and thus are known as endocrine-disrupting chemicals (EDCs).

Steroids and several other EDCs in water, such as bisphenol A, phthalates, disinfection byproducts, pesticides, and estrogens, have various adverse effects in animals and humans (Gonsioroski et al., 2020; Predieri et al., 2022). Once they enter the body, EDCs mimic the naturally occurring hormones, which can bind to their receptors and antagonistically affect hormonal activities or hinder their production (Rashtian et al., 2019). Though their environmental levels are low, as measured by ng/L, they exhibit substantial adverse effects on various organisms' biological organs and processes, including homeostasis, reproductive physiological functions, and metabolism. For example, a study published in 2018 by researchers from Jiangsu Province, China, clearly showed that E2, E3, and EE2 were in high quantities in livestock waste entering the marine environments.

Worldwide presence of the natural and synthetic estrogens in freshwater, groundwater, and wastewater was confirmed by several studies. Estrogens are released into the environment from animal excretions (Zheng et al., 2008), with pigs and poultry industry waste releasing about 58% E2 in feces, while 96% EE2 and

69% E3 are excreted through urine (Adeel et al., 2017). Considering E2 as a reference, it was estimated that the estrogens E1, E2, E3, and EE2 exhibit molar effective concentrations (EC50) of 1.2e^{-12} , 1.8e^{-12} , 7.3e^{-12} , and 1.0e^{-12} ; and the relative potency factor (RPF) is 1.4, 1.0, 0.23, and 1.7, respectively (Bermudez et al., 2012; Conley et al., 2017).

E2, above all, is the most potent natural estrogen and was found at a level exceeding the hazard baseline value (1 ng/L) and higher than the proposed lowest possible observable effect concentration of E2 equivalent quantity (10 ng/L) for aquatic environments. Exposure of aquatic organisms to estrogens present in downstream waters and effluents caused feminized male fish to acquire a high concentration of vitellogenin (an egg yolk protein precursor), loss of secondary sex physical characteristics, decreased testes size, and intersex (Huang et al., 2016).

In human, studies have reported a close association between exposure to estrogens and disorders of the male and female reproductive systems. These chemicals or metabolites can disrupt the hormone-dependent pathways via direct interaction with the receptors or epigenetic changes. Generally, some documented disorders are overall decline in fertility, congenital malformation of gonads development, and an increased incidence of reproductive diseases and cancers (Rashtian et al., 2019).

The increasing worldwide concern about environmental exposure to estrogens has prompted programs to understand and improve their removal from WWTPs, which are the main source of environmental pollution with estrogens (Leech et al., 2009; Yu & Chu, 2009). Some research studies also addressed estrogens removal from drinking water treatment plants, constructed wetlands, sewer, manure, poultry litter and dairy disposal systems (Yu et al., 2013).

2.3. Pollution of the environment in Gulf Cooperation Council (GCC) countries with estrogens

To date, the occurrence of EDCs in the GCC environment, especially aquatic environment, and their impact has not received the proper attention. The Arabian Gulf in general has long been known to be heavily contaminated with diverse pollutants (Al-Sarawi et al., 2015) including petroleum hydrocarbons, crude oil, heavy metals, persistent organic pollutants, and fecal matter. For instance, in Kuwait the major causes of marine pollution include petroleum production and refining, discharges from Shatt Al Arab, untreated domestic and industrial effluents, as well as atmospheric deposition (Smith et al., 2015). The coastal areas in Kuwait have received untreated sewage discharge until 2004 due to insufficient treatment capacity, since then, breakdown, accidents, and down pours have also caused many large discharges of sewage to the coastal areas (Lyons et al., 2015; Saeed et al., 2015).

High throughput GC-MS screening was applied by Smith et al. (2015) to detect over 1000 chemicals, including estrogens and androgens, in contaminated water samples. They also used oyster embryo development screens to assess the biological effect of the contaminants. The authors collected samples from four known hot spots of treated wastewater effluent input, namely, Al Ghazali, Salmiya, Al Bedaa, and Al Mesela. Moreover, they collected offshore samples in Kuwait Bay and along the Gulf coastline adjacent to the city. The authors reported abnormal development of oyster embryo in samples taken from sites close to the outfall. Although the yeast androgen assay, which was used *in vitro* to detect androgenic activities for all samples, was negative, a number of samples, which were also close to the effluent outfall sites, exhibited estrogenic activity in the yeast estrogen assay. This indicates the presence of estrogen-type EDCs in those samples. Chemical screening of the different samples revealed the presence of high concentrations of cholesterol at Al Ghazali sampling site. Moreover, samples from Al Ghazali site contained the xenoestrogens phthalates and bisphenol A in the $\mu\text{g/L}$

range. Similarly, the outfalls at Al Bedaa, Salmiya, and Al Messela had detectable levels of phthalates, and some open water samples had varying levels of both phthalates and bisphenol A.

The authors did not exclude the presence of EE2, the synthetic steroid estrogen, in the samples, albeit below detection limits. The same applies for E1, E2, and E3. However, this study did not provide information on the source or levels of EDCs in Kuwait's coastal areas. In a subsequent study, Saeed et al. (2017) assessed the sources and levels of EDCs in inflows and outflows of wastewater treatment plants as well as water and sediments from Kuwait's coastal areas impacted by sewage discharge. The authors collected samples from Sulaibiya, Um Al-Hyman, and Kabd wastewater treatment plants. Seawater and marine sediments were collected from locations impacted by sewage (Fintas, Sulaibekhat, Al Ghazali) or close to the sewage outlet Fahaheel and Shuwaikh commercial port.

Chemical analysis revealed the presence of phthalates in the inflows of wastewater treatment plants, seawater, and sediment from impacted areas in the range 8.9-78.3 ng/L, 2-16 ng/L, and up to 4590 µg/kg, respectively. The inflows of the wastewater treatment plants contained alkylphenols in the range 0.7-279 ng/L and estrogens at 30-368 ng/L, while seawater contained alkylphenols up to 15 ng/L and estrogens up to 214 ng/L. The sediment samples contained alkylphenols (up to 16.4 µg/kg) and estrogens (up to 36.2 µg/kg). EE2 was the dominant estrogen in all samples. Although the treatment processes in the wastewater plants removed in average 80% of the EDCs, the concentrations remaining in the effluents are still significant and environmentally of concern, since they can exhibit biological activity (Fenlon et al., 2010; Kuster et al., 2009). The predicted-no-effect-concentration (PNEC) values for E2 was proposed to be 1 ng/L and for E1 3 ng/L (Caldwell et al., 2012). The European Commission (2011) proposed a value of 0.4 ng/L as the PNEC for E2. Obviously, the per liter concentrations of estrogens found in seawater or outflows from WWTPs are higher than the proposed PNEC for these

estrogens. Also, worldwide the per liter estrogen concentrations in the effluent of WWTPs are typically in the range of a few ng to several μg (Chen et al., 2010).

These data show the need for further investigations to assess the impact and fate of estrogens and other EDCs in the marine environment of the GCC countries. Moreover, it is important to better understand the microbial biodegradation and biotransformation mechanisms for estrogen to assess the feasibility of intervention in the marine environment with bioremediation approaches in the impacted locations. This should cover the involved microbial communities, catabolic pathways, genes, and enzymes. Comprehensive understanding of estrogens biodegradation and biotransformation will also enable the design of more efficient processes for wastewater treatment towards complete removal of these contaminants. This is particularly important due to the fact that biodegradation by bacteria is the main mechanism by which estrogens are removed from sewage.

2. 4. Removal and fate of estrogens from wastewater treatment plants

Steroidal estrogens produced by humans and animals are micropollutants continuously entering wastewater treatment plants (WWTPs). Though these are naturally occurring hormones, they threaten ecosystems and human health when released into the environment. Removal of these steroid estrogens E1, E2, E3, and EE2 is challenging due to their recalcitrant sterane complex structure and their low aqueous solubility, which were found to be very low as 30, 3.6, 441, and 116 mg/L, respectively (Zhang et al., 2016).

The natural estrogens E1 and E2 and the synthetic hormone EE2 have recently been included in a watchlist of European Decision EU 2015/495 under the European Water Framework Directive (Könemann et al., 2018). Hence, these estrogens must be monitored per their environmental quality standards. Since WWTPs are the primary source of estrogens in surface waters, many studies have focused on

measuring estrogens quantitatively and qualitatively to reflect on the efficiency of the wastewater treatment process.

WWTPs are essential for eliminating these estrogens; however, WWTPs are not designed to eliminate them completely; thus, they are continuously discharged into surface water. Various studies have evaluated estrogens removal from WWTPs using microfiltration, activated carbon adsorption, chemical degradation and advanced treatment such as reverse osmosis. However, despite the relative efficiency of the physical and chemical techniques, there is an emerging approach to using biological-based methods, predominantly the use of biofilms (Gadupudi et al., 2021) (Table 2.6, Figure 2.5).

Table (2.6): Efficiency and disadvantages of estrogen removal methods (Flint, 2019).

Method	Estrogen removal	Disadvantages
Sorption	90% E1, E2, E3 and EE2 by granular activated carbon (GAC) and powdered activated carbon (PAC). 80% EE2 by molecularly imprinted polymer (MIP). 97% E2 by MIP. 96.14% to 98.03% E1 by carbon nanotubes (CNTs) with MIP. 95-98% EE2 by single walled carbon nanotubes (SWCNTs). 50-75% E1, E2 and EE2 by suspended solids (SS).	- Efficiency affected by the presence of natural organic matter. - pH, temperature and method dependent. - Numerous studies with removal of less than 50%.
Membrane filtration	99-100% E1, E2, E3 and EE2 by nanofiltration (NF). 98 – 100% E1, E2, E3 and EE2 by reverse osmosis or NF. 99% E1 and E2 by forward osmosis. ≥99.5% E1 and E2 by direct contact membrane distillation (DCMD).	- Dependent upon equilibrium concentration. - Dependent on physiochemical processes, membrane properties, membrane fouling, pH, temperature, and salinity.
AOP	99-100% E2 and EE2 by Chlorination and ozonation.	- pH dependent. - Production of harmful by- products with estrogenic activity

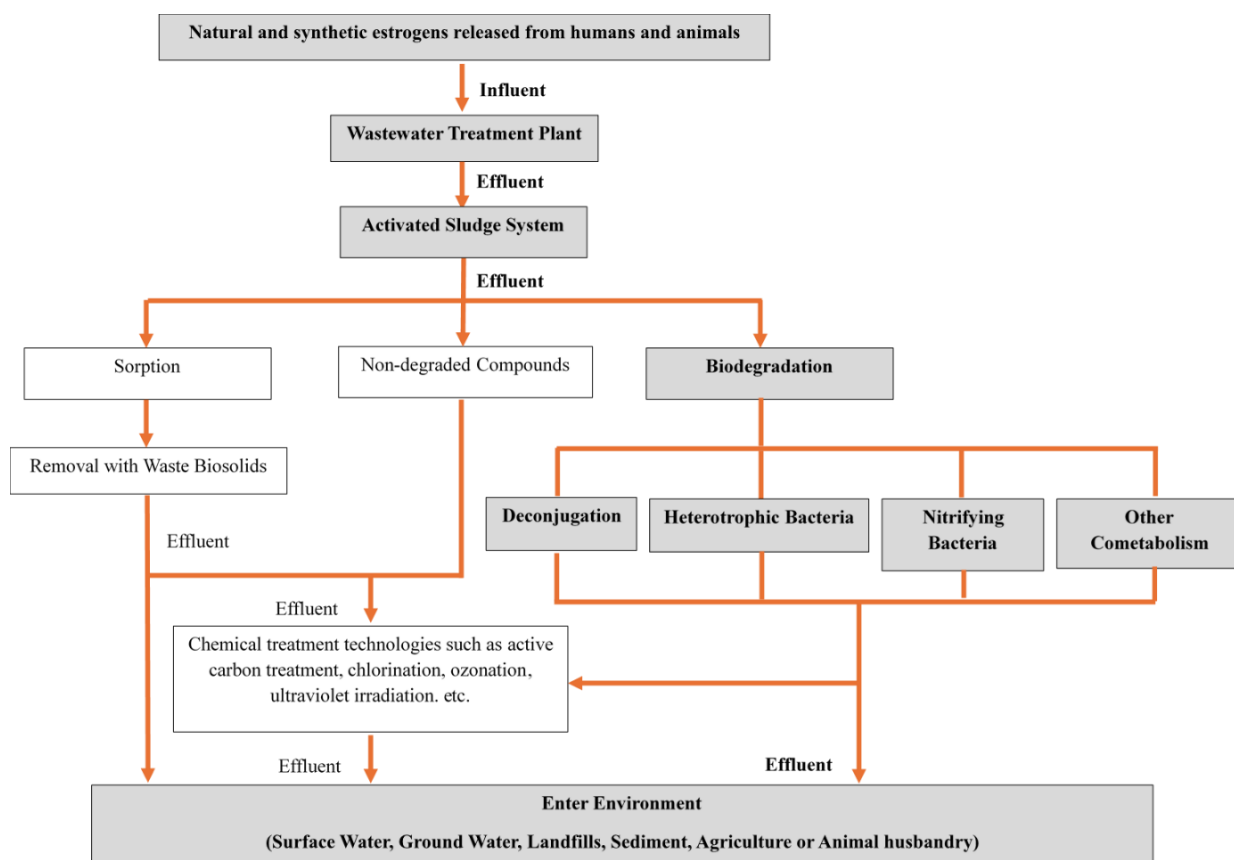


Fig. 2.5: Fate of steroid estrogens in wastewater treatment plants (modified from (Pratish, et al., 2020a; Racz & Goel, 2010).

Estrogen elimination during wastewater treatment has been reported by several studies (Liu et al., 2015; Luo et al., 2014; Racz & Goel, 2010; Xu et al., 2012) (Figure 2.5). Commonly, estrogens are removed in WWTPs during the activated sludge stage either via adsorption onto solids or microbial biodegradation (Racz & Goel, 2010). Biological removal of estrogens in WWTPs is a key route which includes deconjugation, complete degradation by heterotrophic microorganisms, or co-metabolism (Fernández et al., 2017; Ke et al., 2007; Liu et al., 2016). However, the removal efficiency varies from one plant to another and depends on several factors such as operating conditions, geological location, estrogen concentration, and the biological system type (Yu et al., 2013). Estrogen elimination is more efficient in WWTPs equipped with biological treatment systems and having long solid and hydraulic retention times. The latter enables longer

contact time for better estrogen adsorption and sufficient time for growth of slow-growing estrogen degraders (Maeng et al., 2013).

In all cases, it is generally acknowledged that estrogens are challenging substrates for microbial utilization due to their physicochemical properties such as low aqueous solubility, low number of functional groups, the four alicyclic rings, and the presence of two quaternary carbon atoms (Olivera & Luengo, 2019), which may contribute to the various removal efficiencies reported in the literature, ranging between 14–94%, 76–92%, and 83–87% for E1, E2, and EE2, respectively (Baronti et al., 2000). It is also worth noting that steroid estrogens exhibit different susceptibilities to microbial degradation, following the descending order $E3 > E2 > E1 > EE2$ (Coello-Garcia et al., 2019).

In activated sludge, up to 98% E1 can be removed (Coello-Garcia et al., 2019; Racz & Goel, 2010), 99.9% E2 capable of being removed (Coello-Garcia et al., 2019; Heffron et al., 2016; Racz & Goel, 2010), almost 100% E3 can be removed (Coello-Garcia et al., 2019; Racz & Goel, 2010), while EE2 removal varies between 34% and almost 100% (Coello-Garcia et al., 2019; Racz & Goel, 2010). Several studies showed that despite consistency in wastewater flows, the effluent estrogen concentrations fluctuate on daily basis, thus lacking uniform temporal trend (Heffron et al., 2016; Racz & Goel, 2010; Williams et al., 2003). Furthermore, treated wastewater usually contains environmentally significant concentrations of estrogens even after activated sludge treatment. Abusam et al., (2022) reported variations in estrogen removal efficiencies in three major WWTPs in Kuwait, namely, Kabd, Sulaibiya, and Umm Al-Haymann. The concentration of estrogens in the influent streams ranged from 0.0 to 474 ng/L, while that in the effluent streams were between 0.0 to 233 ng/L. Total removal of estrogens were found to be 13%, 79%, 68%, for Kabd, Sulaibiya and Umm Al-Hayman, respectively.

Compared to other natural estrogens, the E1 concentration in surface waters is much higher, and E1 usually accumulates in the aquatic environments (Sami &

Fatma, 2019). This was attributed, at least partially, to less efficient removal of E1 compared to E2, which is perceived as a consequence of the observation that microorganisms that can transform E2 to E1, cannot further metabolize E1 (Khanal et al., 2006). Hence, additional chemical treatments such as chlorination, ozonation, UV irradiation, etc., are usually applied to improve estrogen removal from wastewater ((Bila et al., 2007; Hartmann et al., 2014; Li et al., 2016; Racz & Goel, 2010).

“Estrogens can be degraded by microbial communities present in soil and water under aerobic and anaerobic conditions. The half-life of estrogens varies with environmental conditions and concentration in a particular environment. The half-life of estrogens also depends on their degradation rate and oxygen availability (Adeel et al., 2017). Under aerobic conditions in soil, the half-lives of E1, E2, and E3 were found to be 2.8–4.9, 0.8–0.11, and 0.7– 1.7 days, respectively (Biswas et al., 2013). Whereas in river water under aerobic conditions, the half-life of E1 and E2 were found to be 2–3 and 2–4 days, respectively (Adeel et al., 2017; Jürgens et al., 2002; Ying et al., 2002). Liu et al. 2009 determined the average concentration of steroid estrogens and natural androgens in municipal sewage wastewater treatment plants. The total excretion rate of estrogens E1, E2, and E3 was only 66–82% of total waste excretion” (adapted from Pratush et al., 2020a).

Above all, microbial biodegradation is a promising technology for removing environmental estrogens due to its extreme efficiency, no remaining secondary pollution, and low cost. Thus, more focus is placed on microbial degradation in the subsequent sections.

2. 5. Regulatory aspects

As a consequence of the hazardous environmental and human health impact of steroid estrogens, some countries and organizations have implemented programs or added these and other endocrine-disturbing chemicals to their chemical safety strategies. However, no specific legislation has been implemented to regulate estrogen levels in aquatic environments or drinking water. Nevertheless, due to rising concerns, EE2 and E2 received more attention, leading the European Commission to propose EE2 and E2 as priority substances with proposed EQS of 0.035 ng/L and 0.4 ng/L respectively (Gilbert, 2012; Hecker & Hollert, 2011; Johnson et al., 2013).

In addition, the UK government's Department for Environment, Food, and Rural Affairs proposed an EQS of 0.016 ng/L for EE2 (Gilbert, 2012). Accordingly, EE2 and E2 were included in the Surveillance List of the 2013/39/EU 65 directive passed in August 2013 by the Water Framework Directive (da Cunha et al., 2016). However, opposition by water utility companies such as Thames Water (UK) hindered the implementation of these regulations. This company justified its position by claiming that the studies and proposals presented lack evidence of health risks, have large economic impact, and could lead to higher carbon emissions (Water quality, 2013). In the USA, EE2 and other endocrine disruptors in drinking water are mentioned. In 2009, EE2 and endocrine disruptors were listed on a Contaminant Candidate List for monitoring in the Unregulated Contaminants Monitoring Program of the US- EPA (da Cunha et al., 2016). However, estrogens are not currently monitored in the aquatic environment in the USA.

2.6. Biodegradation of estrogens

2.6.1. Estrogen-degrading microorganisms

Estrogens, like other steroids, are carbon-rich and highly reduced compounds that are abundant and ubiquitous in the environment, and, therefore, they represent a precious carbon and energy source for some microorganisms (Chiang et al., 2020). In fact, some microorganisms (including bacteria, yeasts, fungi and microalgae) have the ability to perform biotransformation reactions on estrogens. However, only dedicated bacteria have the ability to mineralize estrogens (complete degradation of steroids to CO₂) (Bergstrand et al., 2016; Holert et al., 2018). Recent research has focused on identification of steroid-degrading bacteria and elucidation of the biodegradation mechanisms using both culture-dependent and culture-independent approaches to identify steroid hormone-degrading bacteria from different engineered and natural ecosystems.

Bacteria that can degrade estrogens under aerobic conditions have been isolated from various engineered and natural environments such as activated sludge, soil, compost, sandy aquifers, and very little from the marine environment. Although the isolates belong to different phyla, the majority of estrogen-degrading bacteria reported to date belong mainly to the Actinobacteria and Proteobacteria (Tables 2.7 and 2.8) (Bergstrand et al., 2016). The significance of investigating steroids-microbes interactions has increased for four main reasons (Chiang et al., 2020).

First, microbial degradation is crucial for removing steroid sex hormones from polluted ecosystems. Second, microbial transformation of steroids has been exploited in the pharmaceutical industry to produce high-value steroid drugs through biotechnology processes. Third, steroid degradation is important for the virulence of

some bacterial pathogens. Fourth, recent studies suggest that steroid hormones mediate bidirectional interactions between bacteria and their eukaryotic hosts (vom Steeg & Klein, 2017).

Table (2.7): List of known estrogen-degrading bacteria isolated from activated sludge (Modified from Flint, 2019).

Bacterial species	Estrogen substrate spectrum	Estrogen removal (%)
<i>Novosphingobium tardaugens</i> sp. ARI-1	Metabolizes E1, E2 and E3.	40% E1 in 20 d; 99% E3 in 10 d; 60% E2 in 14 d.
<i>Aminobacter aminovorans</i> sp. KC7	Degrades* E1 and E2.	Transformed E2 to E1 in 7 d; 23-31% E1 removed in 7 d.
<i>Aminobacter</i> sp. KC6	Degrades* E1 and E2.	Transformed E2 to E1 in 7 d; 16-26% E1 degraded in 7 d.
<i>Sphingomonas</i> strain KC8	Metabolizes E1 and E2.	Complete mineralisation of E1 and E2 in 7 d.
<i>Brevundimonas diminuta</i> strain NK 2 (EU35276)	Transforms E2 to E1	The residual concentration of E2 was $38 \pm 1\%$ after 168 h of incubation.
<i>Brevundimonas vesicularis</i> KC12	Transforms E2 to E1	Complete transformation within 7 days.
<i>Rhodococcus ruber</i> M2 KC4	Transforms E2 to E1	Complete transformation within 1 day.
<i>Microbacterium testaceum</i> KC5.	Transforms E2 to E1	Complete transformation within 1 day.
<i>Sphingomonas</i> sp. KC9-11	Transforms E2 to E1	Complete transformation within 1 day.
<i>Flavobacterium</i> sp. KC1	Transforms E2 to E1	Complete transformation within 7 days.
Bacteroidetes sp. KC2	Transforms E2 to E1	Complete transformation within 7 days.
<i>Nocardioides simplex</i> sp. KC3	Transforms E2 to E1	Complete transformation within 7 days.
<i>Escherichia coli</i> sp. KC13	Transforms E2 to E1	Complete transformation within 7 days.
<i>Sphingomonas</i> sp. KC14	Transforms E2 to E1	Complete transformation within 7 days.

Table 2.7: Continued

<i>Achromobacter xylosoxidans</i>	Degrades* E1, E2 and transforms E3.	Complete transformation within 2 d.
<i>Ralstonia</i> sp.	Degrades* E1, E2 and transforms E3.	Complete transformation within 2 d.
<i>Pseudomonas aeruginosa</i> sp. TJ1	Metabolizes E2.	Complete mineralisation of E2 in 2 h.
<i>Bacillus subtilis</i> sp. E2Y4	Degrades* E1 and E2.	Transformed E2 completely in 4 days; 40% E1 within 9 d.
<i>Bacillus</i> sp. E2Y1	Degrades* E1 and E2.	Transformed E2 completely in 6 days; 20% E1 within 9 d.
<i>Bacillus amyloqueliciens</i> sp. E2Y2	Transforms E2 to E1.	Transformed E2 to E1 within 4 days.
<i>Bacillus</i> sp. E2Y3	Transforms E2 to E1.	Transformed E2 to E1 within 5 days.
<i>Bacillus cereus</i> sp. E2Y5	Transforms E2 to E1.	Transformed E2 to E1 within 6 days.
<i>Denitratisoma oestradiolicum</i> gen. nov., sp. nov	Metabolizes E2 under anoxic conditions.	Complete mineralisation of E2.
<i>Steroidobacter denitrificans</i> gen. nov., sp. nov	Metabolizes E2 under anoxic conditions.	Complete mineralisation of E2.
<i>Nitrosomonas europaea</i>	Co-metabolizes E1, E2, E3 and EE2 under nitrifying conditions.	>95-100% E1, E2 and EE2 in 8 days; >62.5% E3 in 9 d.
<i>Sphingobacterium</i> sp. JCR5	Metabolizes E1, E2, E3 and EE2.	87% EE2 within 10 d.
<i>Novosphingobium</i> sp. JEM-1	Degrades* E1 and E2.	99-100% E1 and E2 in <1 h.
<i>Rhodococcus zopfii</i> strain Y 50158	Metabolizes E1, E2, E3 and EE2.	81% E2 in 2 h; 91% E1 in 3 h; 96% E3 in 4 h; 70% EE2 in 7 h; Complete mineralisation in 24 h.
<i>Rhodococcus equi</i> strains Y 50155, Y 50156, and Y 50157	Metabolizes E1, E2, E3 and EE2.	>80% E2 in 5 h; 99% E2 in 24 h; >80% E1 in 8 h; 99% in 24 h; 72-95% E3 in 24 h; >70% EE2 in 8 h; >80-95% in 24 h.

Table 2.7: Continued

<i>Pseudomonas citronellolis</i> SS-2	Degradation of E1, E2 and EE2, but not E3	93.6% EE2 removal in 168 hours. 99% E2 in 36 hours.
<i>Pseudomonas citronellolis</i> SJTE-3	Metabolizes E1, E2, E3 and EE2.	Unpublished data
<i>Novosphingobium</i> sp. E2S	Degradation of E2	63.29% of E2 was removed within 7 days.
<i>Rhodococcus</i> sp. DS201	Metabolizes E2 and E1	63.4-100% E2 removed in 3 days, highest removal 100% of 1 mg/L E2 in 3 days. Lowest 63.4% removal of 10mg/L E2 in 3 days.

* Where strains were unable to grow upon estrogen as the sole energy or carbon source, but co-metabolism was not confirmed. All strains are aerobic unless otherwise stated.

Table (2.8): List of known estrogen-degrading bacteria from other sources (Modified from Flint, 2019).

Bacterial species	Estrogen substrate spectrum	Source of bacteria
<i>Phyllobacterium myrsinacearum</i>	Metabolizes E1, E2, E3 and co-metabolizes EE2 in the presence of E1, E2 and E3.	Compost
<i>Ralstonia picketti</i> BP2	Metabolizes E1, E2, E3 and co-metabolizes EE2 in the presence of E1, E2 and E3.	Compost
<i>Pseudomonas aeruginosa</i> BP3	Metabolizes E1, E2, E3 and co-metabolizes EE2 in the presence of E1, E2 and E3.	Compost
<i>Pseudomonas</i> sp. BP7	Metabolizes E1, E2, E3 and co-metabolizes EE2 in the presence of E1, E2 and E3.	Compost
<i>Acinetobacter</i> sp.	Metabolizes E1, E2, E3 and co-metabolizes EE2 in the presence of E1, E2 and E3.	Compost
<i>Sphingomonas</i> sp. CYH	Degrades E1 (aerobic and anoxic) and transforms E2 to E1.	Artificial sandy aquifer
<i>Agromyces</i> sp. LHJ3	Degrades E3 (aerobic), E2 (anoxic) and transforms E2 to	Artificial sandy aquifer

Table 2.8: Continued

	E1.	
<i>Acinetobacter</i> sp. LHJ1	Transforms E2 to E1.	Artificial sandy aquifer
<i>Sphingomonas</i> ED8 and 9.	Metabolizes E1 and E2.	Soil
<i>Rhodococcus</i> ED6, 7 and 10.	Metabolizes E1 and E2.	Soil
<i>Pseudomonas putida</i> strain SJTE-1	Metabolizes E1 and E2	Soil
<i>Leptothrix discophora</i>	Manganese Mn^{2+} - dependent degradation of EE2	Belgian coordinated collections of microorganisms.
<i>Pseudomonas putida</i> MnB1, 6 and 29.	Manganese Mn^{2+} - dependent degradation of EE2	Belgian coordinated collections of microorganisms.
<i>Nitrosomonas europaea</i> ATCC 19718	Co-metabolism and Nitration of EE2	ATCC
<i>Buttiauxella</i>	Metabolism of E2 and Testosterone	Baltic Sea
<i>Vibrio</i> sp. H5	Metabolism of E2 and Testosterone	Baltic Sea
<i>Rhodococcus equi</i> ATCC 13557	Partial degradation of EE2 in the presence of a cosubstrate. EE2 61% $\pm$ 1% in 300h.	ATCC
<i>Rhodococcus erythropolis</i> ATCC 4277	Partial degradation of EE2 in the presence of a cosubstrate. EE2 46% $\pm$ 2% in 300 h	ATCC
<i>Rhodococcus zopfii</i> ATCC 51349	Partial degradation of EE2 in the presence of a cosubstrate. EE2 38% $\pm$ 1% in 300h	ATCC
<i>Bacillus subtilis</i> ATCC 6051	Partial degradation of EE2 in the presence of a cosubstrate. EE2 27% $\pm$ 2% in 300h.	ATCC
<i>Pseudomonas aeruginosa</i> PA01	Partial degradation of EE2 in the presence of a cosubstrate. EE2 34% $\pm$ 2% in 300h.	ATCC
<i>Pseudomonas putida</i> ATCC12633	Partial degradation of EE2 in the presence of a cosubstrate. EE2 21% $\pm$ 2% in 300h	ATCC
<i>Rhodococcus rhodochrous</i> ATCC13808	Partial degradation of EE2 in the presence of a cosubstrate. No EE2 detected after 48 h (possibly co-metabolic)	ATCC

Table 2.8: Continued

<i>Bacillus cereus</i> Socransky 67	Conversion of E2 to unknown metabolites	ATCC
<i>Streptococcus mutans</i> Ingbritt	Conversion of E2 to E1	Dental plaque
<i>Streptococcus mutans</i> NCTC 10449	Conversion of E2 to E1	Dental plaque
<i>Streptococcus mutans</i> NCTC 10904	Conversion of E2 to E1	Dental plaque
<i>Rhodococcus sp. P14</i>	Conversion of E2 to E1	Dental plaque
<i>Nocardia sp. E110</i>	Degradation of E1	Crude oil

ATCC: American Type Culture Collection

2.6.2. Initial degradation and transformation reactions

Biodegradation is defined as the process by which microorganisms transform or alter (through enzyme-catalyzed biochemical reactions) the structure of chemical compounds introduced into the environment (U.S. Environmental Protection Agency, 2010). Microbial biodegradation was reported as the major mechanism which removes estrogens from engineered and natural polluted environments (Huang et al., 2014; Yu et al., 2013). Despite large number of studies on estrogens biodegradation, there are still many knowledge gaps regarding the catabolic pathways. Moreover, the involved enzymes, genes, and their regulation mechanisms are not fully characterized yet. Degradation of estrogens by aerobic bacteria has been reported, and many estrogens-degrading bacteria have been isolated from various environments (Chiang et al., 2020; Olivera & Luengo, 2019; Pratush, et al., 2020a; Yu et al., 2013; Zhang et al., 2016).

Microbial degradation of estrogens can proceed via two different metabolic modes depending on the involved microbe and the prevailing conditions. Some microbes link estrogens utilization to growth via a growth-linked (metabolic) mode, in which case the steroid substrate serves as a carbon and energy source for the

degrading organism. Four wastewater bacterial isolates, *Novosphingobium tardaugens* ARI-1 (Fujii et al., 2002), *Sphingomonas* strain KC8 (hereafter referred as KC8) (Yu et al., 2007), *Sphingobacterium* sp. JCR5 (hereafter referred as JCR5) (Haiyan et al., 2007), and *Pseudomonas aeruginosa* TJ1 (Zeng et al., 2009), were reported to degrade E2 via growth-linked reactions. Among these four strains, KC8 can also grow on E1 and testosterone as sole carbon sources.

The second mode proceeds via co-metabolism where the steroid substrate does not provide carbon or energy for the involved organism. This has to be provided by another primary growth substrate, which is structurally similar to the steroid co-metabolized substrate. Cometabolic degradation of E2 and EE2 by heterotrophic bacteria, ammonia-oxidizing bacteria and nitrifying activated sludge was reported (Pauwels et al., 2008; Skotnicka-Pitak et al., 2009; Yi & Harper, 2007; Yu et al., 2007). Another degradation mode proceeds via abiotic degradation that is mediated by some types of bacteria. For instance, In the presence of high ammonia concentration, *Nitrosomonas europaea* (ammonia-oxidizing) transformed EE2 into nitroestrogens. This nitration reaction is not directly biocatalyzed, it is an indirect abiotic reaction between estrogens and the biogenic nitrite (Gaulke et al., 2008). Moreover, the manganese-oxidizing *Leptothrix discophora* can oxidize Mn^{+2} to form Mn oxides, which facilitate the oxidative cleavage of EE2 (Sabirova et al., 2008).

Various estrogen biotransformation reactions were reported including hydroxylation, dehydrogenation, and ring cleavage steps (Figure 2.6). Most of the studies have focused on the aerobic biodegradation E1 and E2 as model substrates. On the contrary, E3 and EE2 have not been sufficiently investigated. Moreover, the biodegradation of estrogens under anaerobic conditions was reported only very recently (Wang et al., 2020a).

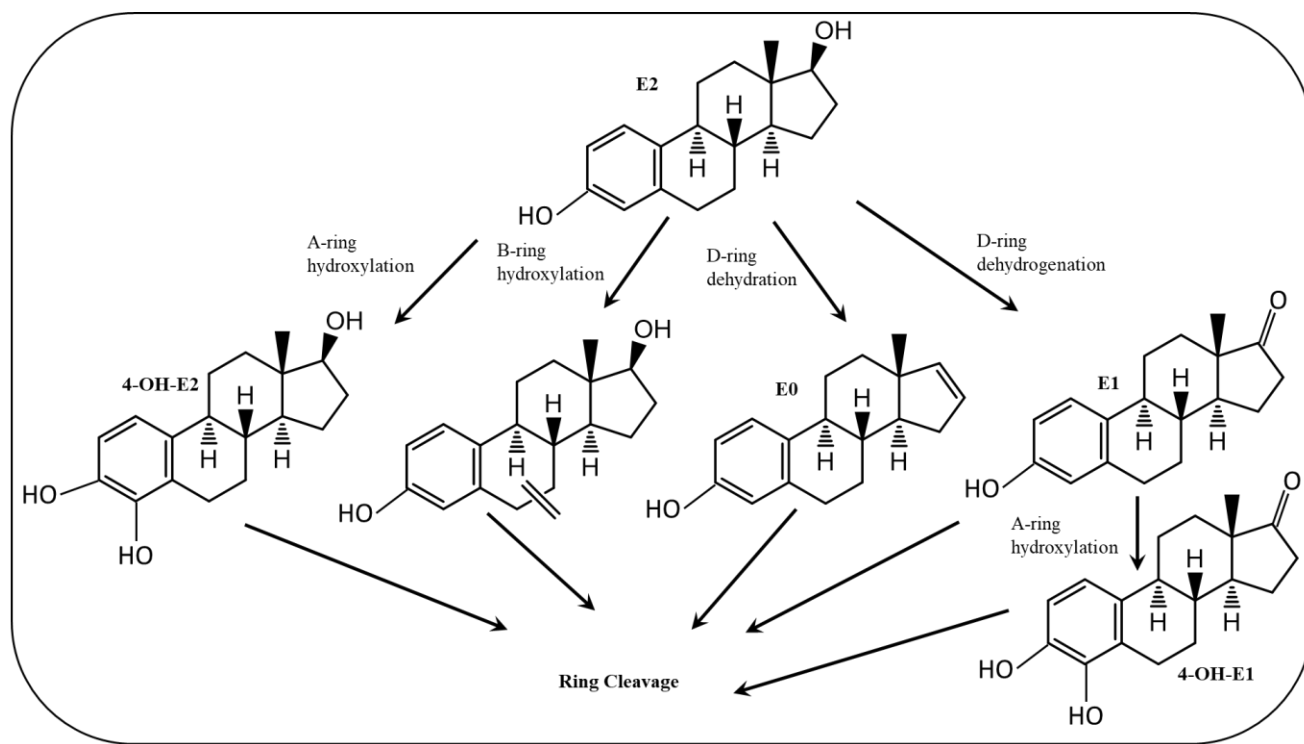


Fig. 2.6: Initial reactions of major E2 degradation pathways (Mensitieri et al., 2023).

As mentioned earlier, after being metabolized in the body, estrogens metabolites and /or conjugates are excreted mainly in urine and feces, which find their way to the WWTPs. During wastewater treatment, conjugated estrogens are cleaved by microorganisms and converted to free estrogens. Recent studies reported that both heterotrophic and autotrophic wastewater microbes can degrade estrogens and some of them can transform estrogens into non-estrogenic products. However, estrogens are not completely removed during wastewater treatment. Hence, effluents from WWTPs are a key source of environmental pollution with estrogens (Hung et al., 2022; Yu et al., 2013).

Despite the high structural similarity among estrogens, a diversity of biotransformation reactions and degradation pathways have been reported in the literature. Some of those reactions/pathways were proposed based on the detection and identification of metabolic intermediates. Another important feature of estrogen

biodegradation is the fact that estrogens are interconvertible, i.e. one estrogen can be transformed to another during biodegradation or biotransformation. To date, three major catabolic pathways for steroid biodegradation have been reported. Under aerobic conditions sterols and androgens are degraded via the 9,10- *seco* pathway, while estrogens are commonly degraded via the 4, 5- *seco* pathway.

On the contrary, under anaerobic conditions sterols and androgens are catabolized via the 2,3-*seco* pathway, while the anaerobic degradation of estrogens has not been studied in detail (Chiang et al., 2020). Regardless of the oxygen availability, the three pathways merge at a common key intermediate, namely HIP (3 α -H-4 α (3'-propanoate)-7 α β -methylhexahydro-1,5-indanedione), which is subsequently degraded via a common central pathway to produce metabolites that are further degraded through central metabolic networks (Figure 2.7). In the following, differed estrogen biodegradation and biotransformation mechanisms will be presented. For clarity, E2 and E1 will be discussed together due to the fact that their degradation pathways are linked. Subsequently, EE2 and E3 degradation will be presented separately.

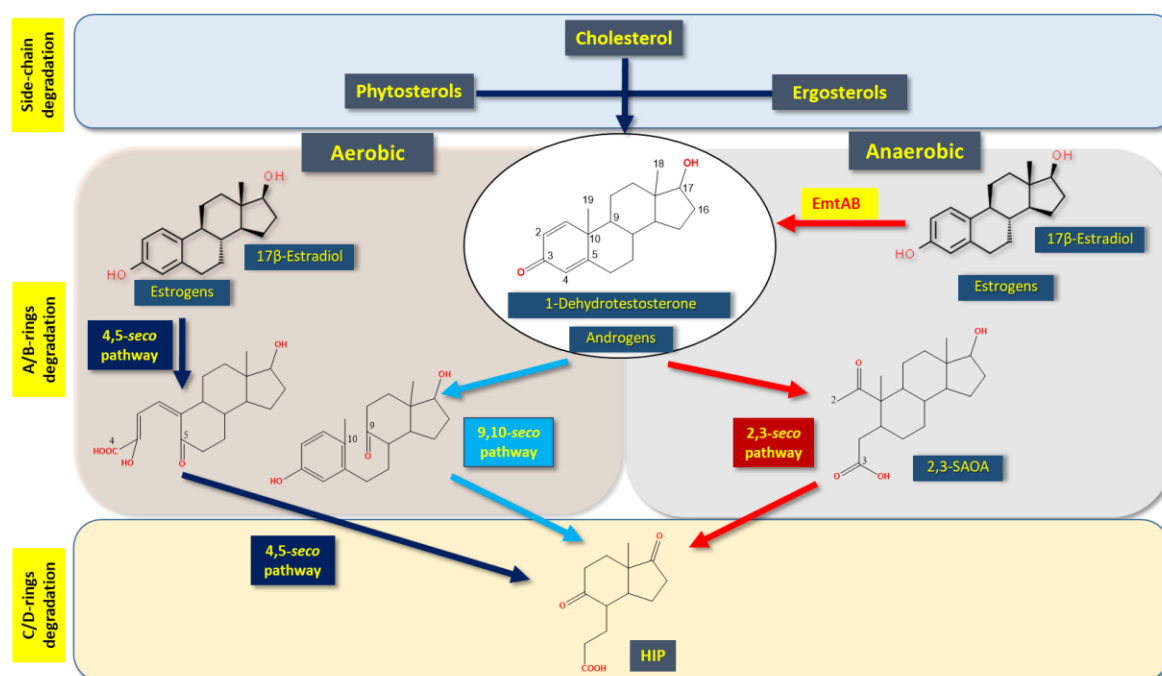


Fig. 2.7: Central pathways for bacterial steroid catabolism. Bacteria adopt a convergent pathway (the 2,3-*seco* pathway) to catabolize different steroids under anaerobic conditions and adopt divergent pathways to catabolize estrogens (the 4,5-*seco* pathway) and other steroids (sterols and androgens; the 9,10- *seco* pathway) under aerobic conditions. All of the 3 steroid catabolic pathways converge at HIP. 2,3-SAOA, 17 β -hydroxy-1-oxo-2,3-seco-androstan-3-oic acid (2,3- SAOA); HIP, 3 α -H-4 α (3'-propanoate)-7 $\alpha\beta$ -methylhexahydro-1,5-indanedione (Wang et al., 2020a).

2.6.3. Aerobic degradation of E2 and E1

In general, the majority of research on estrogen biodegradation focused on E1 and E2. General mechanisms of estrogen biodegradation pathways include modification at one or two rings via oxidation and hydroxylation reactions followed by cleavage of one ring and further metabolism to central metabolites. The first E2-degrading bacterium was isolated from activated sludge and identified as *Novosphingobium tardaugens* sp. nov. (Fujii et al., 2002), and Coombe et al. (1966) proposed the first pathway for E1 degradation by the soil bacterium *Nocardia* sp. E110. The authors proposed that a dioxygenase catalyzes the cleavage of the A-ring of E1. This pathway was included as a part of E2 degradation pathways reported in

subsequent studies, proposing that E2 degradation can be initiated by one of the following reactions: a) hydroxylation of A-ring at C4, b) hydroxylation of a saturated ring, c) dehydration of the D-ring at C17, and d) dehydrogenation of the D-ring at C17 (Yu et al., 2013) (Figure 2.8).

The first mode of degradation was reported by Kurisu et al. (2010) who detected 4-OH-E2 during degradation by the soil isolate *Sphingomonas* sp. ED 8, which suggests that E2 was hydroxylated at C4. The authors proposed that degradation of this hydroxylated product proceeds via *meta* cleavage. Kurisu et al. (2010) detected other products of E2 degradation by the ED8 strain including hydroxy-E2, keto-E2, keto-E1, and 3-(4-hydroxyphenyl)-2-hydroxyprop-2-enoic acid. This latter product suggested that E2 degradation was initiated at a saturated ring. However, the authors did not further investigate how the saturated ring was cleaved.

In another study, Nakai et al. (2011) used *Nitrosomonas europaeae* to degrade E2 and detected a metabolite identified as estra-1, 3, 5 (10), 16 tetraen-3-ol (estratetra enol, or E0), which results from dehydration at C17 of the D-ring. *N. europaeae* further degraded E0 to non-estrogenic compounds.

Transformation of E2 to E1 via dehydrogenation of C17 at the D-ring was also reported (Yu et al., 2007). E1 can be further transformed to 4-OH-E1, which can be further degraded via *meta* cleavage (Kurisu et al., 2010). Lee & Liu (2002) suggested another degradation pathway for E2, when utilized by mixed sewage bacteria, based on the detection of a new metabolite designated XI which contains a lactone structure at D-ring. All those previous studies proposed some biotransformation reactions. On the contrary, at this stage no complete pathway for E2 degradation had been elucidated in terms of metabolic intermediates, genes and enzymes, thus rendering the assessment of the fate and biodegradation potential of estrogens in the polluted environments challenging.

To address these knowledge gaps, Chen et al. (2017) studied E2 degradation by *Sphingomonas* sp. KC8. The authors adopted functional genomic analyses to identify E2 catabolic genes. Furthermore, they purified and characterized two catabolic enzymes and elucidated the chemical structure of some detected metabolites of E2 degradation. Chen et al. (2017) showed that E2 degradation by the KC8 strain starts by oxidation of the C17 hydroxyl group leading to E1. This is followed by hydroxylation at C4 of the A-ring to produce 4-OH-E1. The A-ring is then opened via *meta* cleavage. These reactions constitute the initial steps of the so called 4,5-*seco* pathway for aerobic bacterial degradation of E2/E1 (Figure 2.9). A key feature of this pathway is the abiotic reaction of the ring A cleavage product with NH_4^+ to produce pyridinestrone acid, a typical side product and functional marker of the 4,5-*seco* pathway.

Further characterization of 17 β -E2 dehydrogenase (OecA), which catalyzes the transformation of E2 to E1, and 4-OH-E1 4,5-dioxygenase, which catalyzes the meta cleavage of the A-ring, was conducted by Chen et al. (2017). Moreover, the genes encoding these two enzymes were identified in the genome of the KC8 strain.

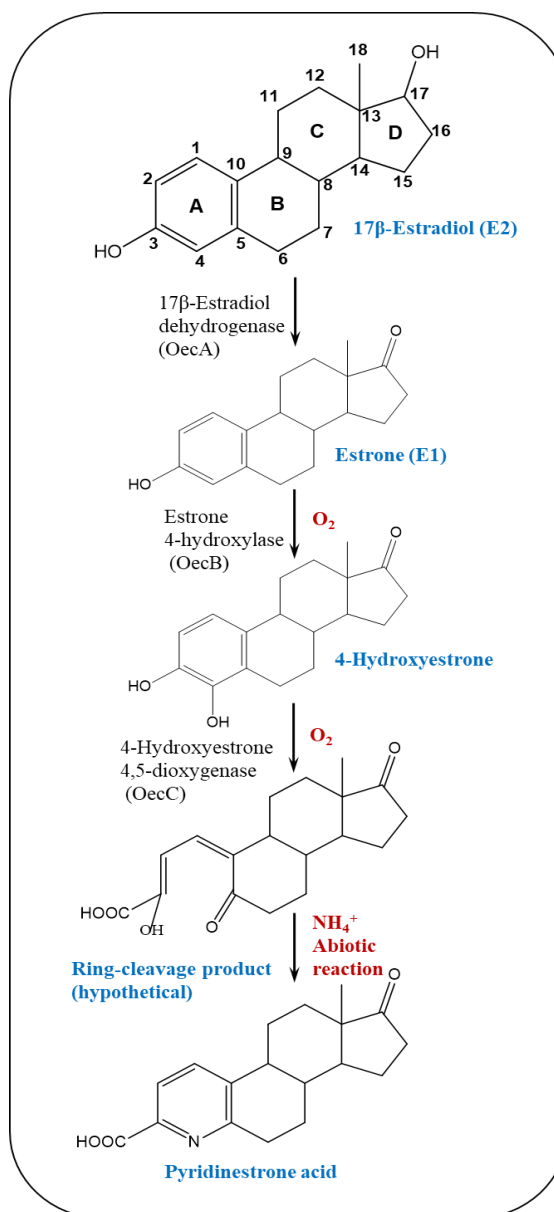


Fig. 2.9: The initial reactions of the 4, 5-seco pathway proposed for aerobic biodegradation of estrogens in *Sphingomonas* sp. strain KC8 (Chen et al., 2017).

Transcriptomics studies showed that two gene clusters were specifically expressed during growth on E2 compared to cultures grown on testosterone. In one of these gene clusters, the authors identified a gene (*oecB*), proposed to encode a putative flavin-dependent monooxygenase, which catalyzes hydroxylation of E1 at C4 of the A-ring. However, this enzyme was not further characterized. Enzymes

encoded by gene cluster II were proposed to catalyze the A/B rings degradation. The authors also identified gene cluster III which was similarly expressed in E2- and testosterone- grown cells. The gene products of this gene cluster exhibit high identity to β -oxidation enzymes and were proposed to be involved in the degradation of the C/D- rings of E2.

In a particular study, Chen et al. (2017) collected activated sludge samples from a WWTP and river water and spiked them with [3,4C-¹³C]E1 and incubated them under aerobic conditions for 10 days. The authors detected pyridinestrone acid after time intervals and accordingly proposed that the 4,5-*seco* pathway might play a role in estrogen degradation in the environment.

Further biochemical and microbiological studies were performed by Chen et al. (2018) to detect additional downstream intermediates of the 4,5-*seco* pathway and to assess the ecological relevance of estrogen-degrading bacteria. In activated sludge samples incubated with 1 mM E1, the authors detected pyridinestrone acid and two ring A/B cleavage products (Figure 2.10). Furthermore, they detected by PCR homologs of the alphaproteobacterial *oecC*, a functional marker of the 4,5-*seco* pathway. Metagenomics analysis revealed that *Novosphingobium* spp. are key estrogen degraders in activated sludge. This finding was confirmed by the isolation of *Novosphingobium* sp. SLCC strain from the E1-amended activated sludge. In batch cultures this strain degraded E1 and produced the A/B rings cleavage products detected in the activated sludge, thus confirming their role in E1 degradation.

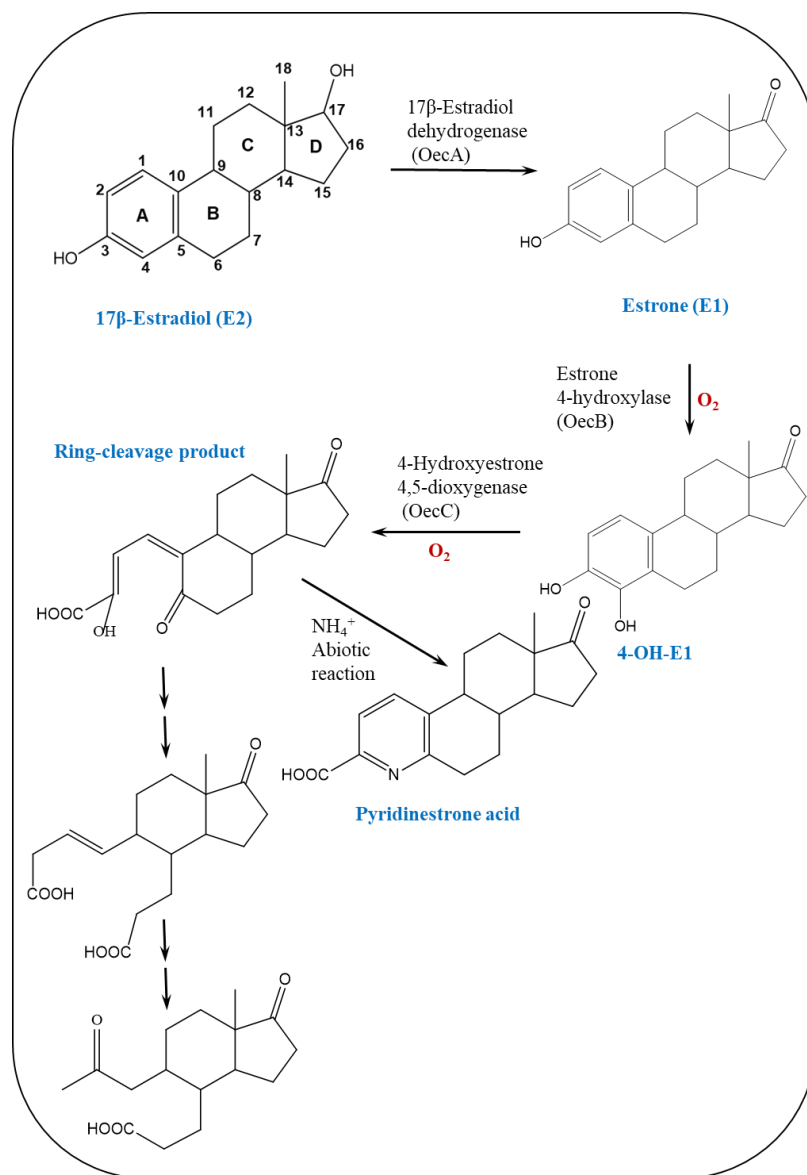


Fig. 2.10: Reactions of the 4, 5-seco pathway for an aerobic biodegradation of estrogens in activated sludge and the α -Proteobacterium *Novosphingobium* sp. SLCC, showing intermediates downstream of the ring A-cleavage product (Chen et al., 2018).

Comparative proteomics on *Pseudomonas putida* SJTE-1 grown with either glucose or E2 as a sole carbon source was performed by Xu et al. (2017). The authors found that 78 proteins were differentially expressed. Of these, 45 proteins were upregulated and 33 proteins were downregulated in the E2 culture. The upregulated proteins were related to stress response, energy metabolism, transport, chemotaxis,

and motility, whereas the downregulated proteins were related to glucose transport and metabolism.

In another comparative proteomics study, Du et al. (2017) studied E1 degradation by *Hydrogenophaga atypica* ZD1 in the presence of different nitrogen and carbon sources. The authors found that E1 degradation rate was higher in the presence of ammonium compared to nitrate and in the presence of acetic or humic acid than in the absence of additional carbon sources. Based on proteomics data, the authors proposed E1 degradation to start by transformation to keto-E1 intermediate, which undergoes saturated rings degradation leading to 4-hydroxyphenylpyruvate. This is followed by a transamination reaction with glutamate to produce tyrosine and 2-oxoglutarate. These assumptions were based on the finding that branched chain amino acid aminotransferase was upregulated in the E1 culture. Moreover, upregulation of 4-hydroxyphenylpyruvate dioxygenase in the E1 culture suggested that 4-hydroxyphenylpyruvate could be transformed to homogentisate and then to fumarate which could merge in the TCA cycle. Metabolism of E2/E1 to tyrosine was also reported by Li et al. (2012) who studied E2 degradation by *Stenotrophomonas maltophilia*.

Further investigations on the 4,5-*seco* pathway were reported by Wu et al. (2019) using *Sphingomonas* sp. KC8 as a model organism who applied NMR and UPLC-HRMS (Ultraperformance Liquid Chromatography-High Resolution Mass Spectroscopy) to identify metabolic intermediates of E1 degradation. In E1 cultures, the authors could detect and identify 4-norestrogen-5(10)-en-3-oyl-coenzyme A (CoA) and the corresponding non-conjugate (4-norestrogonic acid), in addition to the common metabolite of microbial steroid metabolism (HIP). Based on these findings, they proposed that after A-ring cleavage, C4 is removed by oxidative decarboxylation catalyzed by a 2-oxoacid oxidoreductase to produce 4-norestrogen-

5(10)-en-3-oyl-CoA. Then the B-ring is opened by hydrolysis and β -oxidation reactions transform the A/B-ring cleavage product into the common metabolite HIP (Figure 2.11).

E2 biodegradation was studied by Li et al. (2020) using a *Novosphingobium* sp. ES2-1 that was isolated from activated sludge in a domestic sewage treatment plant. Using ^{13}C labeling, NMR and HRMS, and as reported by Chen et al. (2017, 2018) and Wu et al. (2019) the authors proposed that E2 is transformed to E1 then to 4-OH-E1 where the A-ring is opened and pyridinestrone acid is produced. However, ring cleavage appeared to occur by successive monooxygenation reactions leading to long-chain ketonic structures. The authors also observed the production of several pyridine derivatives. In another study by Li et al. (2020), it was reported that tetracycline significantly inhibited E2 biodegradation at concentrations higher than 10 mg/L. Moreover, tetracycline inhibited ring opening and enhanced the production of pyridine derivatives by increasing condensation of some phenolic ring cleavage products with ammonia. However, nothing was reported about genes and enzymes of E2 catabolism.

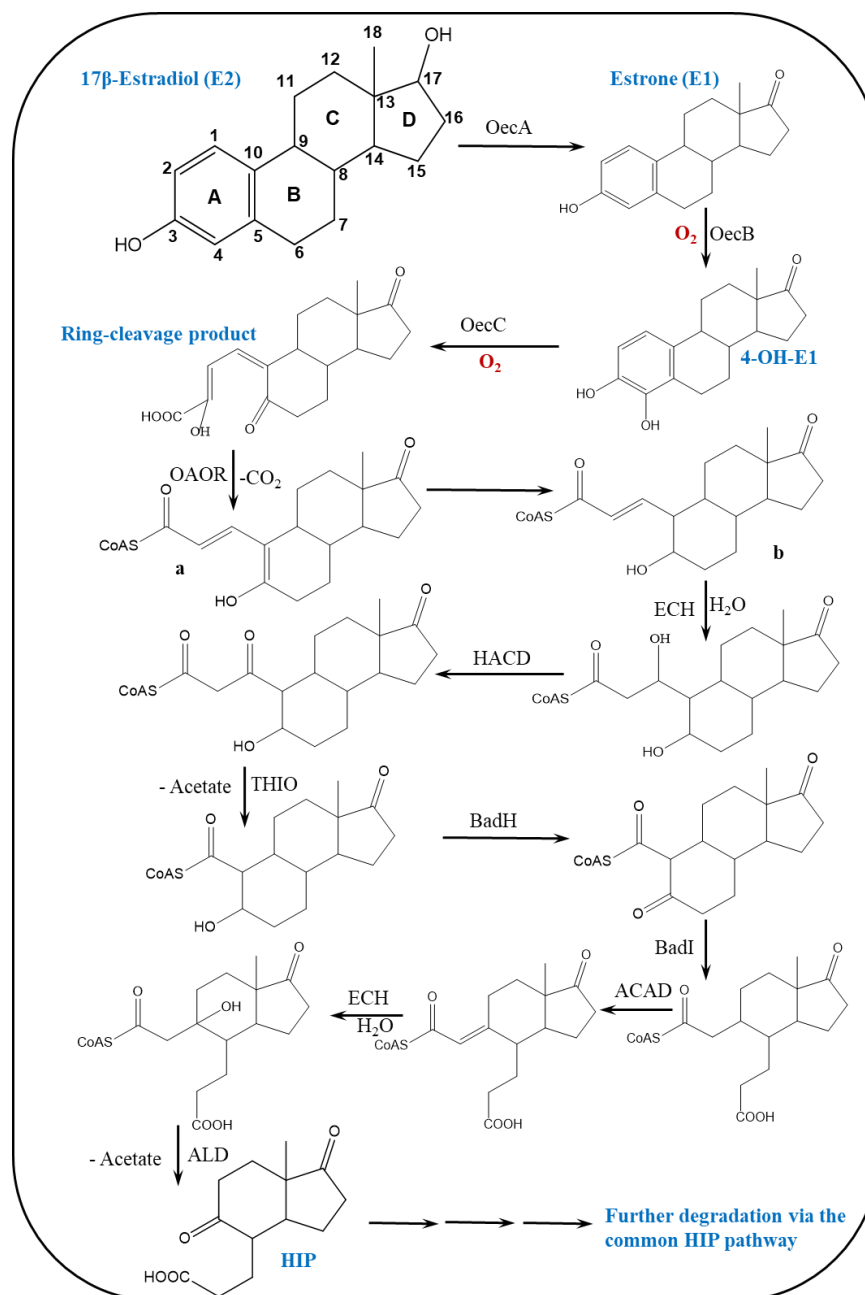


Fig. 2.11: Proposed bacterial degradation pathway of natural estrogens. ACAD, acyl-CoA dehydrogenase; ALD, aldolase; BadH, 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase; BadI, 2-ketocyclohexanecarboxyl-CoA hydrolase; ECH, enoyl-CoA hydratase; HACD, -hydroxyacyl-CoA dehydrogenase; OAOR, 2-oxoacid oxidoreductase; THIO, thiolase (Wu et al., 2019).

Degradation of E2/E1 via the 4,5-*seco* pathway was also reported in *Novosphingobium tardaugens* NBRC 16725 (Ibero et al., 2020). Transcript analysis in this bacterium revealed a gene cluster, designated *edc*, that encodes estrogen degradation proteins. The authors proposed that transformation of E2 to E1 could be catalyzed by a cytochrome P450 monooxygenase and identified a TonB-dependent receptor protein (EdcT) that could be involved in estrogen uptake.

Generally, degradation of estrogens by bacterial consortia has not been often reported. In one study, Wang et al. (2019a) reported different pathways for E2 degradation by a mixed culture of *Rhodococcus equi* DSSKP-R-001 and *Comamonas testosteroni* QYYZ0150409. All the proposed pathways start by transformation of E2 to E1. Then in one pathway, E1 is transformed to 4-OH-E1 which is further hydroxylated at C-16 to give a metabolite that is further degraded via the TCA cycle. In the second pathway, 4-OH-E1 ring A is opened between C4 and C5, the C5 is then oxidized to a carboxyl group, eventually B-ring is cleaved between C9 and C10. In the third pathway, cleavage of E1 starts at C9 of the B-ring by a hydroxylation reaction that forms a keto group. Then the A-ring is hydroxylated at C4 followed by 4, 5- cleavage then cleavage of the bond between C5 and C6. However, no pyridine derivatives or CoA thioesters were reported. Also, the enzymes and genes were not identified.

In another study by Wang et al. (2020b), a *Lysinibacillus sphaericus* DH-B01 was isolated from a contraceptive factory and used to degrade E2. Metabolite analysis revealed that E2 is transformed to E1 and 4-OH-E1 as reported for the *Sphingomonas* sp. KC8 strain. The A-ring is then opened followed by ring-B cleavage. No pyridine or CoA derivatives were reported.

The removal of E1, E2, and EE2 from synthetic wastewater effluent was studied by Wang et al. (2019b) using four microalgae: *Haematococcus pluvialis*, *Selenastrum capricornutum*, *Scenedesmus quadricauda*, and *Chlorella vulgaris*. All three estrogens were removed, and the estrogenic activity was reduced. Chemical analysis confirmed estrogen degradation and the authors proposed metabolic pathways based on the identified metabolites (Figures 2.12 and 2.13).

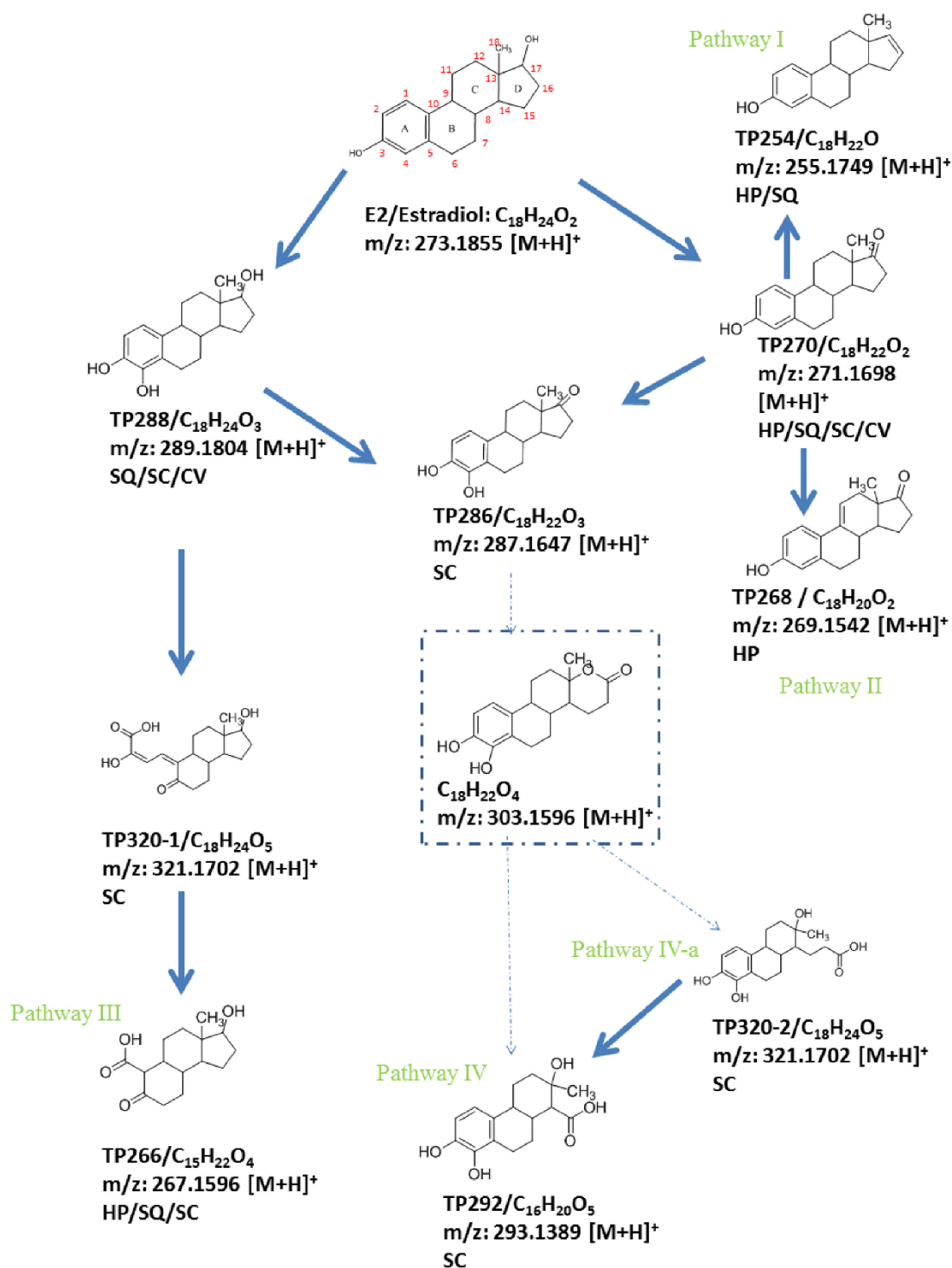


Fig. 2.13: Proposed metabolites and pathways of E2. Solid lines indicated the following proposed metabolites were detected by QTOF; dotted lines and boxes indicated the proposed metabolites were not detected by QTOF due to unacceptable mass difference and instrument intensity (Reproduced from Wang et al., 2019b, with permission from the Elsevier. License number 5735780718428).

It was demonstrated by Tian et al. (2022) that *Rhodococcus equi* can degrade E2 via the 4,5-seco pathway. They also identified some of the genes involved such as those encoding the dehydrogenase (conversion of E2 to E1), the monooxygenase (conversion of E1 to 4-OH-E1) and dioxygenases which catalyze cleavage of the A-ring. *Microbacterium hominis* SJTG1 degraded E2 in minimal medium and artificially polluted soil. A gene encoding 3 β /17 β -hydroxysteroid dehydrogenase was induced by E2, and this enzyme was shown to convert E2 to E1 (Xiong et al., 2023).

Sami et al. (2023) studied E1 biodegradation by the cyanobacterium *Spirulina* CPCC-695 and showed that it utilizes E1 as a carbon and energy source for growth without addition of co-substrates. The degradation intermediates included fatty alcohols, alkanes, and a degradation pathway was proposed which starts with the reduction of E1 to E2. Detection of these intermediates suggested E1 metabolism may include hydrogenation, dehydrogenation, oxidation-reduction, hydroxylation, and side chain cleavage reactions. Moreover, E1 metabolism was proposed to start by reduction of the C17 keto group to produce E2, and the D-ring is cleaved first after formation of E1-formate and E1-propionate. After ring opening, esterification, methylation, and hydrogenation reactions produce diones/diols such as 3-phenylundecane, cyclo-tetradecane, and tetradecane.

After methylation, 4, 6- dimethyldodecane undergoes dehydrogenation to form lauryl alcohol. Further degradation via oxidation reactions produces lauric acid that condenses with CoA. The resulting fatty acyl-CoA is further degraded to acetyl-CoA (Sami et al., 2023). This proposed pathway is completely different from the commonly reported 4, 5- seco pathway and does not converge at HIP. Although these findings suggest the presence of different estrogen degradation pathways in

cyanobacteria, the authors did not present biochemical evidence at the enzyme level. Furthermore, the culture medium contained several other carbon sources in addition to E1, such as acetone (the solvent of E1), citric acid, ferric ammonium citrate, Na-EDTA, and Na-CO₃, which questions the origin of at least some of the detected intermediates. A new strain of *Comamonas testosteroni* was isolated from animal waste by Liu et al. (2021), demonstrating its ability to degrade both E2 and testosterone as sole carbon and energy sources. The authors also sequenced the genome and identified steroid degradation genes via comparative genomics. However, degradation metabolites were not reported.

As mentioned earlier, estrogens are released from human and animal as conjugates of sulfate and glucuronide moieties at C3 and C17. These conjugates are more water soluble and mobile, thus rendering them an environmental risk after hydrolysis to biologically active free estrogens (Nazari & Suja, 2016; Pinto et al., 2014). Ma & Yates (2018) used river water and sediment as an inoculum to study the degradation of E2-3-glucuronide and E2-3-sulfate. In laboratory degradation tests, E2-3-glucuronide was transformed to E2 and E1, while E2-3-sulfate was slowly converted to E1-3-sulfate. Based on the metabolite profile, the authors proposed that E2-3-glucuronide is transformed to E1 via either E2 or E1-3-glucuronide. E1 is then transformed to a lactone at the D-ring or oxidized at the C- or B-ring. On the other hand, E2-3-sulfate is transformed to E1 via E1-3-sulfate or E2. E1 is subjected to similar transformations as reported for E2-3-glucuronide. Alternatively, E1-3-sulfate is hydroxylated at the B-ring followed by either elimination of the sulfate group to produce OH-E1, or oxidation to the corresponding keto structure. However, no ring cleavage products were reported in this study and knowledge on the involved genes and enzymes are lacking.

A microbial strain, BH2-1, was isolated from mangrove sediment by Pratush et al. (2020b), and it was found to utilize E1 as the sole carbon and energy source. The strain was identified as a *Rhodococcus* spp. and the authors detected two non-accumulating E1 metabolites, namely; 3-hydroxyandrosta-5,7,9(11)-trien-17-one and androsta-1,4,6-triene-3,17-dione. Genomic analysis revealed that the strain BH2-1 has 46 genes belonging to 6 major steroid degradation gene classes including cholesterol oxidase, steroid Δ -isomerase, cytochrome P450 monooxygenase, 3 δ -steroid-17-dehydrogenase, 3-steroid-9 α -hydroxylase, and 3 α ,20 β -hydroxysteroid 17-dehydrogenase. The authors reasoned that the steroid Δ -isomerase was involved in the structural rearrangements in E1 which produced 3-hydroxyandrosta-5,7,9(11)-trien-17-one.

Research conducted by Ge et al. (2021) focused on the degradation of E2 by *Candida utilis* CU-2 and *Lactobacillus casei* LC-1. Using LC-MS/MS analysis, the authors detected 12 degradation products in E2 biodegradation experiment and accordingly proposed five degradation pathways (Figure 2.14). In pathway I, degradation proceeds via a dehydration reaction at C17 of the D-ring to produce E0 as proposed earlier by Nakai et al. (2011) In pathway II, E2 is hydroxylated at the D-ring and then methylated. No ring cleavage products were reported for pathways I and II. As reported by several studies, E2 is first transformed to E1 in the other three alternative pathways reported by Ge et al. (2021), and E1 is then oxidized at the C-ring via dehydrogenation, or at the D-ring via oxygen incorporation as reported by Wang et al. (2019b), or at C4 of the A-ring to produce 4-OH-E1. However, further degradation of the intermediate proceeds via opening of the D-ring.

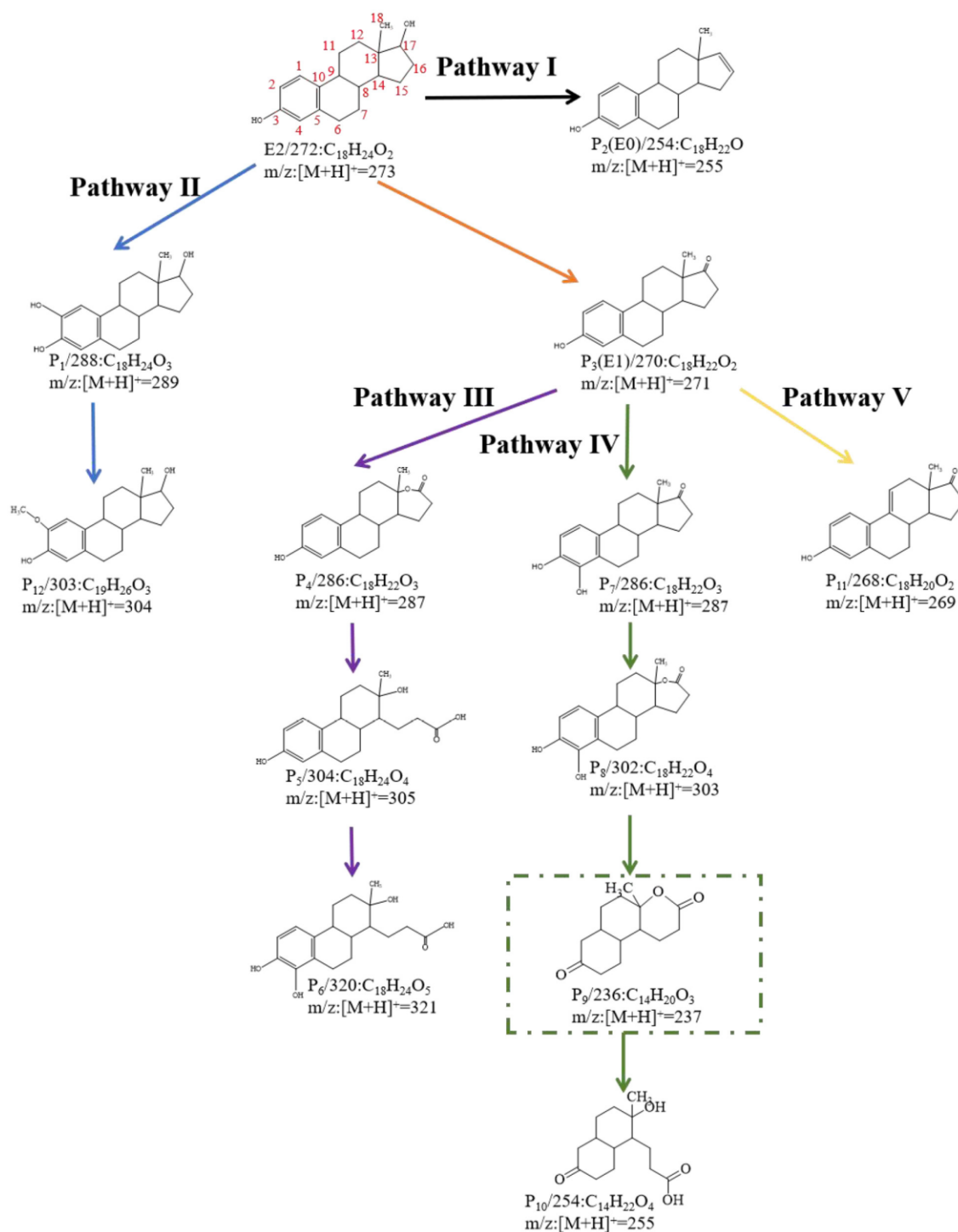


Fig. 2.14: E2 degradation products and predicted biodegradation pathways. The green rectangle indicated the metabolite that was not detected by liquid chromatography tandem mass spectrometry (LC-MS/MS) (the black, blue, purple, green, and yellow lines represent Pathway I, Pathway II, Pathway III, Pathway IV, and Pathway V, respectively) Reproduced from Ge et al., 2021, Open Access Publication under the [Creative Commons Attribution License CC BY](#).

Degradation of E2 and testosterone was studied by Wang et al. (2023) via comparative transcriptomics using *Comamonas testosteroni* JLU460ET. The authors reported upregulation of a large number of genes in the testosterone and E2-grown cultures. A 100 kb steroid degradation gene cluster was identified and included genes for sensing substrates in the environment, steroid uptake, ring-cleavage, and β -oxidation enzymes. Based on the transcriptomic data, the authors proposed E2 and testosterone degradation mechanisms, and E2 was proposed to be degraded via the 4, 5-seco pathway. However, no degradation metabolites were reported from E2-grown cultures.

A study by Hao et al. (2022) isolated *Microbacterium resistens* MZT7 from farm-derived activated sludge and studied its E2 degradation potential. Addition of various carbon (glucose, maltose, lactose, or lactose) and nitrogen (urea, peptone, or beef extract) sources promoted E2 degradation, whereas yeast extract was inhibitory. Based on LC-MS analysis, the authors detected E1, E3, and dehydroepiandrosterone, and accordingly proposed three possible E2 degradation pathways by the MZT7 strain (Figure 2.15).

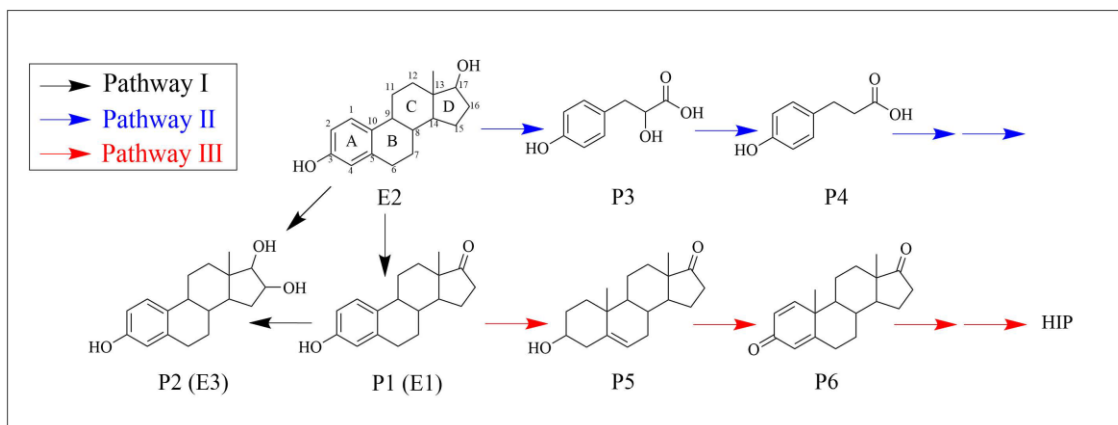


Fig. 2.15: Proposed pathways for E2 degradation by *Microbacterium resistens* MZT7 (Reproduced from Hao et al., 2022, Open Access Publication under the Creative Commons Attribution License CC BY, (<https://creativecommons.org/licenses/by/4.0/>)).

A short-chain dehydrogenase from *Microbacterium* sp. MZT7 was characterized in a study by Hao et al. (2023), showing that it catalyzes the transformation of E2 to E1. They also showed that this enzyme can transform E2 to E1 in synthetic livestock wastewater.

A study by Bergstrand et al. (2016) conducted genome-mining analysis of steroid degradation genes in fungal and prokaryotic genomes from the RefSeq database using hidden Markov models and reciprocal BLAST analysis. The authors identified 265 putative steroid degraders from soil, eukaryotes, and marine and freshwater environments. The identified bacteria were limited to *Actinobacteria* and *Proteobacteria* including 17 genera not previously reported to include steroid degraders. Furthermore, the data suggested that sterols are degraded only by actinobacterial members, while bile salts, and other less complex steroids are degraded by *Proteobacteria*.

In a follow up study, Holert et al. (2018) analyzed the distribution of the 9,10-seco pathway for aerobic steroid degradation in 346 metagenomes from diverse environments. The findings showed the wide occurrence of steroid-degrading bacteria in soil, plant rhizosphere, WWTPs, and the marine environment (including marine sponges). The majority of the 9,10-seco pathway genes were detected in 45 metagenome-assembled genomes. The identified steroid degraders belonged exclusively to *Actinobacteria* and *Proteobacteria*, which coexisted in wastewater, whereas soil and rhizosphere were dominated by actinobacterial members.

A study by Ye et al. (2019) identified a new 17 β -hydroxysteroid dehydrogenase in the marine isolate *Rhodococcus* sp. P14. This enzyme is induced in cultures grown on E3, E2, and testosterone as a sole carbon source. Moreover, it has a dehydrogenation activity which transforms E2 to E1, E3 to 16-hydroxyestrone,

and testosterone to androst- 4-eno-3, 17-dione. Mensitieri et al. (2023) isolated a ring cleavage dioxygenase from the marine isolate *Novosphingobium* sp. PP1Y and showed that it catalyzes the *meta* cleavage of 4-OH-E2 at the A- ring and generates a semialdehyde product.

A study by Liu et al. (2020) studied estrogen degradation by *Gordonia* sp. strain R9 isolated from an enrichment culture of chicken leachate. This strain degraded E2, E1, E3, and EE2 as a sole carbon and energy source. E2 degradation by the strain R9 produced several metabolic intermediates including E1, E3, (3Z)-3-(3-hydroxy-3a-methyl-7-oxododecahydro-6H-cyclopenta[a]naphthalene-6-ylidene) propanoic acid, and 3-hydroxy-3a-methyl-7-oxododecahydro-1H-cyclopenta[a]naphthalene- 6- carboxylic acid. The latter two metabolites suggest that E2 degradation proceeds via cleavage of the A-ring. The authors also reasoned that detection of E1 and E3 in the E2-degrading culture may indicate the presence of alternative estrogen degradation pathways in the R9 strain (Figure 2.16). It is worth noting that no CoA-bound intermediates were reported in this study, in contrast to the studies of Wu et al. (2019) which could be differences in degradation mechanisms by actinobacterial and proteobacterial members.

As forementioned above, the majority of work done on E1 and E2 degradation focused on members of the Proteobacteria and Actinobacteria. However, for Actinobacterial estrogen degraders, less information is available regarding the involved genes, enzymes and pathways, compared to Proteobacteria. Hence, Hsiao et al. performed a series of detailed studies to fill these knowledge gaps. In the first study, Hsiao et al. (2021) used a soil isolate *Rhodococcus* sp. B50 as a model estrogen-degrading strain to study estrogen degradation in *Actinobacteria*. The authors applied metabolomics and gene disruption studies. The B50 strain utilized E1, E2, E3, testosterone, or cholesterol as a sole carbon and electron source. In E1-

fed resting cell assays, UPLC- APCI-HRMS analysis revealed key metabolites of the 4,5-*seco* pathway such as pyridinestrone acid (PEA) and HIP.

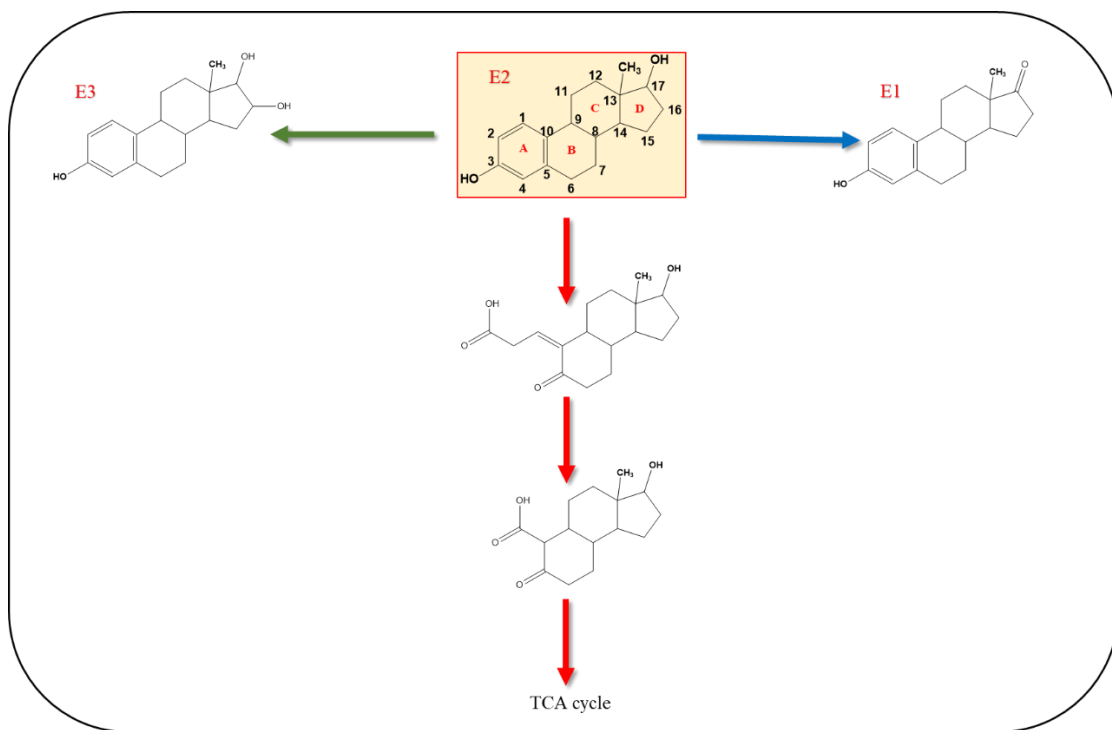


Fig. 2.16: Proposed E2 degradation mechanism in *Gordonia* sp. strain R9 (Reproduced from Liu et al., 2020).

Comparative genomic analysis revealed a putative estrogen degradation gene cluster carried on a mega plasmid. This gene cluster carries a cytochrome P450 E1-4-hydroxylase encoding gene (*aedA*) and a *meta* cleavage dioxygenase (*aedB*) involved in estrogen degradation via the 4,5-*seco* pathway. The function of these two enzymes was validated via gene disruption and phenotype analysis, showing functional similarity with the corresponding homologs from Proteobacteria, EdcA and EdcB (Ibero et al., 2020) and OecA and OecB (Chen et al., 2017). Despite this functional similarity, the amino acid sequence identity between the actinobacterial and proteobacterial homologs is relatively low (< 40%). Subsequently, the authors

used the extracellular metabolites PEA and HIP in addition to primers specific to the actinobacterial 4-hydroxyestrone 4,5-dioxygenase gene (*aedB*) as functional biomarkers to study estrogen biodegradation in urban estuarine sediments. Using E1-spiked mesocosms and PCR-based functional assays, the authors concluded that estrogen biodegradation in the estuarine sediments proceeds via the 4,5-*seco* pathway and Actinobacteria are active estrogen degraders in this ecosystem.

In a follow up study, Hsiao et al. (2022) applied comparative transcriptomics, gene disruption, and metabolite analysis to unravel genes and intermediates of downstream degradation of the estrogen A/B rings using *Rhodococcus* sp. B50 as a model. The authors analyzed the transcriptomes of B50 cultures grown on E1 compared to control cultures on cholesterol or testosterone. The analysis revealed that genes involved in C/D-rings degradation were similarly expressed in all three cultures. On the contrary, the *aed* gene cluster, which was identified by Hsiao et al. (2021) was significantly upregulated in the E1-grown culture. This gene cluster carries genes encoding AedA (E1 hydroxylase), AedB (*meta* cleavage dioxygenase), a putative medium-chain length fatty acid-CoA ligase, genes encoding two sets of β -oxidation enzymes including acyl-CoA dehydrogenase (AedN, AedP), enoyl-CoA hydratase (AedD, AedM, AedO), 3-hydroxyacyl-CoA dehydrogenase, (AedE, AedG) and thiolase (AedF, AedK) (Figure 2.17). The authors then constructed knockouts for the genes AedF and AedK and validated their function via phenotypes and metabolite analysis. Based on the findings, an estrogen degradation pathway for Actinobacteria was established (Figure 2.18). In addition to the common metabolites of the 4,5-*seco* pathway, three novel metabolites were identified including 4,5-*seco*-estrogenic acid, 5-oxo-4-norestrogenic acid, 2, 3, 4-tri norestrogenic acid.

oxidoreductase (EdcC), a member of the indolepyruvate ferredoxin oxidoreductase family, to produce 4-norestrogen-5 (10)-en-3-oyl-CoA. (Wu et al., 2019; Ibero et al., 2020). In the *Rhodococcus* sp. B50, an EdcC homolog is lacking in the genome. Hence, the EdcC function may be carried out by a putative 2-hydroxyacid dehydrogenase (AedG), a decarboxylase (AedH) and a CoA-ligase (AedJ), which awaits further investigations.

Hsiao et al. (2023) continued their investigations on the topic and conducted further comparative genomics, gene disruption, and metabolite analysis studies to validate the proposed function for the actinobacterial enzymes AedGHJ. They first revealed that the genes *aedGHJ* are found exclusively in the genomes of estrogen-degrading *Actinobacteria*. In contrast the α -oxoacid-ferredoxin oxidoreductase EdcC is unique to estrogen-degrading *Proteobacteria* (Figure 2.19). These findings suggested the presence of phylum-specific functional markers that delineate estrogen degradation mechanisms. Gene disruption experiments and metabolite analysis validated the proposed functions for AedGHJ proteins as shown in Figure 2.20. The authors further detected a C₁₇ metabolite (1-hydroxy-5-oxo-4-norestrogonic acid) that accumulated in the wild type.

Based on these findings, the authors concluded and reasoned that activation and degradation of the estrogenic A-ring proceeds via different strategies. In *Proteobacteria*, a single oxidative decarboxylation reaction catalyzed by an α -oxoacid-ferredoxin oxidoreductase EdcC removes the C4 of estrone and produces a CoA-ester C₁₇ metabolite (Ibero et al., 2020; Wu et al., 2019). Homologs of EdcC are lacking in estrogen-degrading *Actinobacteria*, which depend on the enzymes Aed GHJ to degrade the A-ring. Accordingly, the authors used these phylum specific genes as biomarkers to study estrogen degradation in the environment (Figure 2.21).

They designed degenerate specific primers for the *aedJ* gene and *edcC* gene and also used phylum-specific 16S rRNA gene primers.

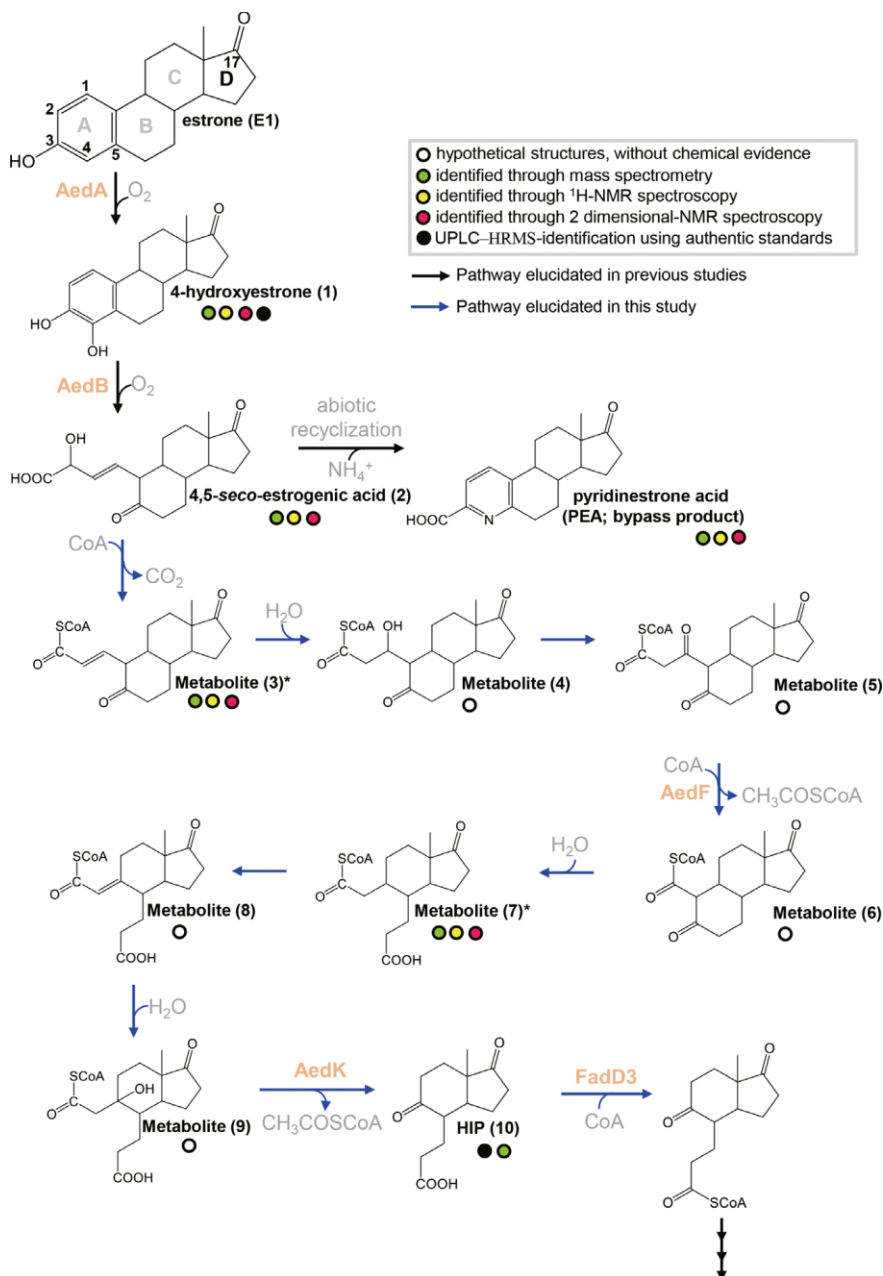


Fig. 2.18: The proposed estrogen degradation pathway of actinobacteria. *, the deconjugated structures have been purified and elucidated in this study. AedF, AedK and fadD3 have been functionally characterized in the study (Reproduced from Hsiao et al., 2022. Open Access Publication Under Creative Commons Attribution-Non-Commercial Use License).

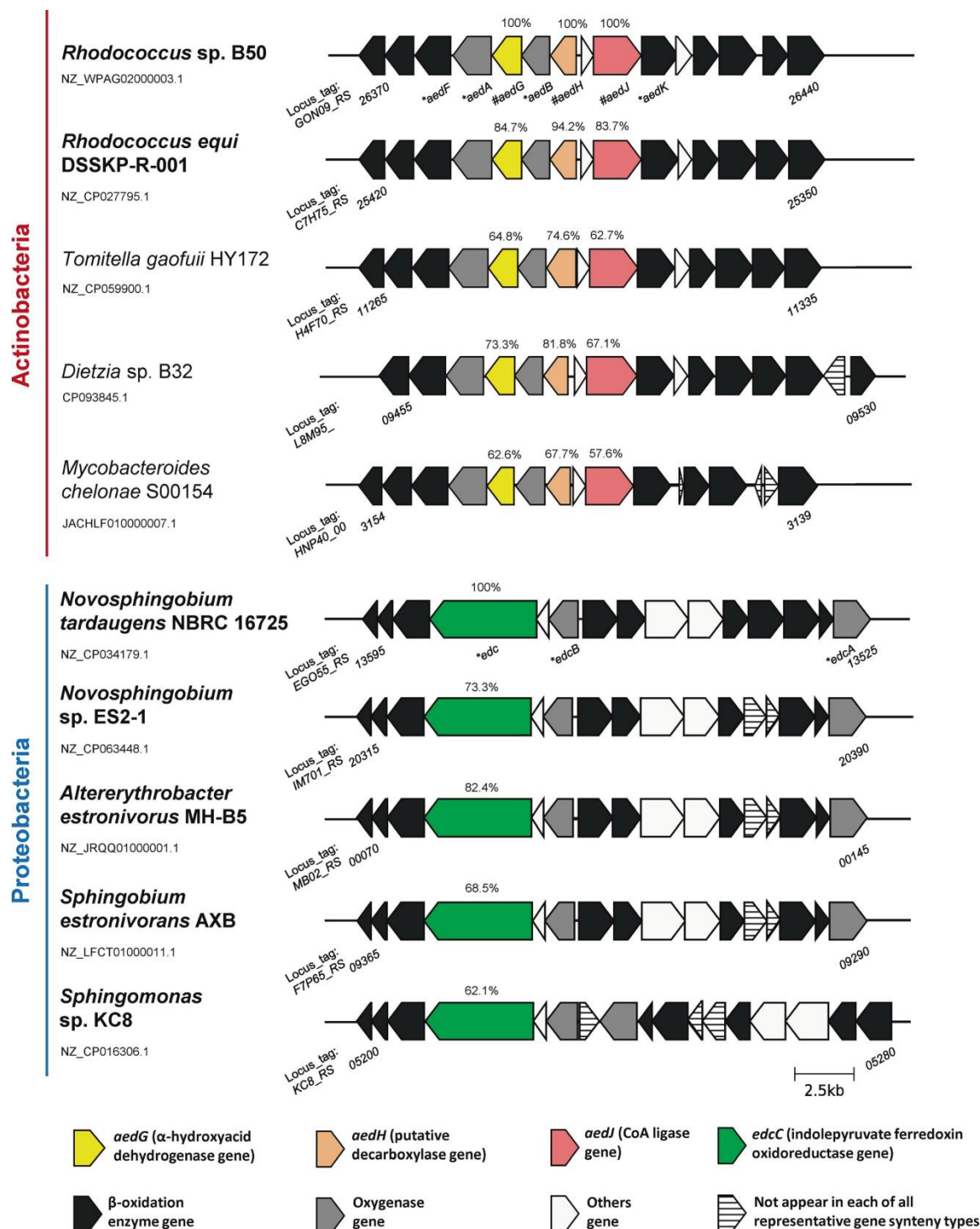


Fig. 2.19: Bacterial gene clusters involved in estrogen degradation. Percentage (%) indicates the identity of the deduced amino acid sequences. Bacterial strains (in bold) are physiologically characterized estrogen degraders (Reproduced from Hsiao et al., 2023. Open Access Publication and the [Creative Commons CC-BY-NC-ND](https://creativecommons.org/licenses/by-nc-nd/4.0/) license <https://creativecommons.org/licenses/by-nc-nd/4.0/>).

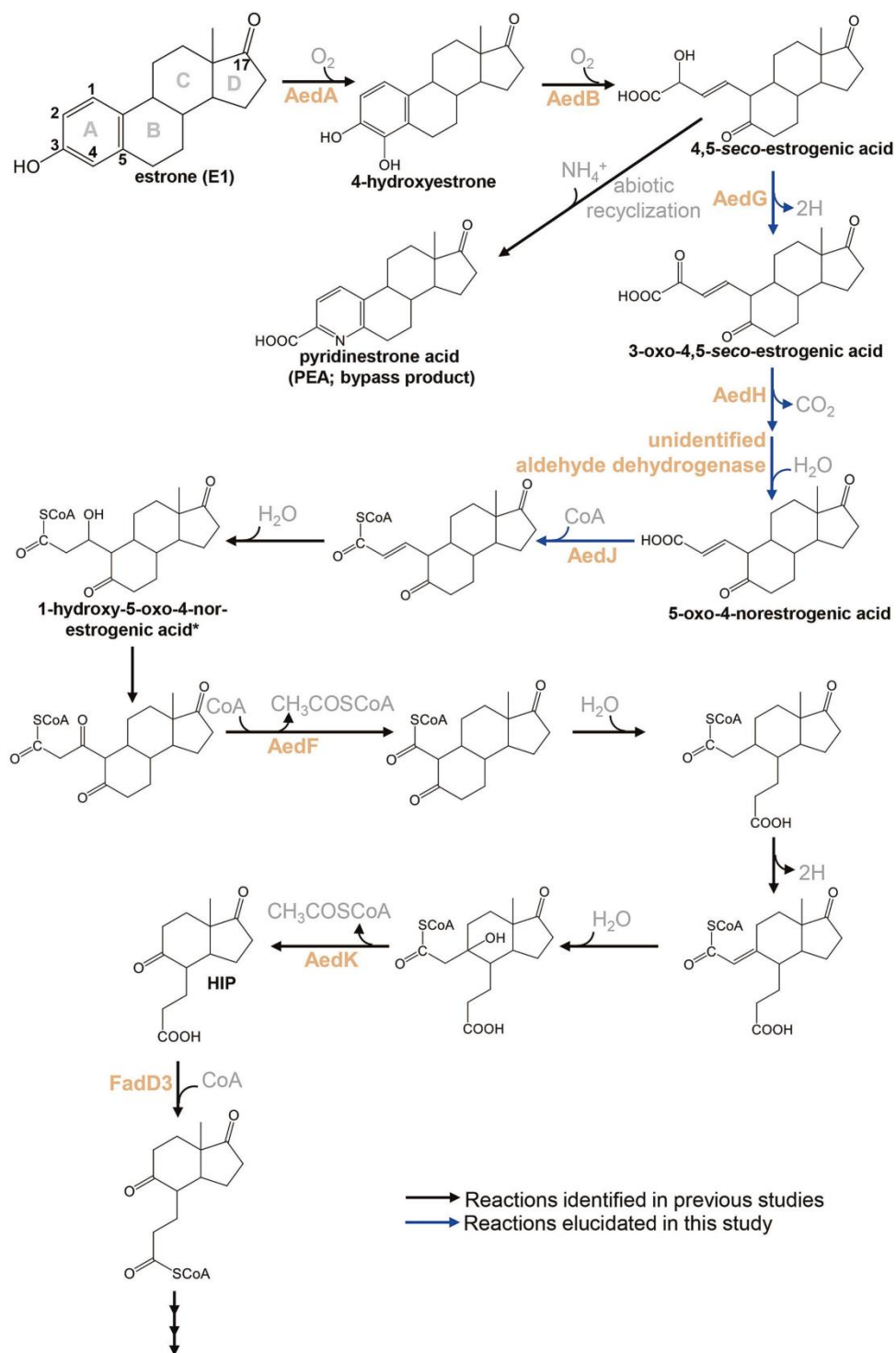


Fig. 2.20: The proposed estrogen degradation pathway of actinobacteria. AedG, AedH, and AedJ were functionally characterized in this study. *, the deconjugated structure (1-hydroxy-5-oxo-4-norestrogenic acid) was identified from the ethyl acetate-extractable samples through UPLC–HRMS (Reproduced from Hsiao et al., 2023. Open Access Publication and the Creative Commons CC-BY-NC-ND license <https://creativecommons.org/licenses/by-nc-nd/4.0/>).

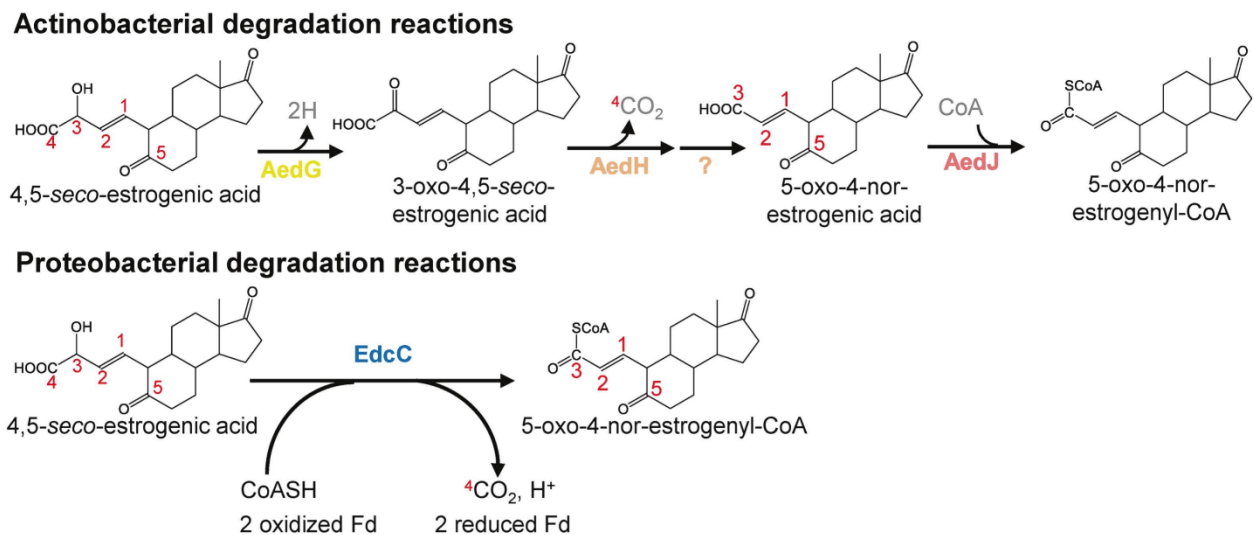


Fig. 2.21: Bacterial taxa-specific enzymes used in estrogen degradation reactions (Hsiao et al., 2023).

Quantitative PCR assay using DNA from various estrogen-contaminated ecosystems in Taiwan revealed that *γ-Proteobacteria* were most abundant in the examined environmental samples. However, the actinobacterial gene *aedJ* was more abundant than the proteobacterial gene *edcC* in most of the examined samples particularly those collected from aquatic ecosystems. These findings suggest that *aedJ* gene can be adopted as a specific biomarker to probe for estrogen-degrading Actinobacteria in the environment due to the following reasons: a) the biochemical function of the AedJ enzyme was characterized and its substrate and product were identified, b) an *aedJ* homolog is lacking in estrogen-degrading *Proteobacteria* and non-estrogen-degrading *Actinobacteria*, c) the long sequence of *aedJ* (1500 bp) enables the design of specific primers, d) *aedJ* is highly expressed only in the presence of estrogens (Hsiao et al., 2022). Similarly, the gene encoding α -oxoacid-ferredoxin oxidoreductase *edcC* can be used as a specific biomarker for estrogen-degrading *Proteobacteria*.

In a recent and unprecedented study in the GCC area, AlDhafiri et al. (2022) studied E1 biodegradation in activated sludge from a major WWTP in Bahrain. Chemical analysis of samples from E1-spiked activated sludge microcosms revealed degradation of more than 90% of E1 within 6 days. Moreover, 4-OH-E1 and pyridinestrone acid were detected, indicating that E1 was degraded via the 4,5-*seco* pathway. Bacteroidia and Proteobacteria dominated both the E1-amended microcosm and a control DMSO-amended microcosm. The alphaproteobacterial genera *Novosphingobium* and *Sphingaurantiacus* were significantly enriched in the E1 microcosm after 2 days, while the Bacteroidete *Pedobacter* was uniquely detected at day 10. Moreover, the relative abundance of *Palygonia* (*Nannocystis*) increased to 10% in the E1 microcosm after 4 days of incubation. The authors enriched a mixed culture from the activated sludge which degraded E1 in batch cultures. From this consortium, an alphaproteobacterial strain *Sphingobium estrogenivorans* was isolated and degraded E1 in batch cultures.

2.6.4. Aerobic EE2 degradation

The fate of estrogens was studied in wastewater treatment process, river water, aquifers, and agricultural soils. Degradation of EE2 was slow in marine sediments (Ying & Kookana, 2003) and aquifer material (Ying et al., 2003). However, it was more rapidly degraded in agricultural soils (Colucci & Topp, 2001) and nitrifying activated sludge (Vader et al., 2000).

EE2 degradation was reported for single bacterial strains, including *Sphingobium* sp. JCR5, *Rhodococcus zopfii*, and *Nitrosomonas europaea* (Aris et al., 2014; Haiyan et al., 2007; Shi et al., 2002; Yoshimoto et al., 2004). EE2 degradation occurs under aerobic and anaerobic conditions, albeit higher degradation rates were reported under aerobic conditions (Aris et al., 2014; Cajthaml et al., 2009). One of the possible degradation pathways starts by transforming EE2 to E1, then E3, but no

further degradation was reported (Li et al., 2013a; 2013b). Transformation of EE2 to E1, followed by ring cleavage, was also reported (Cajthaml et al., 2009). At low concentrations, EE2 could be co-metabolized with E1, E2, or E3 in sludge and wastewater; however, no further metabolites were detected (Cajthaml et al., 2009; Rhee et al., 2014; Writer et al., 2012).

Earlier studies reported the biotransformation of EE2 by some bacteria and microalgae (Della Greca et al., 2008; Haiyan et al., 2007). As discussed for E1/E2, the reported biotransformation reactions for EE2 can be categorized into different mechanisms (Yu et al., 2013). The first proceeds via hydroxylation of the A-ring at C2 (Figure 2.22). Yi and Harper detected 2-OH-EE2 during their studies on EE2 degradation by a nitrifying culture. The authors showed that the A-ring was cleaved before the other rings, probably due to the higher electron density of the A-ring carbon units. These findings suggested that EE2 can be cometabolically transformed under nitrification conditions.

In another mechanism, Della Greca et al. (2008) reported that some algal species can transform EE2 into EE2-glucoside, 3- β -D-glucopyranosyl-2-hydroxy-EE2, 3- β -D-glucopyranoside-6 β -hydroxy-EE2, 17 α -ethinyl-1,4, estradien-10,17 β -diol-3-one, and 6- α -OH- EE2. Thus, hydroxylation, oxidation, and glycosylation reactions were the main biotransformation mechanisms. In another study, Haiyan et al. (2007) isolated *Sphingobacterium* sp. JCR5 strain from activated sludge and showed that it can grow on EE2 as a sole carbon and energy source. Mass spectrometry analysis revealed that EE2 is first transformed to E1, followed by hydroxylation and oxidation reactions at C9, leading to B-ring cleavage. The A-ring is then hydroxylated at C4.

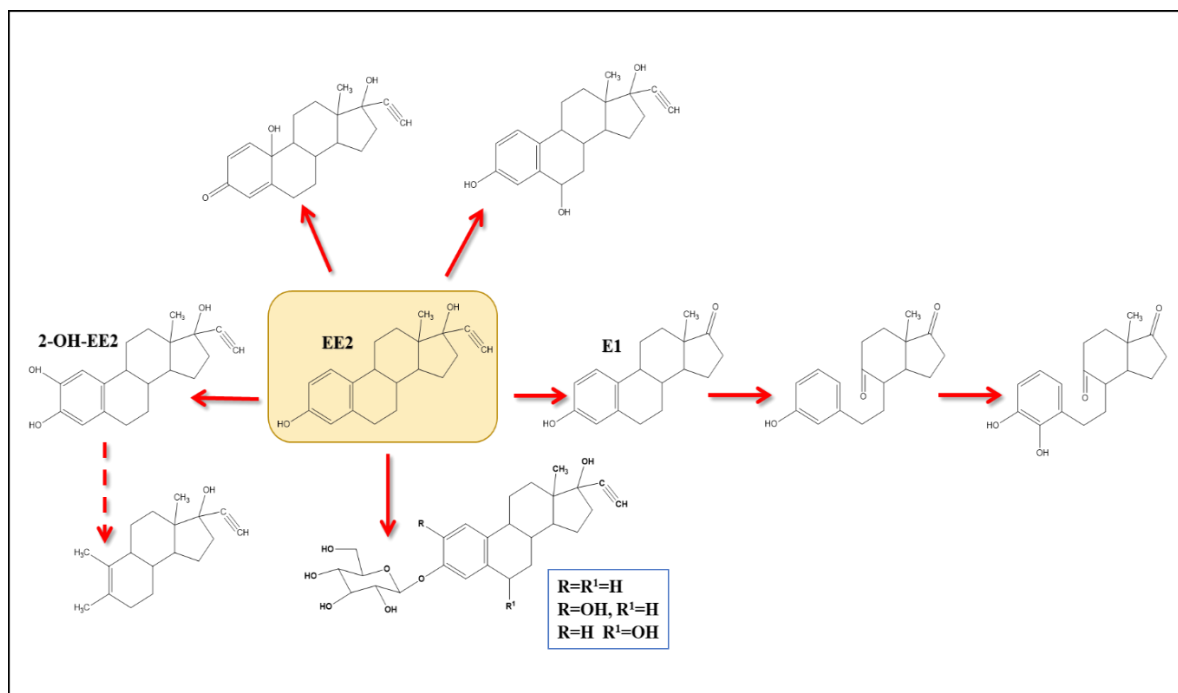


Fig. 2.22: Proposed EE2 degradation pathways by bacteria and algae (Yu et al., 2013).

Co-metabolism of EE2 was studied with several bacteria isolated from compost. The isolated bacteria degraded E1, E2, and E3. However, EE2 was not degraded when used as a sole carbon source, albeit it was degraded in the presence of E2, and in this case E1 was detected as a metabolite. No other metabolites were detected. EE2 removal started after complete removal of E2 and it increased as the initial E2 concentration increased (Pauwels et al., 2008).

In a study by O'Grady et al. (2009), it was shown that *R. erythropolis* and *R. equi* removed EE2 from minimal medium in the presence of co-substrates (adipic acid and glucose). The authors also identified two degradation metabolites as phenol and a derivative of EE2 with a higher molecular mass (331 atomic mass unit) where the ethynyl group was absent.

In a study by Ma et al. (2018), lab-scale percolation columns were used to investigate EE2 biodegradation during groundwater recharge with reclaimed water. Based on metabolite analysis, the authors proposed different pathways for EE2 degradation depending on ground water recharge mode. The dominant pathway in wetting and drying alternative recharge mode started by oxidation of C17 at the D-ring, which is then hydroxylated and cleaved. Thus, EE2 is transformed to E1 → E3 → 7-hydroxy-1-hydroxymethyl-2-methyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene-2-carboxylic acid → 2-hydroxy-2,4-diene-1,6-dioic acid. On the contrary, in a continual recharge mode, EE2 metabolism started by hydroxylation of C4 on the A-ring and then the A- or B-ring is cleaved. The authors proposed that this transition in the degradation mechanism is related to dissolved oxygen and structure of the involved microbial community. They also studied changes in microbial community composition during the two recharging modes.

Recent studies on EE2 degradation in synthetic wastewater by microalgae proposed hydroxylation reactions at C2 or C4, followed by ring opening (Wang et al., 2019b) (Figure 2.23). However, enzymes and genes of the proposed biochemical reactions were not reported. Peng et al. (2023) studied EE2 degradation by *Pseudomonas citronellolis* SJTE-3 and showed that it can grow on and degrade EE2, however, only in the presence of co-substrates like ethanol and glucose. The authors also identified two new degradation metabolites (Figure 2.24). EE2 metabolism was inducible, and 8 genes were significantly upregulated in the presence of EE2, including genes encoding a short chain dehydrogenase, a membrane transporter, and a cytochrome P450 hydroxylase, which were crucial for EE2 metabolism. *Pseudomonas citronellolis* SJTE-3 also degraded EE2 in soil, lake water, and pig manure following bioaugmentation. Moreover, addition of Tween 80 at low

concentration promoted EE2 degradation (Peng et al., 2023). However, this study did not report the degradation metabolites.

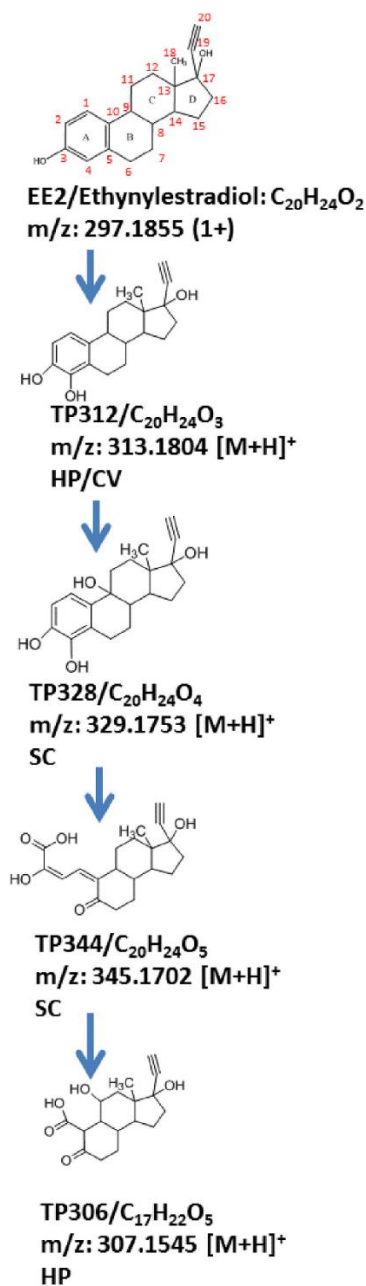


Fig. 2.23: Proposed metabolites and pathways of EE2. Solid lines indicated the following proposed metabolites were detected by QTOF; dotted lines and boxes indicated the proposed metabolites were not detected by QTOF due to unacceptable mass difference and instrument intensity. (Reproduced from Wang et al., 2019b, with permission from the Elsevier. License number 5735780718428).

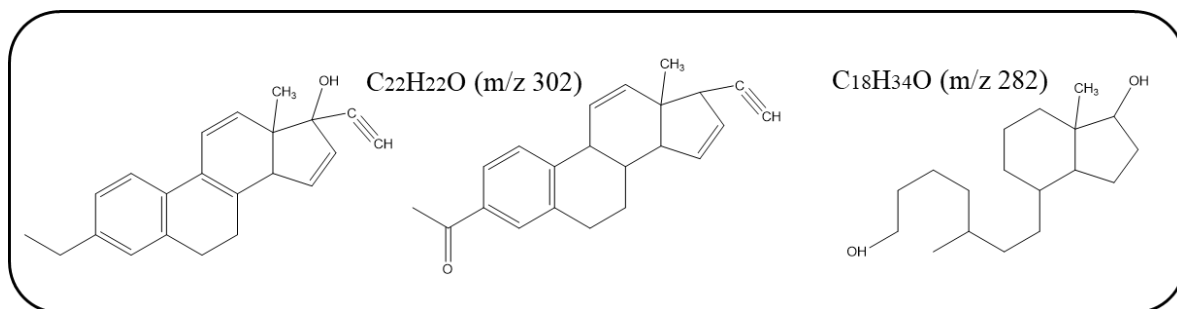


Fig. 2.24: Proposed EE2 transformation products by *P. citronellolis* SJTE-3 grown in minimal medium with EE2 and ethanol (Peng et al., 2023).

A study by Menashe et al. (2020) developed macro-encapsulation method for encapsulation of *Rhodococcus zopfi* and *Pseudomonas putida* F1 to degrade EE2 in a small bioreactor platform. The authors showed that biodegradation of EE2 in minimal medium with the encapsulated *R. zopfii* was better than *P. putida* when EE2 was added as a sole carbon source. A study by Bernardelli et al. (2019) studied estrogen degradation in a sequencing batch reactor using activated sludge and synthetic wastewater. Community structure analysis revealed that *Emticicia*, *Nubsella*, and *Shingobacterium* were key EE2 degraders.

Another study by Guo et al. (2021) studied the effect of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* MIG-N146 on the biodegradation of EE2 in shake flasks containing sediment/water system. The authors reported enhanced biodegradation of EE2 as the rhamnolipid concentration increased, which was attributed to better mass transfer, and stimulation of the indigenous microbial consortium. Three metabolic intermediates were detected including a dehydrogenation product of EE2 at C15-C16 of the D-ring, and a carboxylation product of EE2. This study did not report any data on the involved enzymes or microbial community.

2.6.5. Aerobic E3 degradation

Compared to E1, E2 and EE2, a limited number of studies addressed E3 biodegradation. For instance, Ke et al. (2007) isolated three bacteria from marine sand in microcosms using E1, E2, E3, and EE2 as sole carbon sources. The isolates were identified as members of the genera *Sphingomonas*, *Agromyces*, and *Acinetobacter*. These bacteria, individually or as a consortium, had different substrate spectrum in terms of estrogen degradation. The strain identified as *Acinetobacter* sp. degraded E3 in aerobic cultures and transiently produced 16 α -OH-E1. This compound was reported before as a metabolite of E2 degradation by sewage bacteria (Lee & Liu, 2002) and of E1 degradation by intestinal bacteria (Järvenpää et al., 1980). Moreover, *Staphylococcus aureus* can reduce 16 α -OH-E1 to E3 under anaerobic conditions (Järvenpää et al., 1980).

A study by Shi et al. (2004) showed that nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea* degraded E1, E2, E3 and EE2 in minimal medium. However, no further information was reported on the degradation mechanisms. In his study, Ueda et al. (2012) isolated and characterized a laccase from the mushroom *Pleurotus eryngii* and showed that it can remove E3 from aqueous solutions. A *Comamonas testosteroni* strain isolated from animal waste grew on E3 as a sole carbon and energy source (Liu et al., 2021). Nothing was reported in this study on the specific E3 degradation products, enzymes, or genes. However, genomic analysis revealed the presence of 22 steroid degradation genes. The authors also identified a testosterone degradation gene cluster and a gene encoding 17 β -hydroxysteroid dehydrogenase, which catalyzes the transformation of E2 to E1. In the same context, *Rhodococcus* sp. PI4, a marine isolate, utilized E2, E3, and testosterone as sole carbon sources. This strain contains a 17 β -

hydroxysteroid dehydrogenase whose expression was induced upon growth on E2, E3, or testosterone. This enzyme transforms E3 to 16 α -OH-E1 (Ye et al., 2019).

A *Gordonia* sp. strain R9 isolated from chicken leachates could utilize E3 and other steroid estrogens as sole carbon sources (Liu et al., 2020). Although no information on E3 degradation was reported, the authors detected E3 as a metabolite of E2. E3 was also reported as degradation product during E2 degradation by *Microbacterium resistens* MZT7 (Hao et al., 2022). The authors proposed that E2 is oxidized to E1 which is transformed to E3. However, the authors did not report the involved enzymes and genes, and it was not clear how E3 is further metabolized.

A study by Hsiao et al. (2021) reported that a *Rhodococcus* sp. strain B50 can grow on E3 as a sole carbon source. However, no further information on the mechanism of E3 degradation was reported. Wang et al. (2019c) isolated a *Pseudomonas putida* strain SJTE-1 from activated sludge and showed that it can degrade E1, E2, E3 and testosterone as sole carbon source. Nothing was reported in this study on the E3 degradation mechanisms.

2.6.6. Anaerobic degradation of estrogens

In contrast to the above presented knowledge on the aerobic estrogen degradation, biodegradation of estrogens under anaerobic conditions has been very rarely investigated. The low aqueous solubility (1.5 mg/L at room temperature) of estrogens and stability of the aromatic A-ring render estrogens challenging substrates for microbial utilization (Shareef et al., 2006; Wang et al., 2020a). Therefore, under aerobic conditions, bacteria depend on oxygenases and oxygen to activate and cleave the aromatic A-ring through the 4,5-seco pathway as discussed above (Chen et al., 2017; Chiang et al., 2020). Lack of oxygen in anaerobic environments, such as river and marine sediments, prohibits estrogen degradation

and makes the process slower compared to aerobic degradation. Hence, anaerobic environments are considered as major reservoirs for estrogens (Czajka & Londry, 2006; Hanselman et al., 2003). Only two bacterial species are known to degrade estrogens under anaerobic conditions, namely, *Denitratisoma oestradiolicum* and *Steroidobacter denitrificans* (Fahrbach et al., 2006, 2008).

To elucidate the biochemical mechanisms of anaerobic estrogen degradation, Wang et al. (2020a) isolated a denitrifying bacterium *Denitratisoma* sp. strain DHT3 from a municipal wastewater treatment plant and showed that it can efficiently degrade estrogens under denitrifying conditions. The authors sequenced the genome of this strain and performed comparative transcriptomics to identify potential genes involved in anaerobic estrogen degradation using E2- and testosterone-grown cultures. The authors found that genes involved in the anaerobic 2,3-*seco* pathway are expressed at similar levels in both cultures. On the contrary, genes of transport, salvage, and reductive activation of cobalamin were upregulated in the E2 culture. In addition, a gene cluster encoding a putative methyltransferase (*emtABCD*) was also upregulated in the E2 culture. Gene disruption experiments confirmed that *emtA* plays a role in anaerobic estrogen degradation by the DHT3 strain.

Interestingly, the *emtABCD* gene cluster is present in the genome of estrogen-degrading anaerobes, but lacking in steroid-degrading bacteria that can't utilize estrogens (Wang et al., 2020a). Moreover, metabolite analysis of E1-grown cultures of the DHT3 strain revealed several androgenic metabolites including two ring-cleavage products which are characteristic of the 2,3-*seco* pathway, i.e. 17 β -hydroxy-1-oxo-2,3-*seco*-androstan-3-oic acid (2,3-SAOA) and HIP (Figure 2.7). These findings suggested that anaerobic estrogen degradation in the DHT3 strain proceeds via conversion of the C₁₈ estrogens to C₁₉ androgens through the 2,3-*seco* pathway. The key reaction is the conversion of estrogens to androgens via a

methylation reaction catalyzed by a cobalamin-dependent methyltransferase (*emtABCD*) (Figure 2.25). These findings highlight microbe-host metabolic relationships mediated through the retroconversion of estrogens into androgens, based on the role of sex steroids in bidirectional metabolic interactions between bacteria and their eukaryotic hosts (Karavolos et al., 2013; Markle et al., 2013; vom Steeg & Klein, 2017). The conversion of estrogens into androgens represents the reverse process of estrogen (C_{18}) biosynthesis from androgens (C_{19}) via removal of the C_{19} angular methyl group, leading to formation of the aromatic A-ring (Miyairi & Fishman, 1985). Conversion of estrogens into androgens is thermodynamically challenging and has not been reported in any organism (Wang et al., 2020a).

2.7. Central pathway of bacterial steroid degradation

As mentioned above, HIP, a C_{13} metabolite containing the C/D rings of the steroid substrate, was commonly identified in all bacterial pathways for steroid catabolism (Figure 2.25). Hence it is degraded through a common central catabolic pathway which targets the C/D rings (Figure 2.26). After activation by a specific acyl-CoA synthetase (FadD3), the remaining carbons of the steroid B-ring are removed through β -oxidation. This is followed by hydrolytic cleavage the D-ring by an enoyl-CoA hydratase (EchA20), and another hydrolase (IpdAB) opens the C-ring. Interestingly, gene mining studies revealed the presence of conserved gene clusters for HIP degradation in the majority of experimentally verified steroid-degrading bacteria (Chiang et al., 2020).

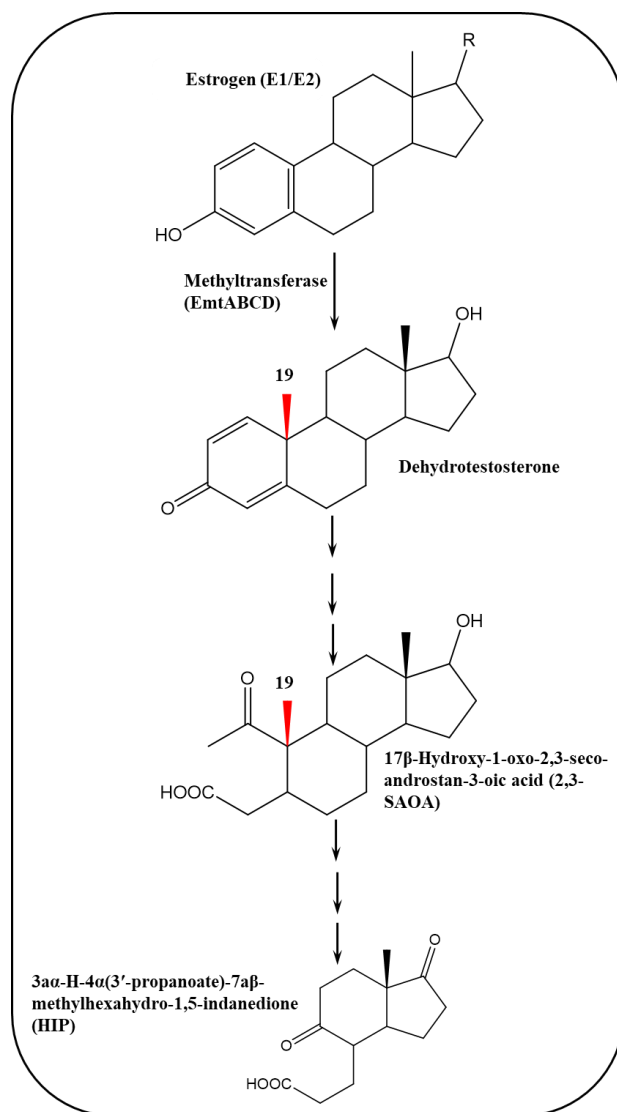


Fig. 2.25: Cobalamin-dependent retroconversion of estrogens into androgens during anaerobic estrogen degradation by *Denitratisoma* sp. strain DHT3 (Wang et al., 2020a).

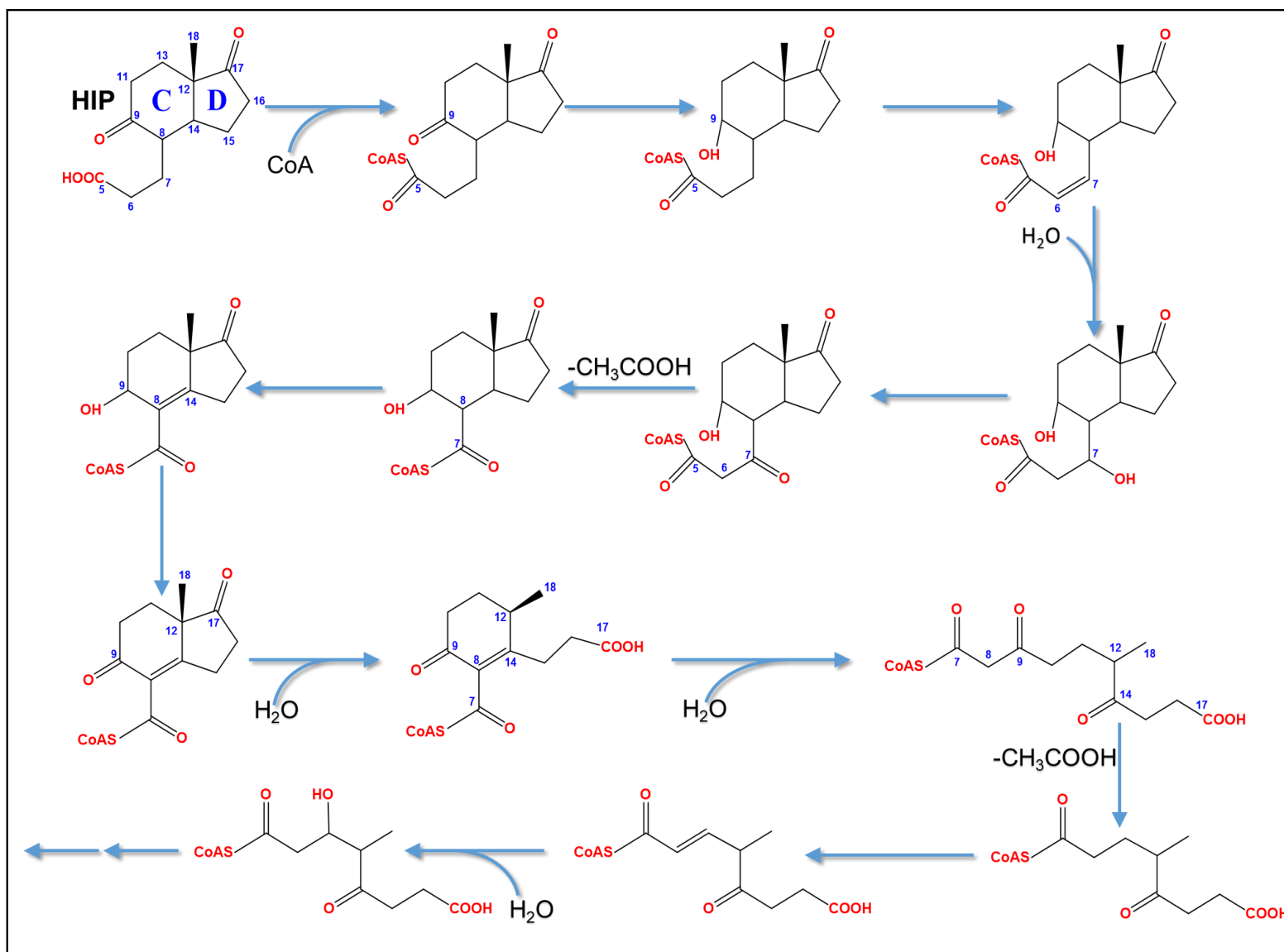


Fig. 2.26: The HIP degradation pathway (Chiang et al., 2020).

2.8. Concluding remarks and future research directions

Steroid estrogens are endocrine-disrupting chemicals that can be harmful to public health and the aquatic environment at extremely low concentrations. It has been recognized that effluents from WWTPs are major source of environmental pollution with estrogens. Hence, proper treatments of wastewater are instrumental to avoid environmental pollution with these hazardous compounds. Microbial biodegradation has been studied as a key process for elimination of estrogens from wastewater. Despite the very low aqueous solubility and relatively inert chemical structure, several bacteria are endowed with metabolic capabilities to utilize these challenging substrates as carbon and energy source for growth. Others can only perform specific biotransformations on estrogens. Estrogen-degrading bacteria have been isolated from engineered and natural environments such as activated sludge, soil, compost, and sandy aquifers, and the majority of them belong mainly to *Actinobacteria* and *Proteobacteria*. The majority of studies focused on the aerobic degradation of E1 and E2, and the involved enzymes, genes and pathways were investigated in some detail. On the contrary, EE2 was much less studied and even fewer studies are available for E3.

Despite the high structural similarity between steroid estrogens, diverse pathways for their degradation have been reported. A common feature of those pathways is that they start by modification of a certain ring of the estrogen substrate followed by cleavage of that ring. However, for most of the proposed degradation pathways or biotransformations, biochemical and molecular evidence at the level of the involved enzymes and genes is lacking. Exceptionally, only the 4,5-*seco* pathway for aerobic estrogen degradation has been thoroughly investigated. Interestingly, biochemical and molecular studies on the 4,5-*seco* pathway revealed some

differences between *Actinobacteria* and *Proteobacteria*, which can be exploited as functional biomarkers for environmental and ecological assessment of estrogen biodegradation. In contrast to the aerobic pathways, anaerobic estrogen degradation has been rarely studied, and key mechanisms have been only recently elucidated, which indicates that anaerobic estrogen degradation proceeds through the 2,3-*seco* pathway after a unique initial retro conversion of estrogens into androgens.

Regardless of oxygen availability, both the aerobic and anaerobic pathways converge at a common central metabolite of steroid catabolism by bacteria; namely, HIP. The latter is then degraded through a common central metabolic pathway. One of the characteristic features of estrogen biodegradation is interconversion of one compound to another as shown in Figure 2.27. This feature might have some implications on the environmental fate of estrogens due to changes in their concentrations and potential substrate preference differences among the involved microbial communities, particularly in the presence of estrogen mixtures.

It was clear from the literature review that E3 and EE2 need further studies to unravel their degradation mechanisms and the involved microbial communities. Furthermore, future research should focus more on microbial consortia as they are prevailing in natural and engineered ecosystems. In addition, more work is needed to elucidate estrogen biodegradation process in environmental matrices to gain more realistic and conclusive data for in-situ applications.

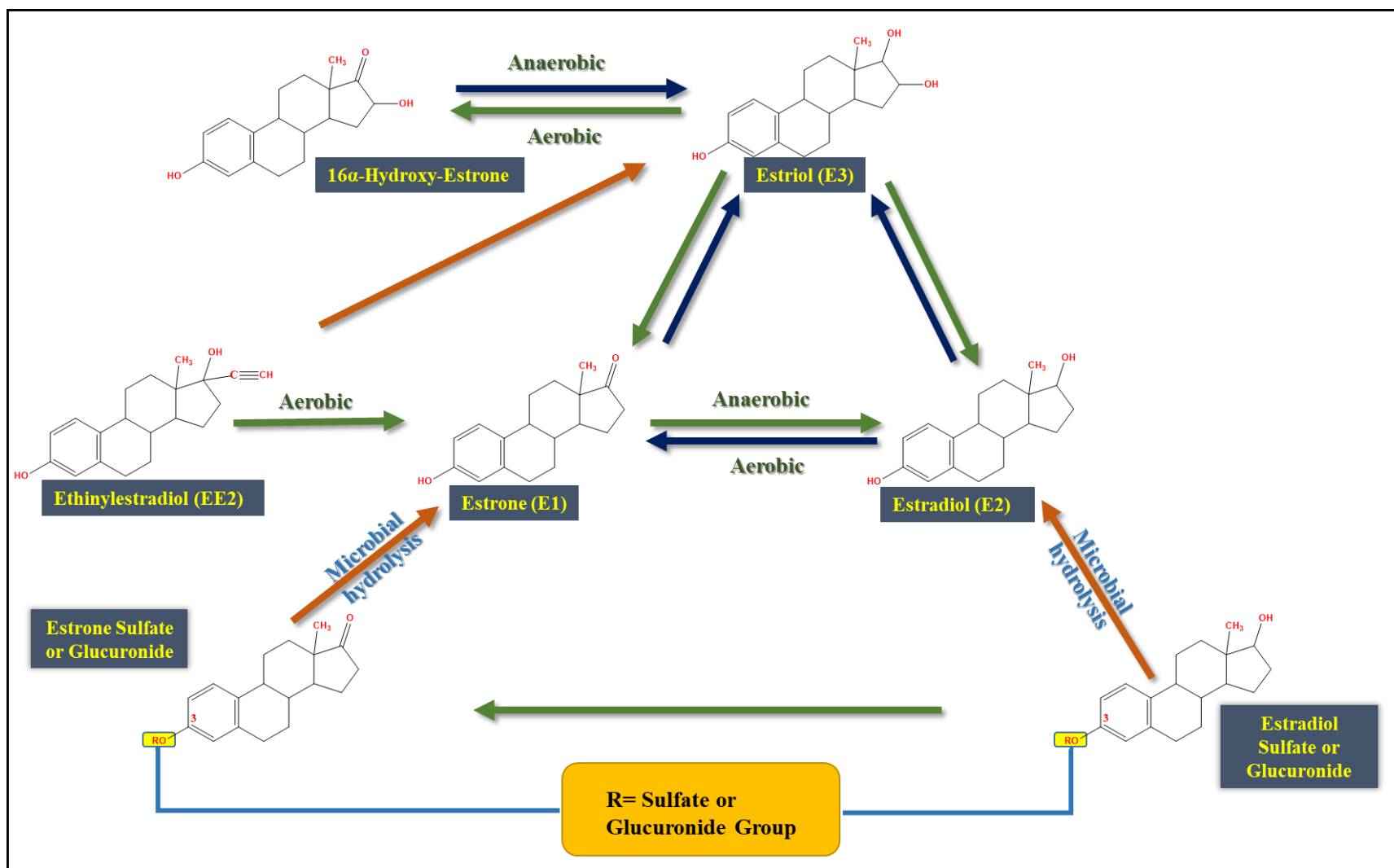


Fig. 2.27: Interconversions between different steroid estrogens.

3. Materials and Methods

3.1. Chemicals, reagents, and kits

Chemicals, biochemical, and microbiological media were from Fluka (Switzerland) Sigma Aldrich (USA), and Difco (France). Luria Bertani (LB) agar and LB broth media were from Difco-France and prepared in distilled water as per the manufacturer's instructions. The steroidal estrogens E3 (>98% purity), E1 (>98% purity), E2 (>98% purity), and EE2 (>98% purity), and the organic solvent dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich-USA. Stock solutions of E1, E2, E3 and EE2 were prepared as 100 mM in DMSO and stored at room temperature, wrapped with aluminum foil to protect from light. Ethyl acetate of GC-MS grade was purchased from Thermo Fisher Scientific (USA). Total genomic DNA was isolated using the ab288102-Bacterial Genomic DNA isolation kit (Abcam-UK) and DNeasy UltraClean Microbial kit (Qiagen-Germany). Primers were obtained from Biolegio (Nijmegen-Netherlands). All other molecular biology reagents and kits were from Qiagen (Germany).

3.2. Chemically defined medium

A chemically defined medium (CDM) was used to enrich and isolate estrogen-degrading bacteria from inflow, activated sludge, and thickened sludge samples. The CDM was prepared in deionized water and supplemented with steroid estrogen (1 mM or 0.1 mM), glucose (10 mM), glycerol, or DMSO as a carbon source, as the experiment deemed. The CDM consists of a basal buffer (water, ammonium chloride, and phosphate buffer) which is complemented with vitamins, trace elements, iron, CaCl_2 , and MgSO_4 (Table 3.1). The overall composition of CDM per liter of deionized water was: KH_2PO_4 , 1.08 g; K_2HPO_4 , 5.6 g; NH_4Cl , 0.54 g;

CaCl₂.2H₂O, 0.044 g; FeCl₂.4H₂O, 1.5 mg; vitamins (cyanocobalamine 0.2 mg, pyridoxine-HCl 0.6 mg, thiamin-HCl 0.4 mg, nicotinic acid 0.4 mg, *p*-aminobenzoate 0.32 mg, biotin 0.04 mg, Ca-pantothenate 0.4 mg) and trace elements (ZnCl₂.7H₂O 70 µg, MnCl₂.4H₂O 100 µg, CuCl₂ 20 µg, CoCl₂.6H₂O 200 µg, Na₂MoO₄.2H₂O 40 µg, NiCl₂.6H₂O 20 µg, H₃BO₃ 20 µg). Glucose, trace elements, and vitamins stock solutions were sterilized by filtration using 0.22 µm sterile filters (Stericups, Nalgene). The basal buffer, which consists of phosphate buffer, NH₄Cl, and water, was autoclaved, and the other components were added from sterile stock solutions. Liquid cultures were routinely prepared in 250 mL Erlenmeyer baffled flasks containing 100 mL of CDM. All cultures were incubated at 30°C in an orbital shaker at 180 rpm.

Table (3.1): Preparation of chemically defined medium from stock solutions.

Element	Stock solution concentration	Final Concentration	Volume added (mL) per L of medium
Phosphate Buffer			
KH ₂ PO ₄	21.8 g/L	1.08 g/L	50 mL
K ₂ HPO ₄	112 g/L	5.6 g/L	
NH ₄ Cl	54 g/L	0.54 g/L	10 mL
MgSO ₄ .7H ₂ O	24.647 g/L	0.1 g/L	10 mL
CaCl ₂ .2H ₂ O	44.08 g/L	0.044 g/L	1 mL
FeCl ₂ .4H ₂ O*	1.5 g/L	1.5 mg/L	1 mL
Trace Elements			
ZnCl ₂ .7H ₂ O	70 mg/L	70 µg/L	

Table (3.1): Continued

MnCl ₂ .4H ₂ O	100 mg/L	100 µg/L	1 mL
CuCl ₂	20 mg/L	20 µg/L	
CoCl ₂ .6H ₂ O	200 mg/L	200 µg/L	
Na ₂ MoO ₄ .2H ₂ O	40 mg/L	40 µg/L	
NiCl ₂ .6H ₂ O	20 mg/L	20 µg/L	
H ₃ BO ₃	20 mg/L	20 µg/L	
Vitamins			
Cyanocobalamin	0.2 g/L	0.2 mg/L	1 mL
Pyridoxamine-HCl	0.6 g/L	0.6 mg/L	
Thiamin-HCl	0.4 g/L	0.4 mg/L	
Nicotinic acid	0.4 g/L	0.4 mg/L	
<i>p</i> -aminobenzoate	0.32 g/L	0.32 mg/L	
Biotin	0.04 g/L	0.04 mg/L	
Ca-pantothenate	0.4 g/L	0.4 mg/L	
*FeCl ₂ .4H ₂ O was prepared by dissolving 0.15 g of FeCl ₂ .4H ₂ O in 5 mL of HCl (25%) + 95 mL deionized water and sterilized by filtration			

3.3. Instrumentation

The equipment used in this study and their applications are listed in Table (3.2).

Table (3.2): Equipment used in the study.

Equipment	Application
Sorvall Lynx 6000 Centrifuge–Thermo Scientific	Harvest of biomass
Orion Aquamate 8000–UV/VIS Spectrophotometer–ThermoScientific	Measurement of optical density at 600 nm
Class IIA/B3 Biological Safety Cabinet	Culturing and sample collection
Orbital shaker set at 30°C with 180 RPM	Incubation of liquid cultures
Veriti 96 Well Thermal Cycler–A.B. Applied Biosystems	PCR
Vacuum Concentrator–SpeedVac	Evaporation of organic solvents to concentrate extracts
Fluorimeter (DeNovix) Qubit 4 Fluorometer (Thermo Fischer Scientific)	Quantification of nucleic acids
Nanodrop 2000/2000C–Thermo Scientific	Quantification of nucleic acids
Ultraperformance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS)-Thermo Fischer Scientific	Analysis of estrogen degradation intermediates
Gas Chromatography system (GC 2010 Plus)-Mass Spectrometer (MS-QP2020)- Shimadzu	Analysis of estrogen degradation

3.4. Description of the study area

By the year 1984, the newly constructed Tubli wastewater treatment plant (WWTP) was operated. It is the largest wastewater treatment plant in the Kingdom of Bahrain, located east of Manama, the capital of Bahrain (Figure 3.1) and receives municipal wastewater from Manama, Muharaq, Isa Town, Hamad Town, Bani-Jamrah, and Budaiya. Tubli WWTP is a treated sewage effluent production facility that aims to get rid of sewage and produce high-quality water suitable for the agricultural irrigation systems and to minimize the impact of the sewage on the environment. The plant capacity covers a population of 700,000 through a daily flow of 200,000-350,000 m³/day.

The sewage is pumped to Tubli WWTP from five central pumping stations, where it enters the inlet for processing. The principal sewage treatment processes consist of primary, secondary, and tertiary phases. The primary treatment starts with hydraulically screening the sewage for large object disposal and grit removal, and then the sewage is discharged into four circulation channels. The tanker waste facility consists of a vector waste-collecting pit, screening, detraction, oil exclusion, preliminary aeration, and removal of odorous gases. After the primary treatment, the sewage passes to the aeration tanks, where the essential secondary treatment process occurs. The sewage enters the aeration tanks, which are long and deep, consisting of three mixing chambers and two oxygenation chambers.



Fig. 3.1: The geographical location of the Tubli wastewater treatment plant (Google Earth, 2024).

The effluent enters the aeration tanks (Figure. 3.2a), where the sewage is aerated with a mixture of microorganisms (activated sludge) that use the organic impurities as a nutrient source, while carbon dioxide gas is the main by-product. The sewage and activated sludge mixture are kept aerated and mixed by mechanical surface aerators at a low speed, maintaining the mixing of the tank contents and causing oxygen to be absorbed. It takes 12-15 hours for the sewage to flow through the aeration tanks, where all impurities are removed, leaving water with microorganisms.

In the tertiary phase, the water and microorganisms are separated in the final clarifiers (settling tanks) (Figure. 3.2b). The flowing mixture's speed is reduced to a low level so that the microorganisms (activated sludge) are settled at the bottom of the tanks and removed.

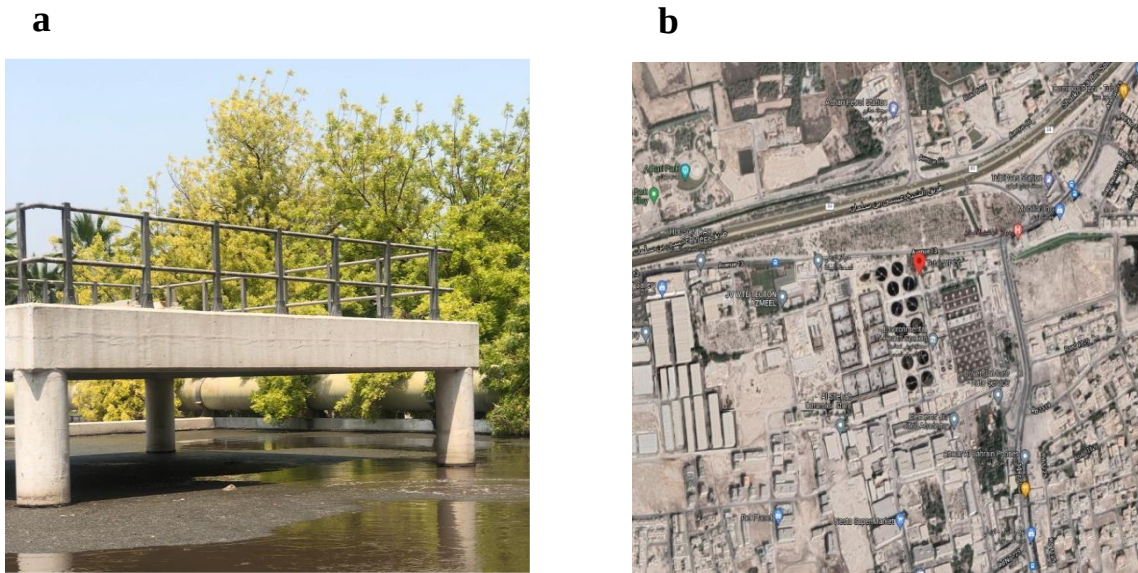


Fig. 3.2: Photos of Tubli wastewater treatment plant. (a) an aeration tank, (b) showing a top view of the plant and the clarifiers.

The removed sludge floods through pipes to the sludge pumping station and is recycled into the aeration tanks. A portion of the surplus sludge passes through a flow meter into four gravity-thickening tanks, thickening the sludge from 0.5% to 3% solid content. The thickened sludge is removed and pumped into drying concrete beds for air drying, stored in the site, or further processed with a particular chemical and spun in a high-speed centrifuge to produce a harmless dry solid sludge recycled for use in government farms as a soil conditioner.

On the other hand, the clear treated effluent is either discharged from the clarifiers to Tubli Bay or further treated with ozone to kill bacteria and other pathogens, inactivate viruses, and filtered. Then, after adding a small amount of chlorine to maintain sterility, the product is pumped into transmission pipes to reservoirs to supply water for irrigation projects.

3.5. Collection of samples from the WWTPs

Three types of wastewater samples were collected: inflow sample from the primary phase (screening phase), activated sludge from the secondary phase (aeration tank 2) and thickened sludge sample from the tertiary phase settling tanks. In addition, similar samples were obtained from another WWTP, Sitra WWTP. The samples (0.5 L) were collected in sterile 1 L glass bottles, labelled with the location, date, and time of collection and transported to the laboratory within 30 minutes at an atmospheric temperature. The thickened and activated sludge samples were processed immediately to inoculate enrichment cultures, while the inflow samples were kept at 4 °C (for 2 days) for later processing.

3.6. Collection of soil samples

Soil samples (dry and wet) from the Arabian Gulf University (AGU) garden were collected in sterile containers from the upper part (10-20 cm) of the soil's surface and then transported to the laboratory. Upon arrival, 10 grams of each sample were weighed and added to a CDM flask containing 0.5 mM EE2 dissolved in DMSO.

3.7. Enrichment and isolation of EE2-degrading bacteria

Activated sludge samples collected from Tubli and Sitra WWTPs were used as inocula to isolate EE2-degrading bacteria. Flasks containing 100 mL CDM were prepared for the initial enrichments and were supplemented with EE2 (dissolved in DMSO) at a concentration of 1 mM as a carbon and energy source and each was inoculated with 2 mL of activated sludge. Another flask was inoculated with 2 mL autoclaved activated sludge and spiked with 1 mM EE2 (the abiotic control).

Further enrichments were also tested with 0.5 g/L EE2 only (without DMSO) to rule out the effect of the estrogen solvent on bacterial growth. These enrichments were similarly inoculated with 2 mL activated sludge. All the cultures were incubated at 30°C with 180 rpm shaking for 7 days. Throughout the incubation period, the cultures were inspected visually daily for bacterial growth. After 7 days of incubation, all cultures were analyzed chemically via extraction and gas chromatography-mass spectrometry (GC-MS) analysis as described later.

To isolate EE2-degrading bacteria from microcosms of activated sludge from the Tubli and Sitra wastewater treatment plants, 50 mL of each sample was spiked with 1 mM EE2 as a carbon and energy source. Abiotic controls were prepared by autoclaving the activated sludge samples before spiking with EE2. The cultures and the controls were incubated for 23 days then extracted with ethyl acetate and analyzed by GC-MS.

Furthermore, different bacterial strains from the AGU culture collection were screened for EE2 degradation. Each strain was incubated overnight in 50 mL Luria-Bertani (LB) broth medium, and the next day centrifuged to get the pellet. The pellet was then washed and resuspended in 0.1 M phosphate buffer, and 5 mL of the cell suspension was used as an inoculum for 100 mL of CDM containing 0.5 mM EE2 as a carbon and energy source. Culture samples were extracted after 15 days of incubation with ethyl acetate and analyzed by GC-MS.

To isolate EE2-degrading bacteria from the collected soil samples, 10 g from each sample were used to inoculate 100 mL of CDM containing 0.5 mM EE2. Uninoculated medium was included as abiotic controls. Culture samples were extracted after 15 days of incubation with ethyl acetate and analyzed by GC-MS.

3.8. Enrichment and isolation of E3-degrading bacteria

To enrich E3-degrading bacteria, a 10 mL inoculum of fresh samples of each inflow, thickened sludge, and activated sludge were added individually to 250 mL Erlenmeyer flasks containing 100 mL CDM and supplemented with 1 mM E3 (dissolved in DMSO) as a source of carbon. All cultures were incubated for 7 days at 30 °C in an orbital shaker at 180 rpm. From initial enrichment cultures that showed growth (visually assessed by change in turbidity and or color), 5 mL were then transferred to new CDM cultures containing 1 mM E3 and incubated under the same conditions for three days. This subculturing step was repeated three to five consecutive times. Uninoculated CDM supplemented with E3 was included at all steps and incubated under the same conditions to serve as abiotic and sterility controls. At the end of each enrichment culturing, samples from each enrichment were extracted and analyzed by GC-MS (described later in detail) to verify that E3 biodegradation phenotype was still retained after repeated subculturing.

Samples (1 mL) from the initial enrichment and each subculture therefrom were mixed with 1 mL sterile 50% glycerol and kept at -80°C. At this stage, those frozen stocks were considered to have bacterial consortia and were retrieved later for phenotype confirmation.

From the third subculture, 100 µL were taken and serially diluted in sterile normal saline to isolate axenic bacterial cultures. Aliquots of 100 µL from the different dilutions were spread on LB agar plates and incubated for two days at 30 °C. At the end of the incubation period, single morphologically different colonies

were selected and purified by repeated three consecutive streaking on LB agar plates to obtain pure isolates. All enrichments and isolated bacteria were then tested for growth on and degradation of E3 as described later.

3.9. Confirmation of growth and E3 biodegradation by the enriched consortia

Frozen stocks of the enriched bacterial consortia were thawed on ice for 15 min and, after gentle mixing, 500 μ L of each were used to inoculate 100 mL CDM containing 0.1 mM E3. All cultures and abiotic controls were incubated at 30 °C with shaking (180 rpm) and the optical densities were measured every 24 h. Furthermore, culture samples were retrieved for GC-MS analysis after various time intervals (30 h, 54 h, 7 days).

3.10. Growth of the isolated bacteria on E3

To amplify the inoculum of the selected isolates (39 isolate), a single colony from each was picked from the respective LB agar plates, inoculated in 20 mL LB broth medium and kept in the incubator for 1-3 days under the same incubation conditions. Once growth was established, the whole culture volume was poured into 50 mL sterile centrifuge tubes and the biomass was harvested by centrifugation for 10 min at 4 °C and 6000 rpm. The cell pellet was resuspended in 10 mL complemented CDM (without the E3), and the entire cell suspension was inoculated into CDM containing 1 mM E3. All cultures and abiotic controls were incubated and checked visually at time intervals. Furthermore, culture samples were extracted and analyzed by GC-MS at various time points.

Following this initial screening of the isolates, 5 strains that showed E3 degradation potential were selected for further investigations. These strains originated from different sources: strains TH-11, TH-16, and TH-30 were isolated from the thickened sludge, whereas strain IF-20 from the inflow wastewater, and strain AS-31 from activated sludge. Those strains were cultured in CDM containing 0.1 mM E3 following the above-mentioned procedures. Growth was monitored by measuring the OD at 600 nm (OD_{600}) every 24 h over 2 weeks, and culture samples were retrieved at time intervals to be analyzed by GC-MS as described later.

To test whether the selected 5 isolates can grow on E3 without adding the solvent DMSO, the same procedures were followed as described above to prepare inoculum which was used to inoculate CDM containing 50 mg/L E3 as solid crystals. Abiotic controls were included throughout. Growth was monitored by measuring the OD at 600 nm (OD_{600}) every 24 h and culture samples were retrieved at time intervals to be analyzed by GC-MS as described later.

3.11. Growth of the isolated bacteria on DMSO

To test whether the selected isolates can utilize DMSO, the used solvent of E3, as a sole carbon source, they were initially grown in LB broth to prepare inoculum as described earlier. The isolates were then inoculated in 100 mL CDM containing 1 mL of autoclaved DMSO as a sole carbon source, and all cultures and abiotic controls were incubated under the same conditions. Growth was monitored visually and by measuring the OD at 600 nm (OD_{600}) every 24 h.

3.12. Identification of the isolates

Single colonies of the different isolates were picked from fresh LB-agar plates and suspended in 20 μ L of sterile nuclease-free water in an Eppendorf tube. A 2 μ L aliquot of the resulting cell suspension was then utilized in colony PCR for 16S rRNA gene amplification. The 16S rRNA gene was amplified from the genomic DNA of the strains using the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-GGTTACCTTGTTACGACTT-3'). The PCR was run for six samples (5 strains plus the negative control) containing a master mix that has been customized as per Table (3.3).

Table (3.3): Reaction setup using Taq PCR master mix.

Component	Volume
Taq PCR Master Mix (2x)	50 μ L
10x primers (2 μ M of each primer)	10 μ L each
DNase-free water	28 μ L
Template DNA	2 μ L
Total reaction volume	100 μ L

The PCR cycles were performed as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1.5 min., and final elongation at 72 °C for 10 min. Samples from the PCR tubes were analyzed by electrophoresis at 80 volts for 40 min on 1% agarose gels. After verification, the target PCR products were purified using the protocol and instructions of the QIAquick PCR purification kit (Qiagen, Germany). DNA quantification was conducted using Nanodrop spectrophotometer and a Qubit

4 Fluorometer. Finally, the purified DNA was stored at -20 °C for later sequencing and phylogenetic analyses to identify the bacterial strains.

3.13. Growth of the E3-degrading consortia on glycerol

To verify that growth observed in the E3 cultures of the enriched bacterial consortia was solely due to E3 utilization, it was important to check whether these consortia can utilize glycerol (existing in the frozen stocks) as a carbon source for growth. This experiment commenced by thawing one tube of each frozen stock for the three isolated consortia, from the inflow, thickened sludge, and activated sludge samples, on ice for 15 min. After gentle mixing, 500 µL of each thawed mixed culture was inoculated into its respective flask containing 100 mL of CDM. The CDM was complete but no other carbon sources were added. All cultures and abiotic controls were incubated under the same conditions and growth was monitored visually and by measuring the OD₆₀₀ at various time points.

3.14. Preparation of precultures and the inoculum

Precultures and inoculum of bacterial consortia were routinely prepared from glycerol stocks frozen at -80°C. Initially, and as described above (3.8), the frozen stocks were thawed on ice, mixed gently, and then directly inoculated into CDM. However, after discovering that the bacterial consortia can utilize glycerol (found in the frozen stocks) as a carbon source, the procedure was modified. A frozen 2 mL vial was retrieved from -80°C and thawed on ice for 15 min. Following gentle pipetting to ensure thorough mixing, the content of the vial was centrifuged at 10,000 rpm for 5 min in a pre-cooled centrifuge at 4°C. The supernatant was discarded, and the resulting pellet was washed once with 1 mL of ice-cold 0.1 M phosphate buffer (pH=7), followed by centrifugation and decantation of the supernatant. This

procedure serves to eliminate glycerol which can be used as a carbon source by the enriched bacterial consortia. The washed pellet was resuspended in 1 mL of the same buffer, and the cell suspension was divided into 0.5 mL portions to inoculate two flasks of 100 mL CDM containing 0.1 mM E3 each. These cultures and abiotic controls were then incubated at 30°C with shaking at 180 rpm for 5 to 6 days.

After measuring the OD₆₀₀, the biomass was harvested in 250 mL sterile centrifuge tubes at 10,000 rpm for 10 min at 4°C. After discarding the supernatant, the cell pellet was washed once with 25 mL of ice-cold 0.1 M phosphate buffer, transferred to a sterile 80 mL centrifuge tube, centrifuged again and resuspended in 30 mL of the same buffer to yield a homogeneous cell suspension. This cell suspension was used as an inoculum and a portion was retained to measure OD₆₀₀ and dry weight. The inoculum size was standardized to start with a biomass load of ~ 200 mg /L (dry cell weight) of culture medium.

3.15. Growth profile of the E3-degrading consortia

The growth profile of each isolated consortium (inflow, thickened sludge, and activated sludge) on E3 dissolved in DMSO was studied in 100 mL CDM containing 0.1 mM E3 under the same conditions (at 30 °C with shaking at 180 rpm). The precultures were prepared as described under (3.13) after eliminating glycerol from the frozen stocks. The incubation times of the preculture of the inflow, thickened sludge, and activated sludge consortia were 5 to 6 days. From each preculture, 1 mL was used to inoculate the cultures which were incubated and monitored for growth at time intervals by measuring the OD₆₀₀ until they reached the stationary phase, and culture samples were analyzed by GC-MS. All cultures were run in biological triplicates.

3.16. Substrate spectrum of the inflow-consortium

The E3-degrading bacterial consortium obtained from the Tubli WWTP inflow wastewater samples was subjected to further investigations. First, the substrate spectrum of this consortium (hereafter referred to as “IF-consortium”) was studied in CDM containing other estrogens like E1, E2, or EE2 (0.1 mM each), individually and as a mixture with E3 (one mixture contained E1, E2, and E3, another mixture contained E1, E2, E3, and EE2). In addition, growth of this consortium was tested on glucose (10 mM) and DMSO (1%, solvent of the steroid estrogens). The preculture and inoculum were prepared in CDM containing 0.1 mM E3 as described (3.13). All cultures were grown in 100 mL CDM supplemented with the different substrates, inoculated with 1 mL of the inoculum cell suspension, and together with the abiotic controls were incubated under the same conditions. Growth was monitored by measuring the OD₆₀₀, and samples from the estrogen cultures were retrieved at time intervals and analyzed by GC-MS to monitor estrogen biodegradation.

3.17. Degradation metabolites of E3 and the inflow-consortium community structure profiling

A large-scale biodegradation experiment was conducted to elucidate the E3 degradation pathway and investigate temporal and substrate-associated compositional shifts in the structure of the IF-consortium to unravel key members involved in E3 degradation. The preculture and inoculum were prepared as described above, with a little modification where E3 concentration was 1 mM. The inoculum was suspended in 20 mL ice-cold phosphate buffer (pH 7) and 1 mL of this cell suspension was used to inoculate the different cultures.

The IF-consortium was cultured in 400 mL CDM (in 1 L Erlenmeyer baffled flasks) containing various carbon sources. Two cultures contained E1 or E3 at 1 mM final concentration. Another culture contained 10 mM glucose and 1% DMSO. Glucose and E1 cultures were included to facilitate the identification of key E3-degrading members of the IF-consortium via comparative analysis of the temporal and substrate-dependent shifts in the community structure. All cultures were in biological triplicates and were incubated together with the abiotic controls under shaking (180 rpm) at 30 °C for 14 days. Culture samples (20 mL) were retrieved at day zero (after 2 h incubation) and then every 48 h. From the collected 20 mL, 4 mL aliquots of each were transferred into 15 mL sterile tubes and kept at -80 °C for DNA isolation and processing later, whereas the remaining 16 mL were kept at -20 °C for extraction of the E3 degradation metabolites and chemical analysis.

3.17.1. Identification of E3 degradation metabolites by UPLC-ESI-HRMS

The frozen samples were thawed at 37 °C for one and a half h and mixed gently by inverting the tube 3-5 times. Then 10 mL of each sample were mixed with 20 µL of EE2 stock solution in DMSO (as an internal standard) to a final concentration of 50 µM. The mixture was then transferred to a separating funnel and extracted with 10 mL of GC-MS grade ethyl acetate. The organic phase, along with the interphase, were pooled and evaporated in a rotary evaporator to reduce the volume to approximately 0.5 mL, followed by a complete dryness in a vacuum concentrator at 34°C for 75 min, and lastly stored at -20 °C. A list of the samples prepared for chemical analysis is shown in Table (3.4).

Table (3.4): Description of the samples retrieved from the growth of the inflow consortium on the estriol (E3).

No.	Sample Code	Description
<u>Day 0</u>		
1	E3-0a	E3, replicate a
2	E3-0b	E3, replicate b
3	E3-0c	E3, replicate c
4	CE3-0a	E3, abiotic control, replicate a
5	CE3-0b	E3, abiotic control, replicate b
<u>Day 2</u>		
6	E3-2a	E3, replicate a
7	E3-2b	E3, replicate b
8	E3-2c	E3, replicate c
9	CE3-2a	E3, abiotic control, replicate a
10	CE3-2b	E3, abiotic control, replicate b
<u>Day 4</u>		
11	E3-4a	E3, replicate a
12	E3-4b	E3, replicate b
13	E3-4c	E3, replicate c
14	CE3-4a	E3, abiotic control, replicate a
15	CE3-4b	E3, abiotic control, replicate b
<u>Day 6</u>		
16	E3-6a	E3, replicate a
17	E3-6b	E3, replicate b
18	E3-6c	E3, replicate c
19	CE3-6a	E3, abiotic control, replicate a
20	CE3-6b	E3, abiotic control, replicate b
<u>Day 8</u>		
21	E3-8a	E3, replicate a
22	E3-8b	E3, replicate b
23	E3-8c	E3, replicate c
24	CE3-8a	E3, abiotic control, replicate a
25	CE3-8b	E3, abiotic control, replicate b

<u>Day 10</u>		
26	E3-10a	E3, replicate a
27	E3-10b	E3, replicate b
28	E3-10c	E3, replicate c
29	CE3-10a	E3, abiotic control, replicate a
30	CE3-10b	E3, abiotic control, replicate b
<u>Day 12</u>		
31	E3-12a	E3, replicate a
32	E3-12b	E3, replicate b
33	E3-12c	E3, replicate c
34	CE3-12a	E3, abiotic control, replicate a
35	CE3-12b	E3, abiotic control, replicate b
<u>Day 14</u>		
36	E3-14a	E3, replicate a
37	E3-14b	E3, replicate b
38	E3-14c	E3, replicate c
39	CE3-14a	E3, abiotic control, replicate a
40	CE3-14b	E3, abiotic control, replicate b

All samples were analyzed through UPLC-ESI-HRMS on a UPLC system connected to an ESI mass spectrometer. Separation was achieved on a reversed-phase C18 column (Acquity UPLC® BEH C18, 1.7 μ m, 100 x 2.1 mm; Waters) with a 0.4 mL/min flow rate at a 35 °C column oven temperature. The mobile phase comprised a mixture of two solvents: solvent A (2% vol/vol acetonitrile containing 0.1% vol/vol formic acid) and solvent B (methanol containing 0.1% vol/vol formic acid). Separation was achieved using a gradient of solvent B from 10% to 90% in 8 min. ESI-MS analysis was performed using a Thermo Fisher Scientific™ Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer (Waltham, MA, USA). Mass spectral data were collected in positive ESI mode in separate runs on a Thermo Fisher Scientific Orbitrap Elite Hybrid Ion Trap–Orbitrap Mass Spectrometer

(Waltham, MA, USA) that was operated in scan mode at 50–500 *m/z*. The source voltage was set at 3.2 kV; the capillary and source heater temperatures were 360 °C and 350 °C, respectively; and the sheath, auxiliary, and sweep gas flow rates were 30, 15, and 2 arbitrary units, respectively. The predicted elemental composition of individual intermediates was calculated using Xcalibur™ Software Mass Spectrometry Software (Thermo Fisher Scientific, Waltham, MA, USA). Structural elucidation was performed as described (Chen et al., 2017; 2018; Wu et al., 2019).

3.17.2. Isolation of total genomic DNA

Seventy-two frozen samples were collected from the IF-consortium cultures grown on E1, E3, and glucose at different time points (every 48 hrs. in triplicates, 8 time points) and stored at -80 °C. The samples were thawed on ice and centrifuged for 10 min at 4 °C, 6000 rpm. The supernatants were removed, and the pellets were used to isolate the total genomic DNA following the procedure described in the ab288102-Bacterial Genomic DNA Isolation kit (Abcam, UK). The isolated DNA was visualized by gel electrophoresis and quantified using the NanoDrop spectrophotometer and a Qubit4 fluorometer.

3.17.3. Dynamics of the bacterial community structure

The isolated DNA was sequenced at the BIOTOOLS company (www.toolsbiotech.com, Taiwan) via the PacBio sequencing platform. 16S rRNA amplicon sequencing was carried out as outlined below. DNA concentrations were determined by Qubit 4.0 Fluorometer (Thermo Scientific) and were adjusted to 1 ng/μL. Two ng of DNA were used for amplification of full-length 16S rRNA genes (V1-V9 regions) using the barcoded 16S rRNA gene-specific primers and KAPA HiFi HotStart ReadyMix (Roche), following the manufacturer's instructions. The PCR conditions included a hold at 95°C for 3 min, 30 cycles of 95°C for 30 sec,

57°C for 30 sec, 72°C for 60 sec, second hold at 72°C for 5 min followed by storage at 4°C. The PCR products were analysed by 1% agarose gel electrophoresis. Samples with strong amplicon of ~1,500 bp were purified using the AMPure PB Beads.

The purified PCR products were pooled in equimolar concentrations and up to 1 mg was used for end-repairing/A-tailing and ligation to universal hairpin adapters, followed by purification with AMPure PB beads to remove the adapter dimer. The library was incubated with Sequel II primer 3.1 and sequel II Binding Kit 3.1 for the primer annealing and polymerase binding. Sequencing was performed in the circular consensus sequence (CCS) mode on a PacBio Sequel IIe instrument to generate the HiFi reads with Predicted Accuracy (Phred Scale) of 30.

The circular consensus sequence (CCS) reads were determined with a minimum predicted accuracy of 0.9 and the minimum number of passes set to 3 using the PacBio SMRT Link software. Only CCS reads with a quality score greater than Q30, referred to as Q30 HiFi reads, were proceed to the next stage. After demultiplexing, the HiFi reads were analysed by DADA2 version 1.20 for quality filtering, dereplication, application of the dataset-specific error model, inference of amplicon sequence variants (ASVs) and chimera removal (Callahan et al., 2016; Quin et al., 2018). The reads were trimmed and filtered with a maximum of two expected errors per read (maxEE = 2). DADA2 algorithm resolves ASVs with single-nucleotide resolution from the full-length 16S rRNA gene with very high accuracy. The number of reads after quality control varied between 9123 – 20045 (Table 3.5) that were used for further analyse.

Table (3.5): Sequencing data quality control and noise reduction*.

Sample Name	Raw HiFi-Read (#)	Filtered Read (#)	Denoised Read (#)	Non-chimera Read (#)
E1.10a	12318	11335	11312	11312
E1.10b	13064	12153	12092	12092
E1.12a	15535	14484	14463	14463
E1.14a	21536	20081	20045	20045
E1.14c	16800	15371	15295	15295
E3.10a	14375	13033	12932	12910
E3.10b	19927	18238	18191	18191
E3.10c	14972	13548	13494	13494
E3.12a	13168	11962	11890	11890
E3.12b	10420	9387	9333	9333
E3.12c	14097	12749	12703	12703
E3.14a	15903	14348	14303	14303
E3.14b	13290	11873	11795	11790
E3.14c	12720	11609	11545	11542
E3.4a	11670	10616	10534	10505
E3.4b	10273	9221	9126	9123
E3.4c	12428	11172	11063	11022
E3.6a	12114	10706	10583	10552
E3.6b	15131	13667	13532	13493
E3.6c	16640	15028	14934	14934

*Raw HiFi is the original number of sequences; Filtered read refers to the number of sequences that passed the quality control parameters; Denoised read refers to the number of sequences DADA2 denoising; and non-chimera read refers to the number of sequences after removing chimeras.

Taxonomic classifications for representative sequences were obtained from the NCBI 16S ribosomal RNA database using feature-classifier (Bokulich et al., 2018) and classify-consensus-vsearch (Rognes et al., 2016) algorithms in QIIME2 v2022.11 (Bolyen et al., 2019). The sequence similarities among ASVs were analysed using alignment tool MAFFT (Katoh & Standley, 2013) within QIIME2 workflow. A phylogenetic tree was constructed for representative ASVs using the FastTree algorithm (Price et al., 2009, 2010). QIIME package was used for

calculating α and β diversities and R package ggplot2 was used to generate the plots. Bray-Curtis dissimilarity matrices were generated using microeco package in R. To visualize patterns in multidimensional data, dimensionality reduction methods NMDS (Noval Rivas et al., 2013) were used.

For statistical analysis, significance of all species among groups at various taxonomic levels was detected using differential abundance analysis with a zero-inflated Gaussian (ZIG) log-normal model as implemented in the “fitFeatureModel” function of the R Bioconductor metagenomeSeq package (Paulson et al., 2013). The Welch’s t-test was performed using the stat package in R. Statistically significant biomarkers were identified using the LEfSe analysis (Segata et al., 2011). In brief, LEfSe is an approach based on an algorithm that performs the non-parametric Kruskal-Wallis test and Wilcoxon rank-sum test to identify bacterial taxa whose relative abundance is significantly different between the control and tests. LEfSe applies LDA to those bacterial taxa identified as significantly different and further assesses the effect size of each differentially abundant taxon. In this study, taxa with LDA score ($\log_{10}$) > 2 were considered significant.

For functional prediction analysis, 16S rRNA gene sequencing data were analyzed with FAPROTAX (v1.2.6). These tools predicted functional abundances with different reference databases. FAPROTAX is a manually constructed database based on the literature on cultured representatives. To find the important functional profiles across groups, R package ggplot2 and microeco were used to generate the plots and metastat statistics.

3.18. Estrogen biodegradation in wastewater microcosms

In this experiment, biodegradability of estrogens was studied in wastewater microcosms containing either activated sludge (AS) or inflow (IF) wastewater samples and spiked with varying estrogen concentrations. Freshly collected samples from Tubli WWTP were transferred to the laboratory within 30 min at an ambient atmospheric temperature, where they were processed immediately as soon as they reached the laboratory. For each type of wastewater sample, three microcosms were prepared in 2 L sterile Erlenmeyer flasks each containing 500 mL of either AS or IF samples. The three microcosms were: 1) A microcosm spiked with E1, E2 and E3 (0.1 mM each), designated as “high-concentration (HC) microcosm”, 2) A microcosm spiked with E1, E2 and E3 (0.01 mM each), designated as “low-concentration (LC) microcosm”, and 3) A microcosm spiked with E1, E2 and E3 (0.1 mM each) after autoclaving the wastewater sample designated as “abiotic control (AC) microcosm”. The experimental design aimed to investigate estrogen biodegradation by the endogenous microbiome of the wastewater samples at two different concentrations.

Another microcosm experiment was designed to investigate estrogen biodegradation in the wastewater samples after bioaugmentation with the IF-consortium which was isolated and investigated in this study. The general design of the microcosms was identical to that described above and for each wastewater type three microcosms were prepared: 1) A microcosm spiked with E1, E2 and E3 (0.1 mM each) and inoculated with the IF-consortium, designated as “augmented high-concentration (AHC) microcosm”, 2) An autoclaved microcosm spiked with E1, E2 and E3 (0.1 mM each) and inoculated with the IF-consortium, designated as “autoclaved augmented high-concentration (AAHC) microcosm” and 3) An autoclaved microcosm spiked with E1, E2 and E3 (0.01 mM each) inoculated with

the IF-consortium, designated as “autoclaved augmented low-concentration (AALC) microcosm. All microcosms were incubated at 30°C in an orbital shaker (180 rpm) for 2 h, after which samples (20 mL) were collected for GC-MS analysis (day 0). The microcosms were further incubated for two weeks and samples were retrieved every 48 h for GC-MS analysis.

To prepare the inflow-consortium for the bioaugmentation microcosms, a preculture was prepared in 100 mL CDM containing 0.1 mM E3 as described above. After 5 days of incubation, this preculture was used to inoculate (10%) two 1-L baffled Erlenmeyer flasks each containing 400 mL CDM with 0.1 mM E3. After incubation for 5 days, the biomass was harvested from the whole culture by centrifugation at 10,000 rpm for 10 min at 4°C (Sorvall Lynx 12000 centrifuge) washed with 50 mL of 0.1 M ice-cold phosphate buffer and resuspended in 50 mL of 0.1 M phosphate buffer. This cell suspension was used to inoculate the microcosms with 5 mL, and the OD₆₀₀ and cell dry weight were also determined.

A microcosm experiment was conducted to study the biodegradation of EE2 by indigenous bacteria present in the inflow wastewater and activated sludge samples from the Tubli WWTP. Four sterile 1L baffled Erlenmeyer flasks were set up with two flasks designated for the inflow microcosm (culture and control) and two for the activated sludge microcosm (culture and control). Each flask was supplied with 250 mL of freshly collected samples from the respective sources. The control flasks were autoclaved to eliminate indigenous microorganisms and then cooled to room temperature. Both the culture and control flasks were spiked with 0.1 mM of EE2 and incubated at 30 °C in an orbital shaker at 180 rpm. Aliquots were collected after 2 h of incubation and subsequently at 48-h intervals, up to day 14. Samples were extracted and analyzed using GC-MS.

3.19. Stability of the E3-degrading consortium

Precultures of the IF-consortium were prepared as described. Then 1 mL was inoculated in 100 mL CDM containing 0.1 or 1% autoclaved DMSO. The cultures and controls were incubated at 30°C in an orbital shaker at 180 rpm, and OD₆₀₀ was recorded at 24 h intervals for 8 days to monitor growth. To assess the stability of the IF-consortium after its growth on DMSO, aliquots (1 mL) from those DMSO cultures were retrieved after 3 days and reinoculated in 100 mL CDM containing 0.1 mM E3 (duplicate cultures). All cultures and controls were incubated under the same conditions and growth was monitored by measuring the (OD₆₀₀) after 2 h and then at 24-h intervals for 8 days. Additionally, GC-MS analyses were conducted on samples collected on days 4, 5, and 12 of incubation.

3.20. Analysis of estrogen biodegradation by GC-MS

To investigate estrogen degradation by the isolated bacteria, mixed cultures (consortia), and wastewater microcosms, culture samples were analysed by Gas Chromatography-Mass Spectrometry (GC-MS) at various time intervals. Routinely, samples (10 or 25 mL) were retrieved from the respective cultures/microcosms and extracted by equal volume of GC-grade ethyl acetate in a separating funnel. After mixing for 5 minutes, the mixture was allowed to settle till the two phases are separated. The aqueous phase was discarded, scarifying the interphase, while the organic phase was collected and concentrated to 1 mL in a rotary evaporator and analyzed by a Shimadzu gas chromatography system (GC 2010 plus) coupled to a Shimadzu MS–QP2020 mass detector, using a column Rxi–5SILms (30 m, 0.25 mm id, 0.4 µm df, Restek, USA). The oven temperature program was set to 1 min at 50°C with increments of 20°C/min up to 200°C where the oven was held at this temperature for 3 min, followed by 6°C/min up to 280°C, and finally the temperature increased to 290°C with a rate of 30°C/min and held for 12 min. The GC was run in

the split mode (split ratio 16.0) with a flow of 1 mL/min of helium as a carrier gas. The MS conditions were set to have the ion source temperature at 250°C and the interface temperature at 280°C. The scan interval was 0.3 sec, scan speed was 2000 and the mass range was 50–600 m/z. The collected mass spectra were matched with the NIST spectral database.

3.21. Sanger sequencing and phylogenetic analysis

Sanger sequencing was performed at DNA Sequencing Core Facility of the Institute of Biomedical Sciences, Academia Sinica. Briefly, 16S rRNA gene templates amplified with 27F/1492R primers were fluorescence labeled with BigDye™ Terminator v3.1 Cycle Sequencing kit (4337457, Applied Biosystems™) then purified with BigDye XTerminator™ Purification kit (4376484, Applied Biosystems™). The fluorescence-labeled DNA was detected with ABI 3730XL DNA ANALYZER and further analyzed with Sequencing Analysis Software v5.3.1 (4440845, Applied Biosystems™). The sequencing results were examined and pairwise-aligned for generating 16S rRNA gene amplicons with Geneious prime Bioinformatics Software for Sequence Data Analysis. Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999). The consensus sequences were manually adjusted in accordance with the 16S rRNA secondary structure model (Winker & Woese, 1991). Phylogenetic trees were constructed based on almost complete 16S rRNA gene sequences (>1300 bp). Sequences used in the phylogenetic analysis were obtained from GenBank database (Benson et al., 1999). Positions of sequences and alignment ambiguity were omitted, and pairwise evolutionary distances were calculated using the method of Jukes and Cantor (1969). A dendrogram was constructed using the neighbor-joining method (Saitou & Nei, 1987) with the Molecular Evolutionary Genetic Analysis (MEGA) program version

6 (Tamura et al., 2013). Confidence in the tree topology was determined using 100-bootstrapped trees (Felsenstein, 1985).

3.22. Measurement of biomass dry weight

Three empty Eppendorf tubes were weighed, and the weights were recorded. The cell suspension was mixed, and 1 mL was transferred to each Eppendorf tube. The tubes were centrifuged at 14,000 rpm for 5 minutes, and the supernatants were discarded. The tubes were placed in a hot air oven at 100°C overnight (24 h) to ensure their dryness. The next day, the Eppendorf tubes were reweighed, and the differences between the pre-dryness and post-dryness measurements showed the dry weight of the cell suspension and multiplied by 1000 to get the weight as gram per liter.

4- Results

4.1. Analysis of authentic estrogen samples

Stock solutions of authentic estrogen samples were prepared in DMSO to be used for the enrichment culturing and subsequent biodegradation experiments. As GC-MS was the main analytical tool, aliquots of the stock solutions were analyzed to have baseline data on their retention times and mass spectra, individually and as a mixture. As shown in Figures 4.1- 4.5, the various estrogens appeared as single peaks at different retention times based on their molecular masses. However, EE2 appeared earlier than E3 although it has a higher molecular mass (E3: 288.4 versus EE2: 296.41). The mass spectra of the different estrogens matched the corresponding mass spectra from the NIST library.

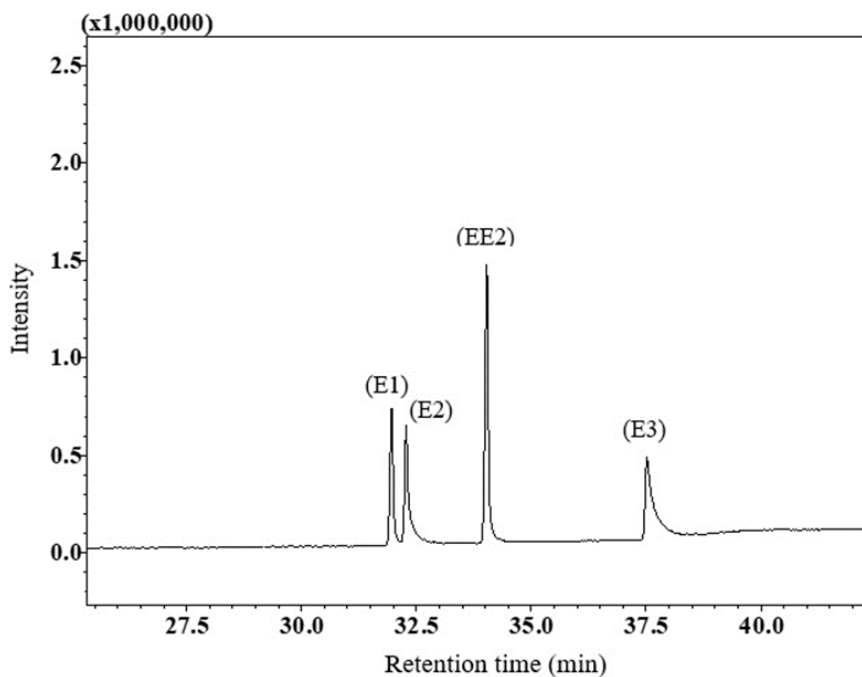


Fig. 4.1: Total ion chromatogram of a mixture of estrogens from standard stock solutions in DMSO (1mM each).

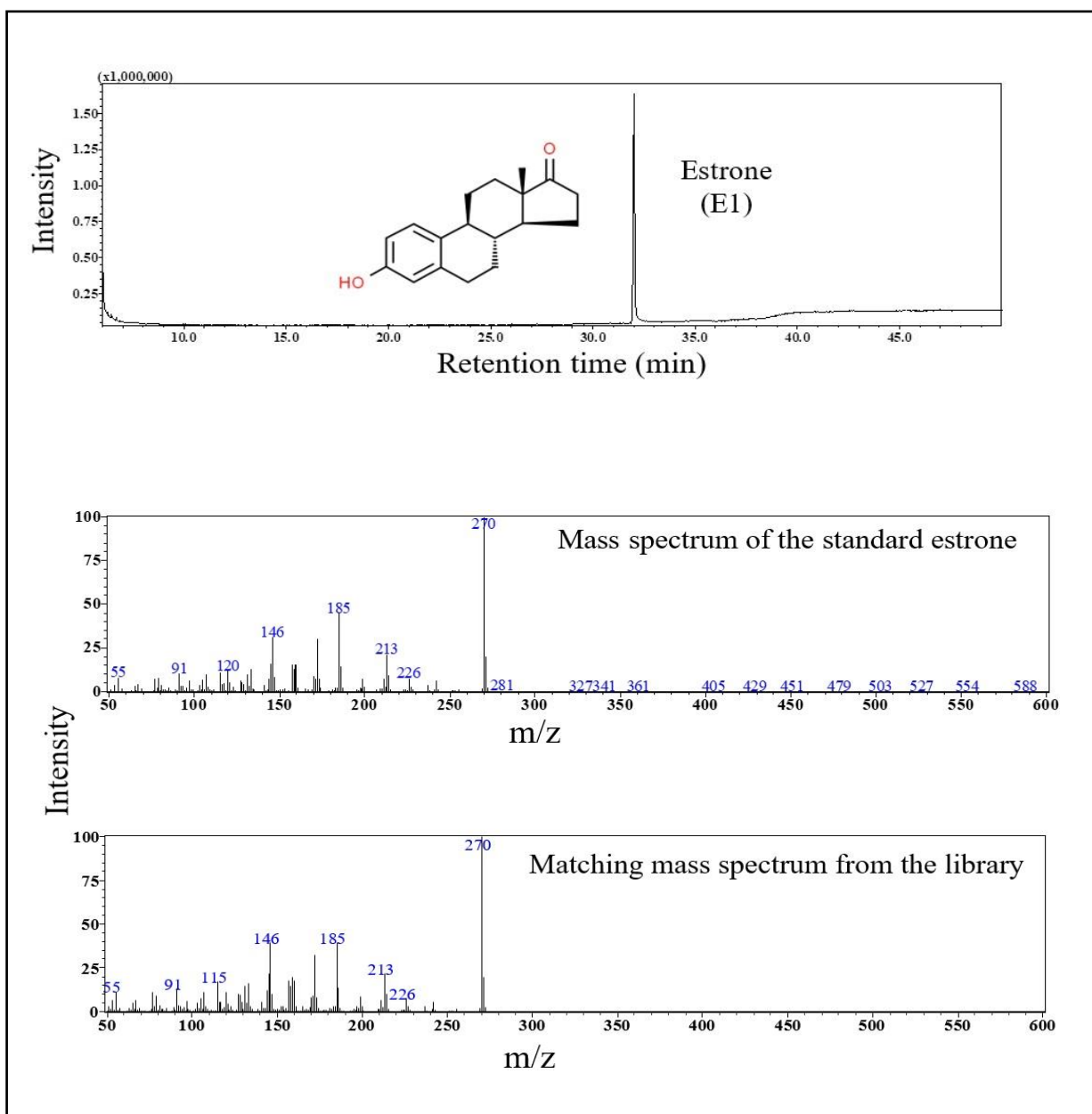


Fig. 4.2: Total ion chromatogram and mass spectrum of a standard sample of estrone (1 mM).

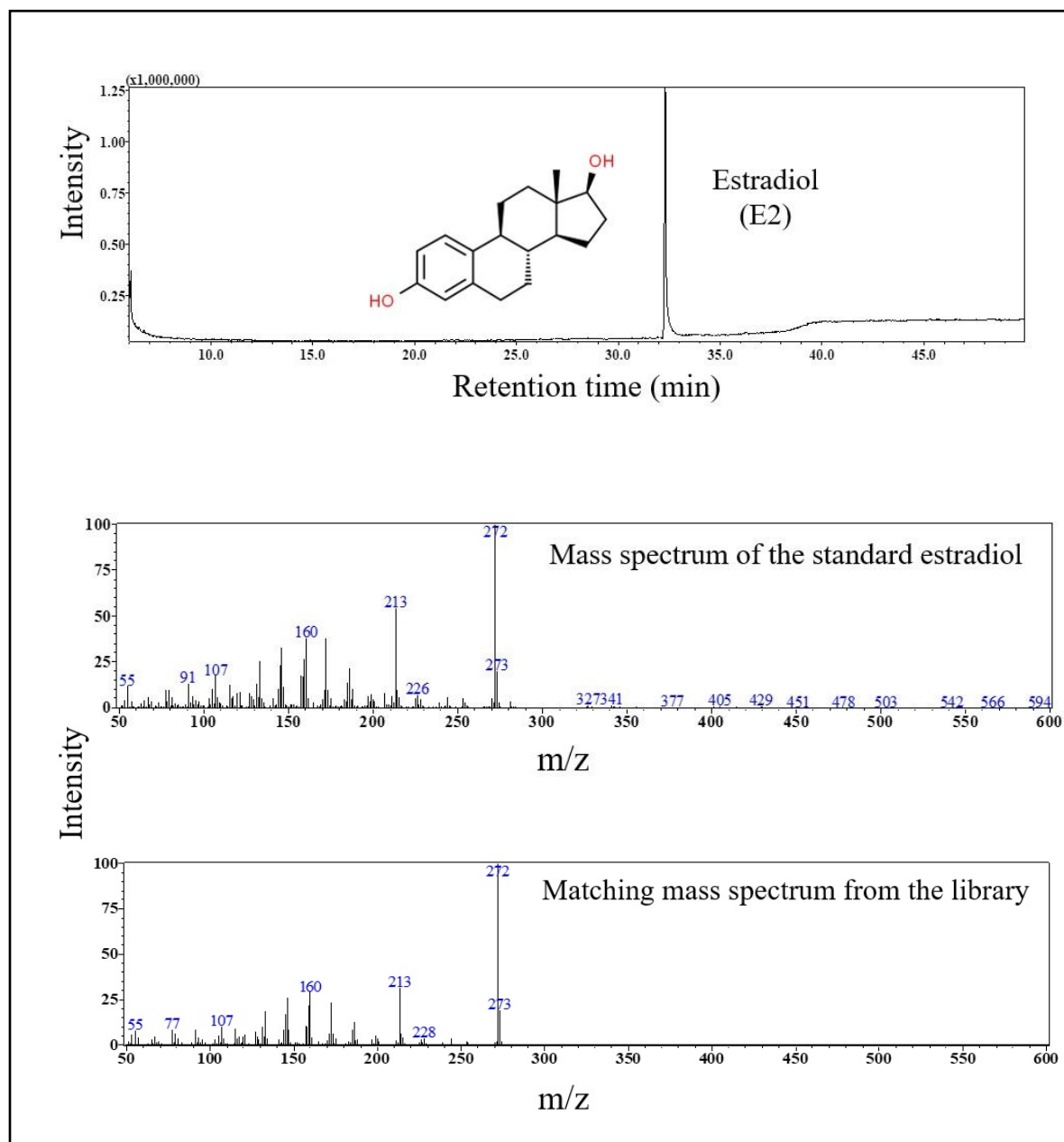


Fig. 4.3: Total ion chromatogram and mass spectrum of a standard sample of estradiol (1 mM).

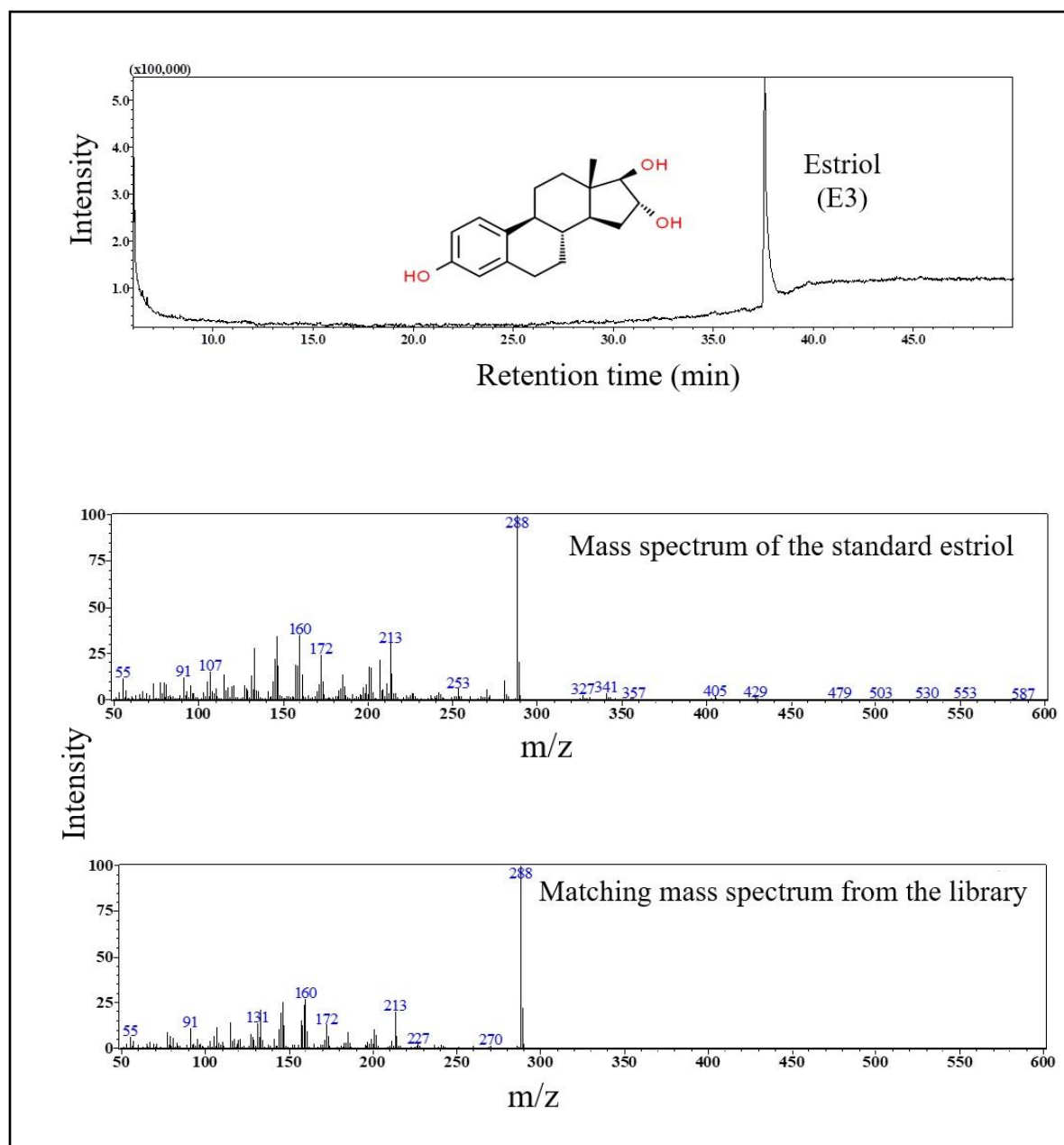


Fig. 4.4: Total ion chromatogram and mass spectrum of a standard sample of estriol (1 mM).

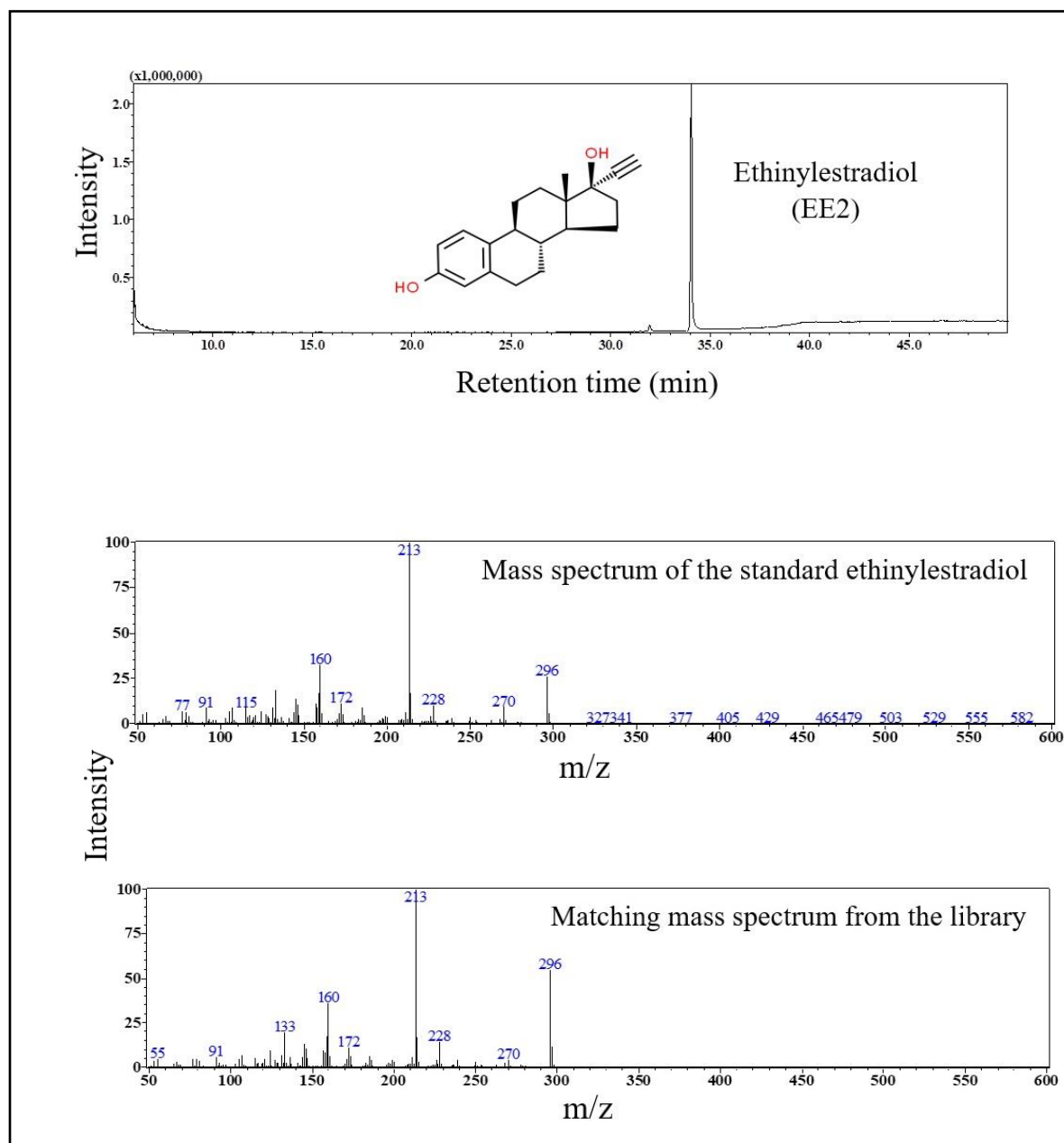


Fig. 4.5: Total ion chromatogram and mass spectrum of a standard sample of ethinylestradiol (1 mM).

4.2. Enrichment of EE2-degrading bacteria

Activated sludge samples were collected from two WWTPs and used as inocula in enrichment cultures supplemented with EE2 either from a DMSO stock or as solid crystals (no solvent). As shown in Figures 4.6- 4.8, EE2 was not degraded in any of those enrichments. This agrees with the lack of change in the turbidity of the enrichments with time, thus indicating no growth. Instead of using CDM for the enrichment culturing, other enrichments were performed directly in activated sludge microcosms by spiking them with 1mM EE2. However, even this approach failed to enrich EE2-degrading bacteria after three weeks of incubation (Figure 4.9). Several bacterial strains from AGU culture collection at the Environmental Biotechnology Laboratories were screened for EE2 biodegradation in CDM. However, none of the strains could grow on EE2 and GC-MS analysis revealed no remarkable decrease in EE2 after 15 days of incubation (Figure 4.10). A last attempt to isolate EE2-degrading bacteria was done by using garden soil as an inoculum in CDM containing EE2. As shown in Figure 4.11, EE2 was not utilized after 15 days of enrichment culturing.

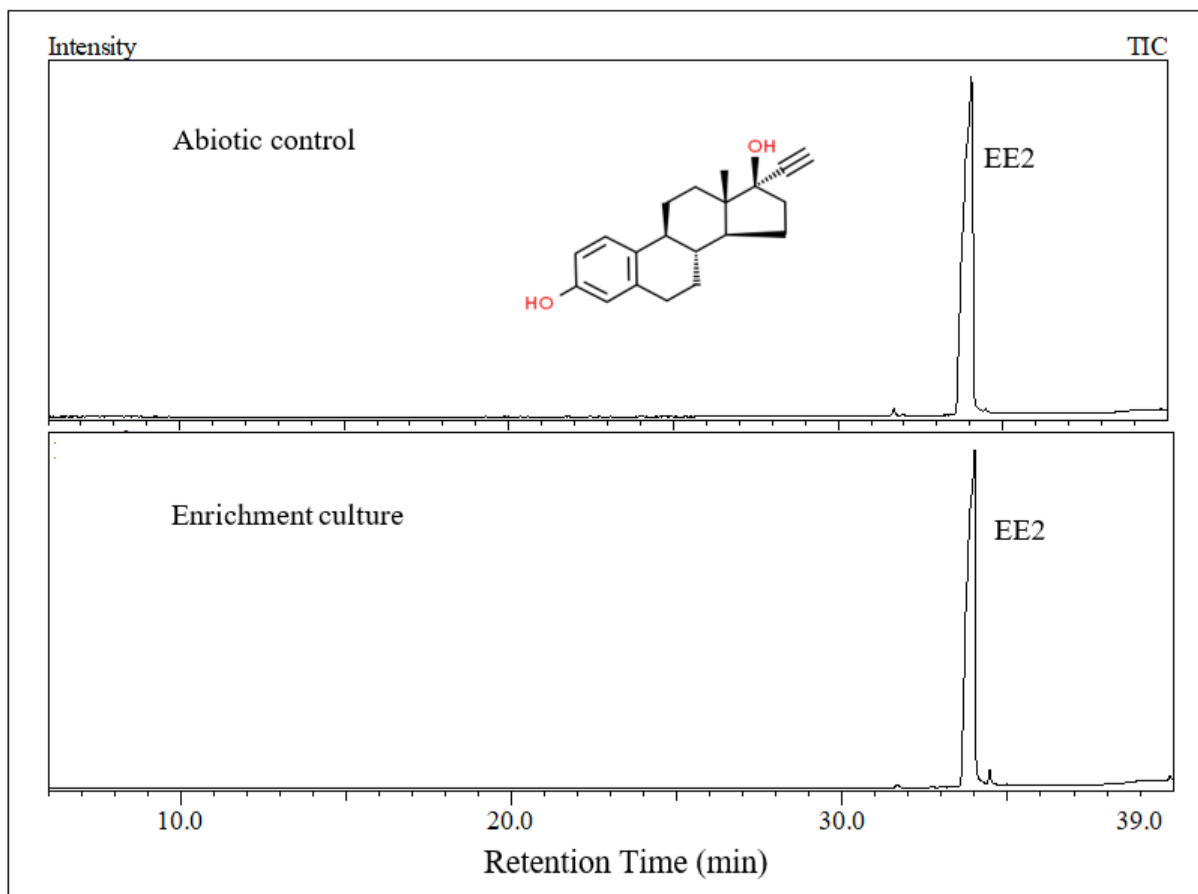


Fig. 4.6: GC-MS analysis showing total ion chromatograms of the abiotic control and enrichment culture containing 1 mM EE2 and inoculated with activated sludge from Tubli wastewater treatment plant after seven days of incubation. EE2 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

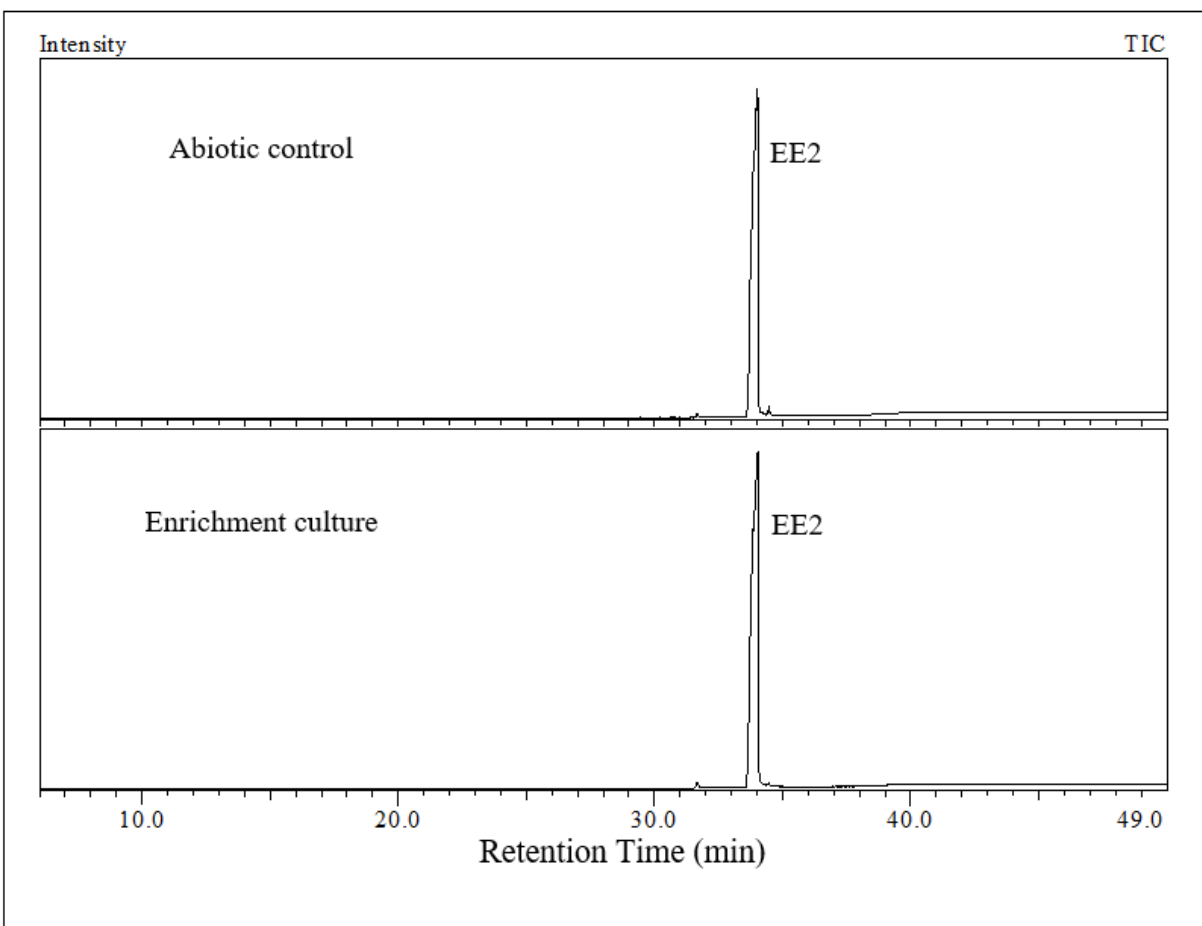


Fig. 4.7: GC-MS analysis showing total ion chromatograms of the abiotic control and enrichment culture containing 1 mM EE2 and inoculated with activated sludge from Sitra wastewater treatment plant after 16 days of incubation. EE2 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

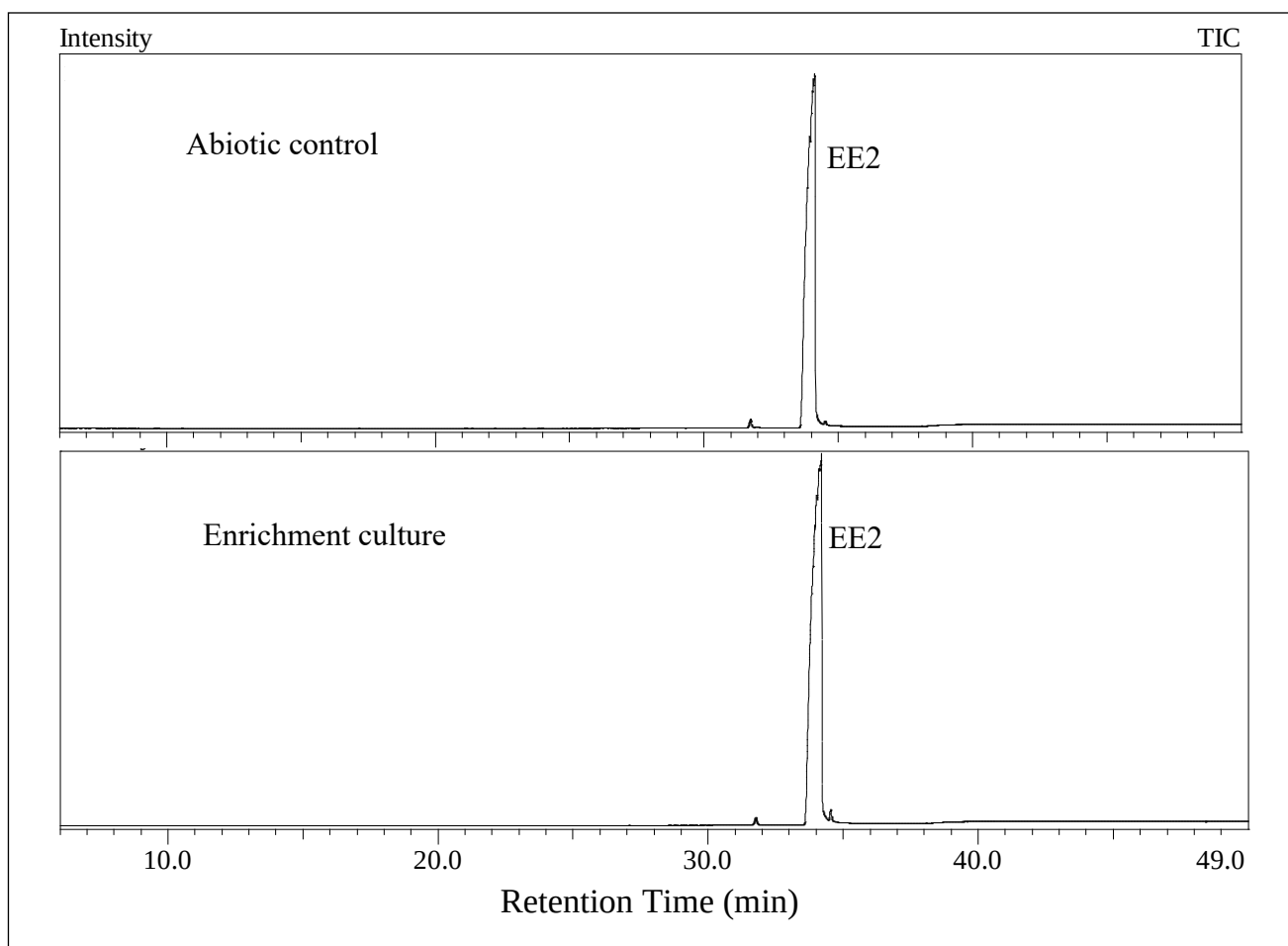


Fig. 4.8: GC-MS analysis showing total ion chromatograms of the abiotic control and enrichment culture containing 50 mg EE2 and inoculated with activated sludge from the Sitra wastewater treatment plant after 14 days of incubation. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

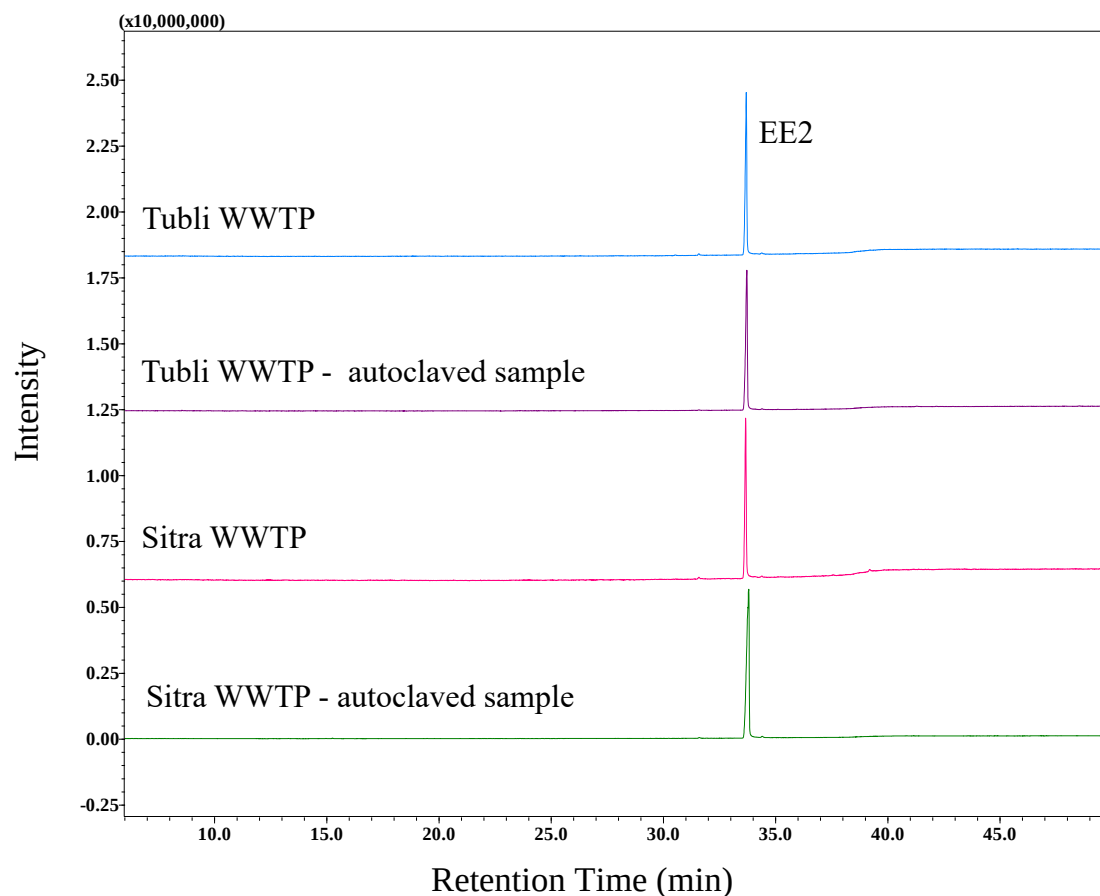


Fig. 4.9: GC-MS analysis showing total ion chromatograms of the abiotic controls and enrichment cultures containing 1 mM EE2 in 50 mL activated sludge from Tubli or Sitra wastewater treatment plants after 23 days of incubation. EE2 was added from a stock solution in DMSO. All microcosms were incubated at 30 °C in an orbital shaker (180 rpm).

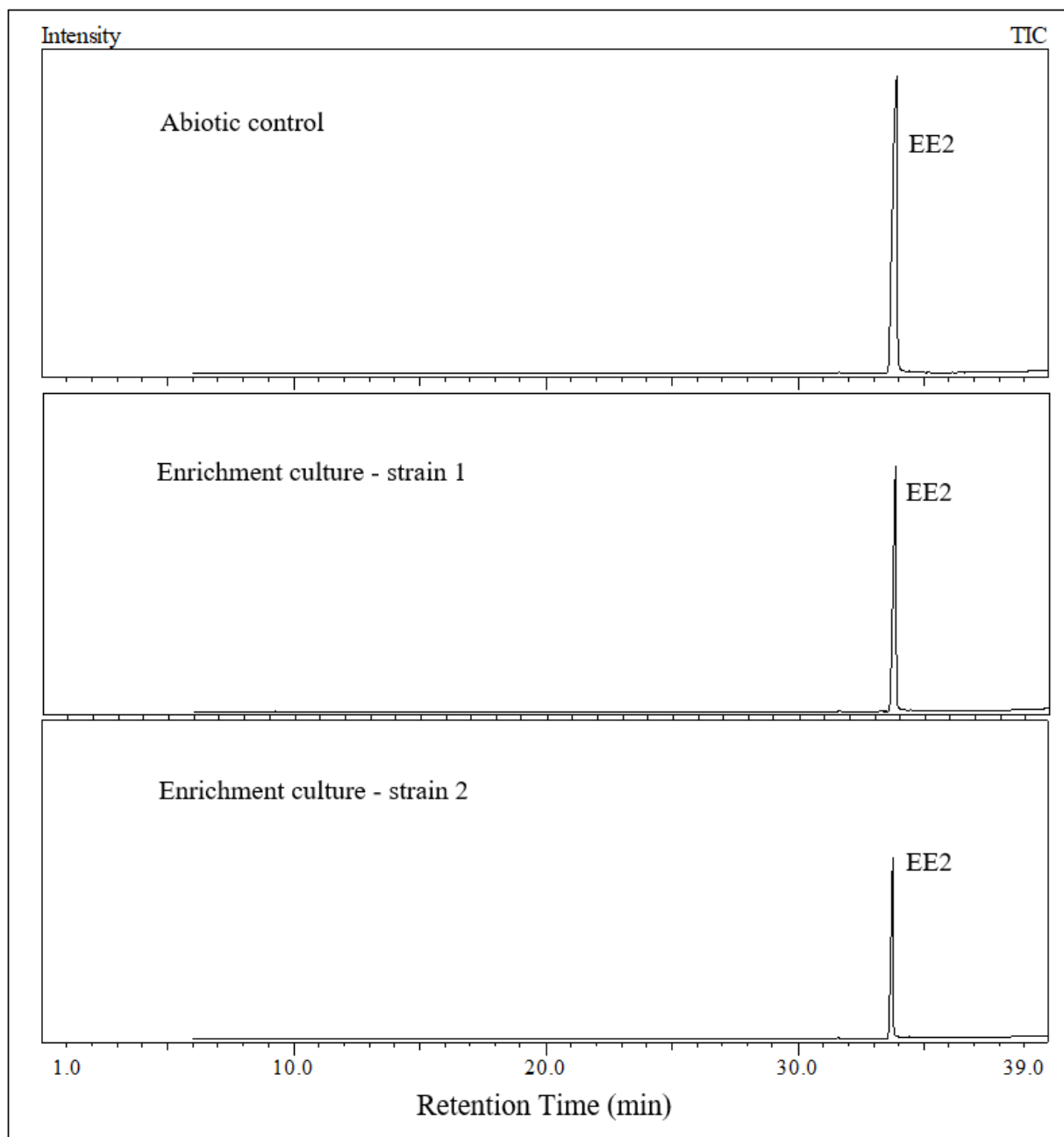


Fig. 4.10: GC-MS analysis showing total ion chromatograms of the abiotic controls and cultures containing 0.5 mM EE2 in 100 mL CDM inoculated with pure bacterial cultures from AGU culture collection after 15 days of incubation. EE2 was added from a stock solution in DMSO. All microcosms were incubated at 30 °C in an orbital shaker (180 rpm).

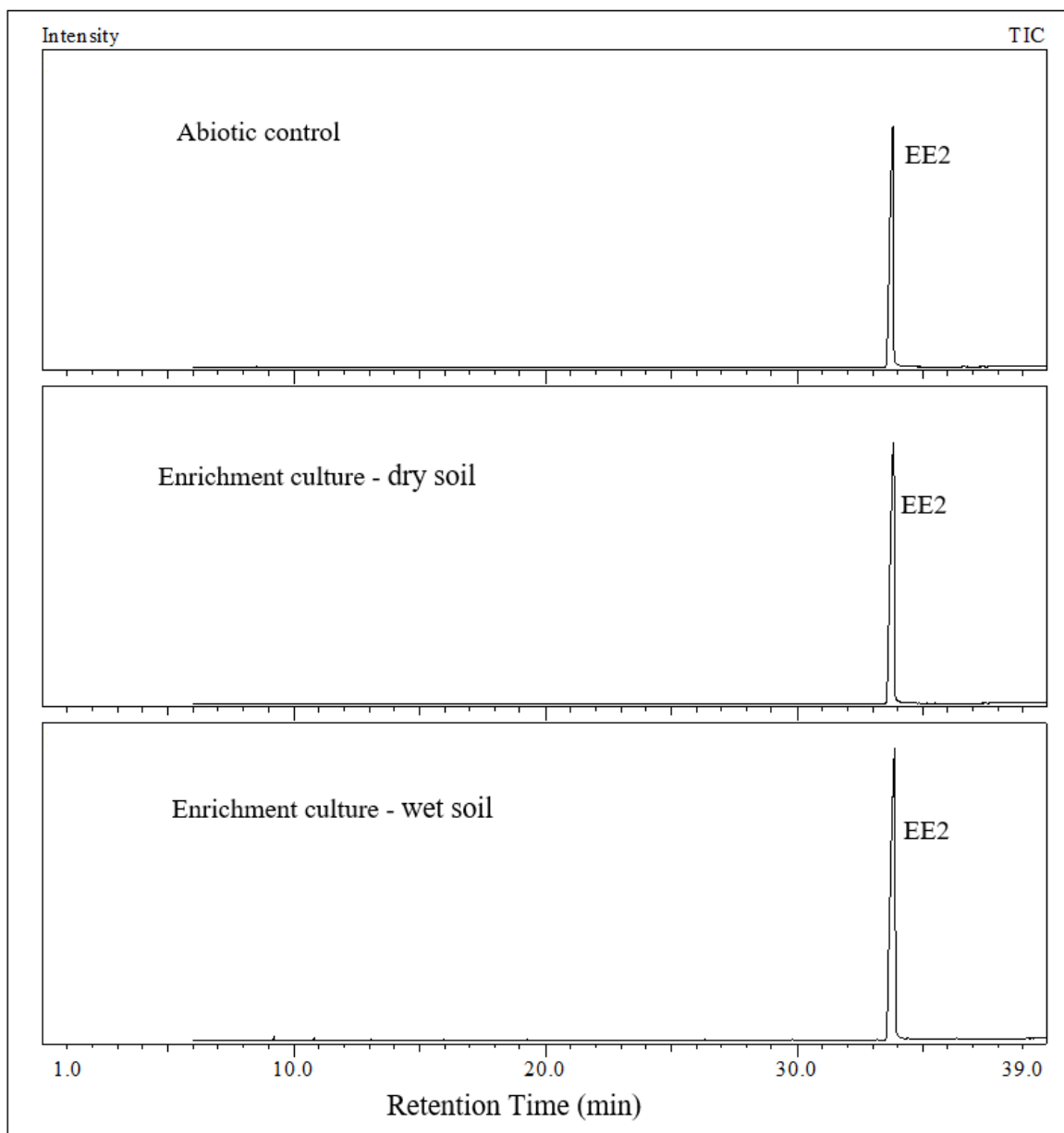


Fig. 4.11: GC-MS analysis showing total ion chromatograms of the abiotic controls and enrichment cultures containing 0.5 mM EE2 in 100 mL CDM inoculated with 10 g of different soil samples after 15 days of incubation. EE2 was added from a stock solution in DMSO. All microcosms were incubated at 30 °C in an orbital shaker (180 rpm).

4.3. Enrichment of E3-degrading consortia

Another estrogen, namely E3, was selected and was added as a carbon source in CDM inoculated with samples of raw sewage (inflow wastewater), activated sludge, and thickened sludge collected from a domestic WWTP. Interestingly, the turbidity of the enrichment cultures increased with time (visual inspection) and GC-MS analysis revealed E3 degradation within 7 days of incubation (Figure 4.12). Those initial enrichments retained E3 biodegradation capacity even after repeated subculturing (Figures 4.13 - 4.15). It is worth noting that the GC-MS analysis did not reveal E3 degradation products or intermediates, although E3 was fully utilized. These findings indicate that the wastewater and sludge samples contained E3-degrading bacteria, and these were enriched after culturing in CDM containing E3. At this stage, aliquots from the various enrichments and subcultures were frozen with glycerol at -80 °C to preserve the enriched cultures for subsequent studies.

To confirm E3 degradation by the enriched cultures, aliquots from the frozen stocks of the three enrichments were thawed and directly inoculated into CDM containing E3. All three enrichments grew in this medium and the growth profiles were different (Figure 4.16). However, no further growth was observed after 48 h of incubation. As these cultures contain different carbon sources (DMSO, E3, glycerol), GC-MS analysis was performed on culture samples at time intervals to reveal whether E3 was utilized or not. As shown in Figures 4.17 - 4.19, E3 was completely utilized within 7 days of incubation in all three cultures. However, E3 utilization or degradation appears to have occurred faster in the thickened sludge and activated sludge cultures compared to the inflow wastewater cultures. Moreover, one degradation product was detected only in the thickened sludge cultures (Figure 4.18). This product was designated product-1 and was detected in culture samples analyzed after 54 h of incubation. It appeared in the GC chromatogram before E3 at

34.5 min, indicating that its molecular mass is smaller than that of E3. Mass spectral database search revealed that product-1 has a molecular mass of 286 and the base peak appeared at m/z 213. This indicates that product-1 is two mass units smaller than E3.

Based on these criteria, it was assumed that product-1 is an oxidation/dehydrogenation product of E3. Indeed, mass spectral library search revealed the highest matches are oxidation products of E3 where two hydrogens are lost from the OH- group on either C16 or C17 (Figure 4 - 20), thus predicting product-1 to be 16-keto-E2 or 16 α -OH-E1, respectively.

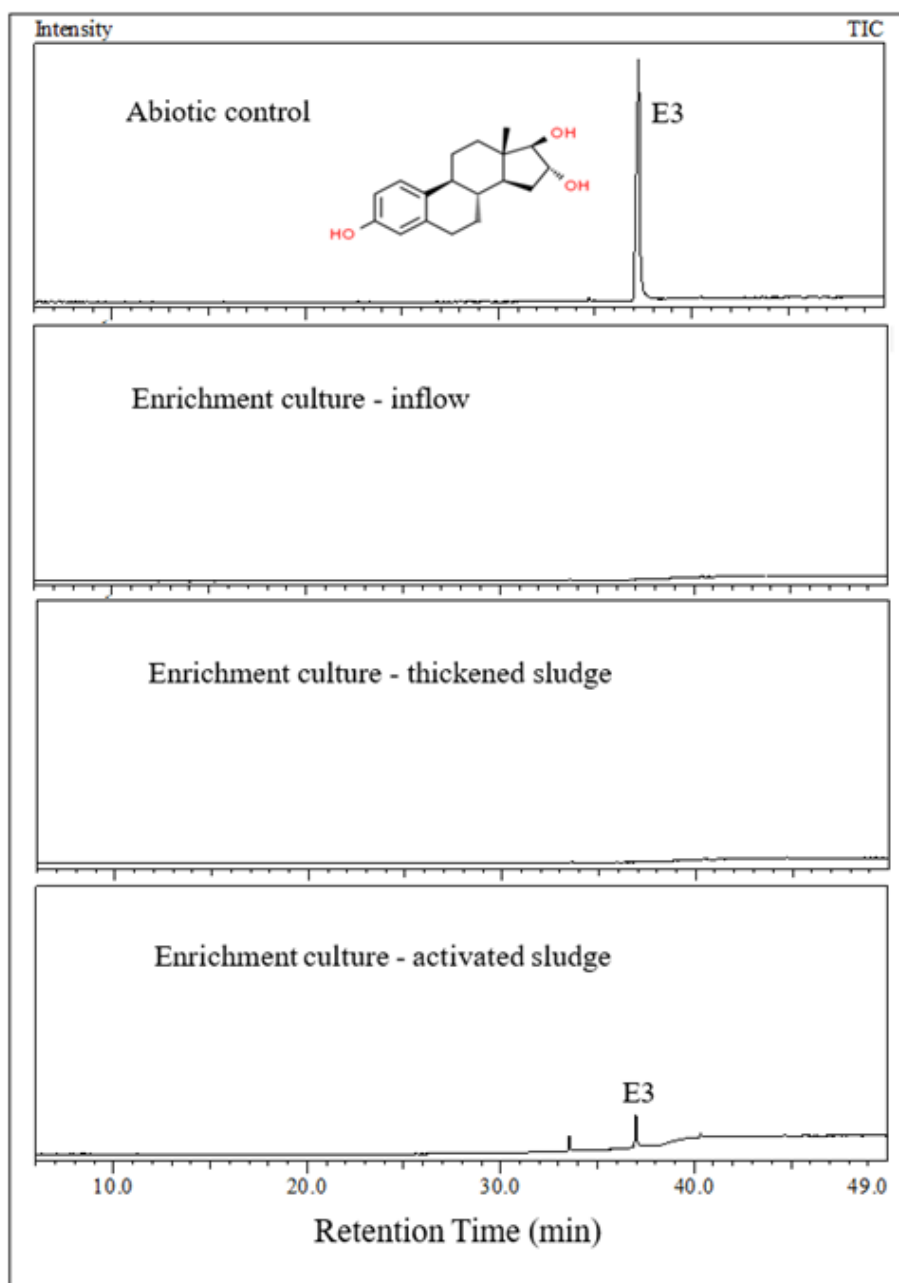


Fig. 4.12: GC-MS analysis showing total ion chromatograms of the abiotic control and enrichment cultures containing 1 mM E3 and inoculated with 10 mL of either activated sludge, thickened sludge, or inflow wastewater samples from Tubli wastewater treatment plant after 7 days of incubation. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

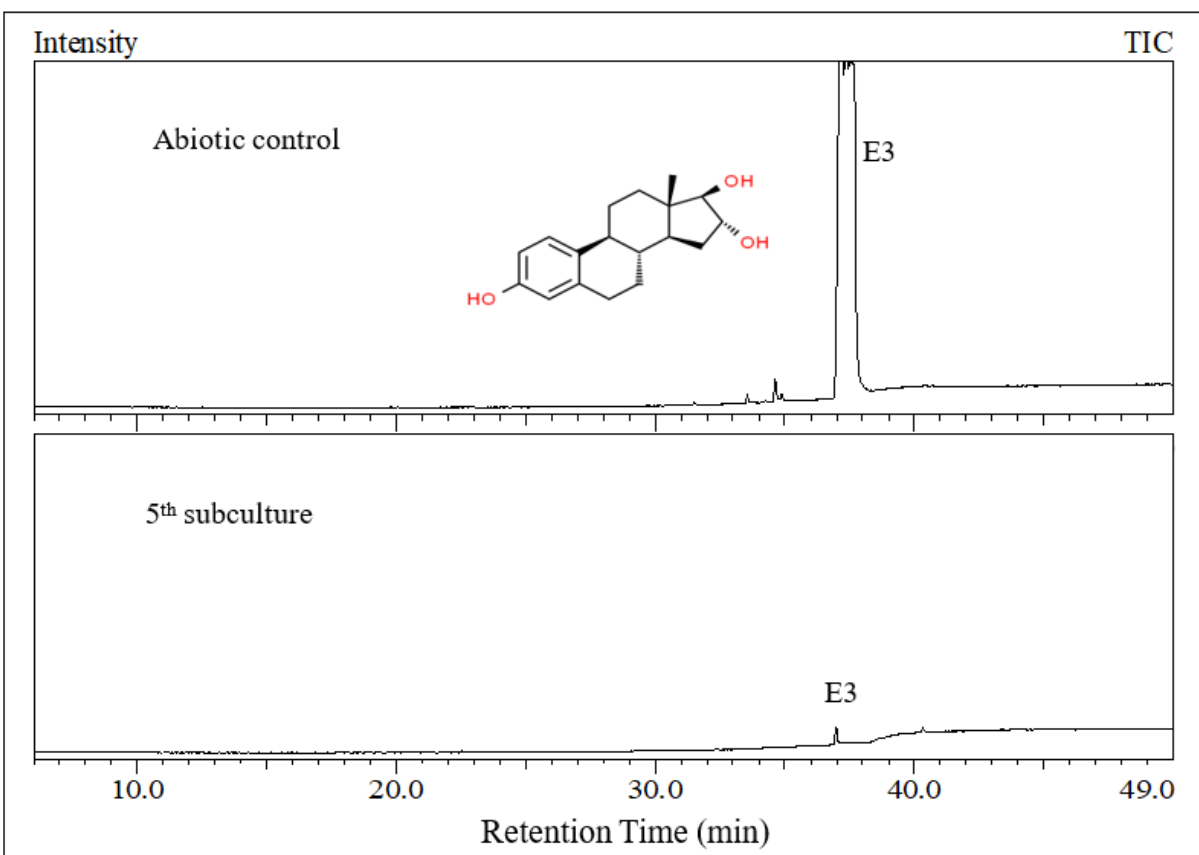


Fig. 4.13: GC-MS analysis showing total ion chromatograms of the 5th subculture containing 1 mM E3 and inoculated with inflow wastewater samples. Culture samples were extracted after 4 days of incubation and analyzed by GC-MS. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

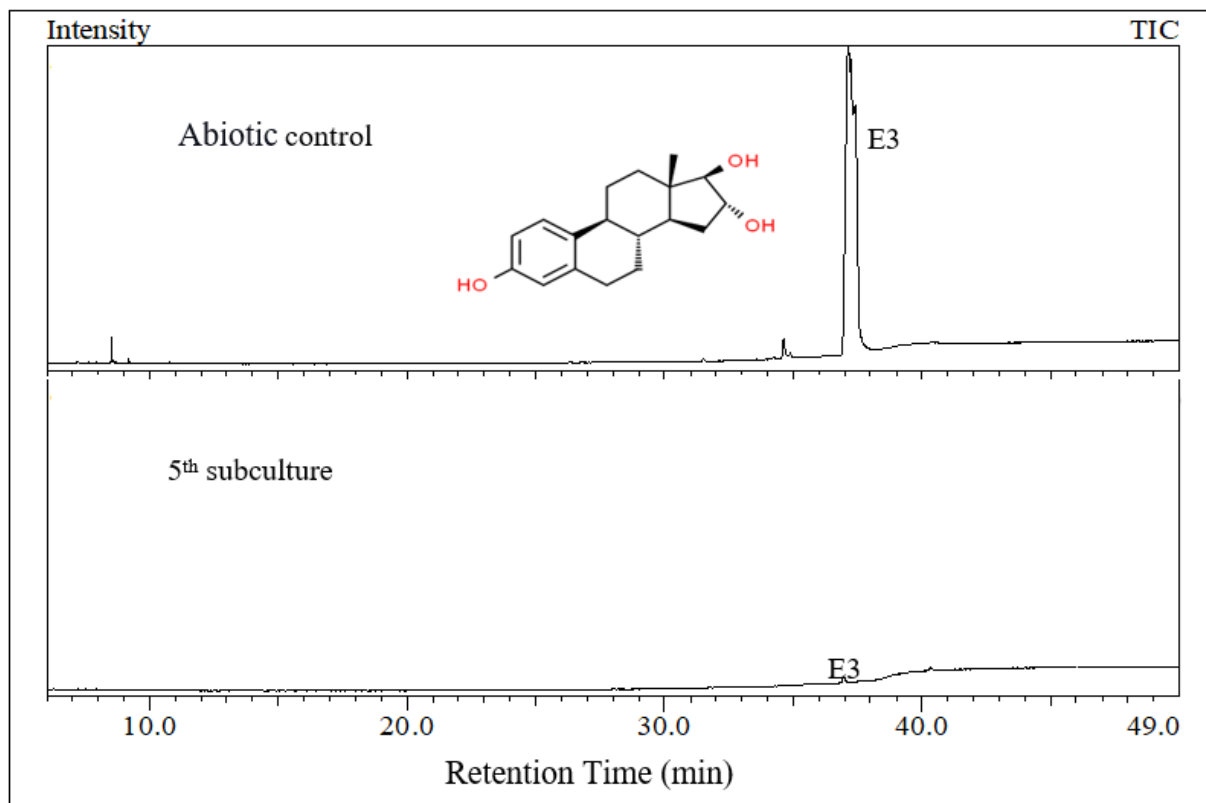


Fig. 4.14: GC-MS analysis showing total ion chromatograms of the 5th subculture containing 1 mM E3 and inoculated with thickened sludge samples. Culture samples were extracted after 4 days of incubation and analyzed by GC-MS. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

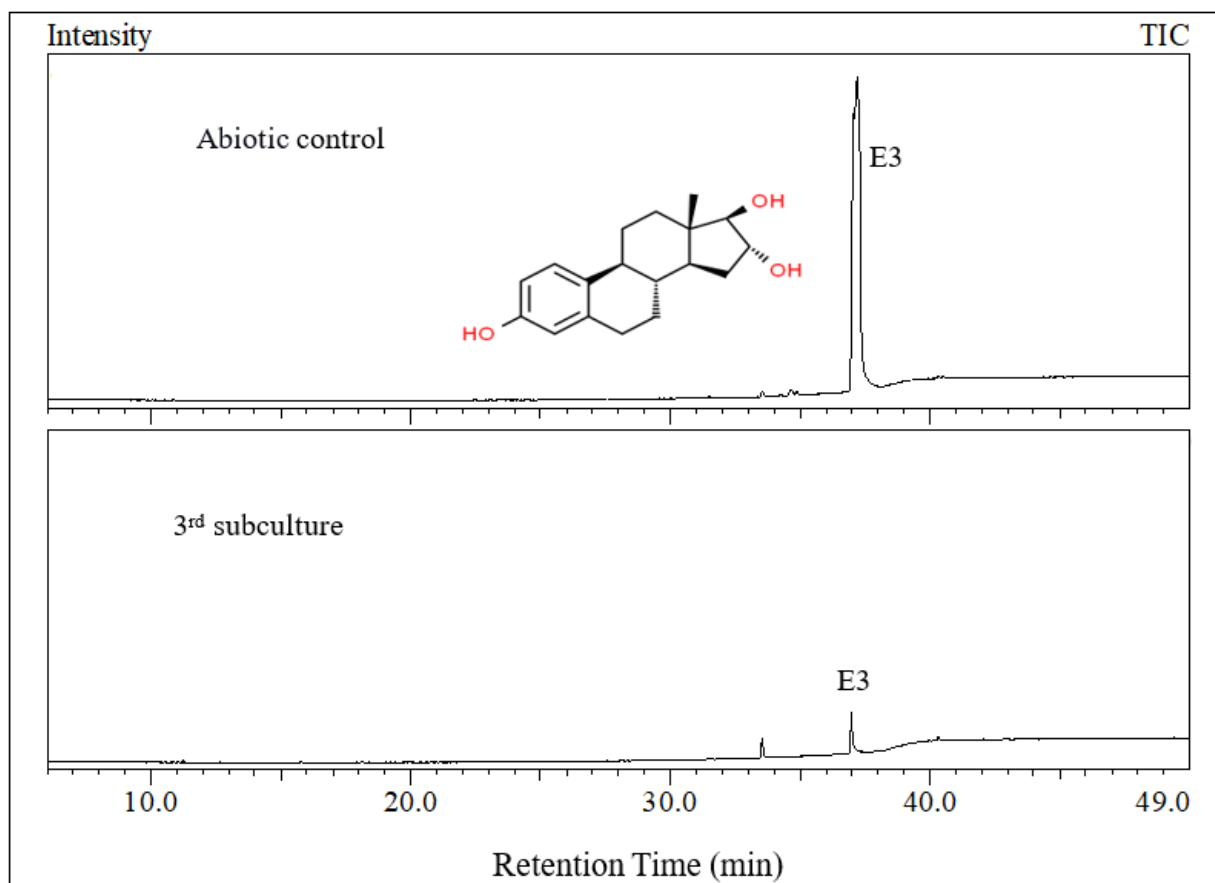


Fig. 4.15: GC-MS analysis showing total ion chromatograms the 3rd subculture containing 1 mM E3 and inoculated with activated sludge samples. Culture samples were extracted after 4 days of incubation and analyzed by GC-MS. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

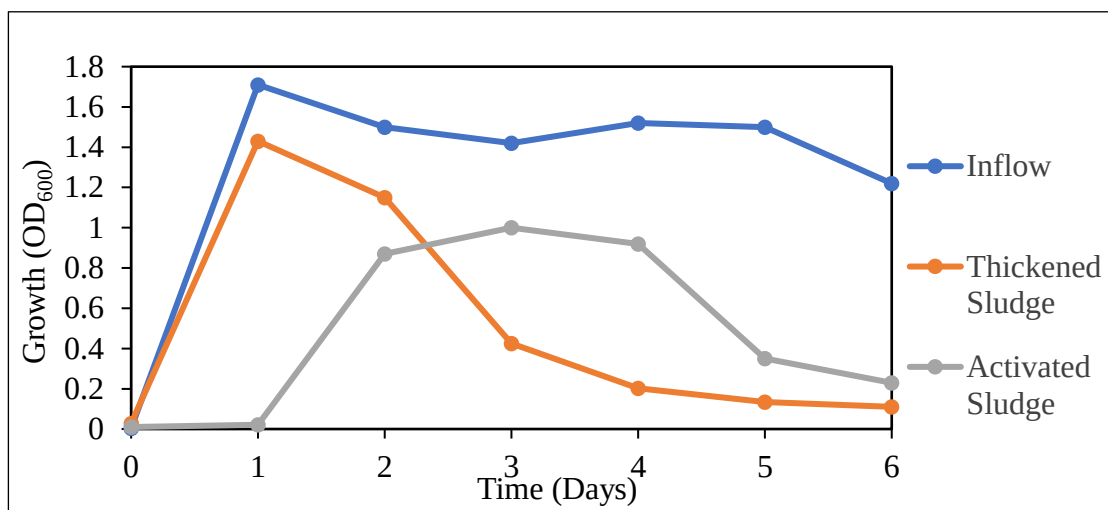


Fig. 4.16: Growth curves of E3-degrading consortia on 0.1 mM E3. All cultures were grown in CDM inoculated with E3-degrading bacterial consortia obtained from inflow wastewater, thickened sludge, or activated sludge. E3 was added from a DMSO stock solution. The inocula were obtained directly from frozen stocks containing glycerol. All cultures were incubated at 30 °C in an orbital shaker (180 rpm).

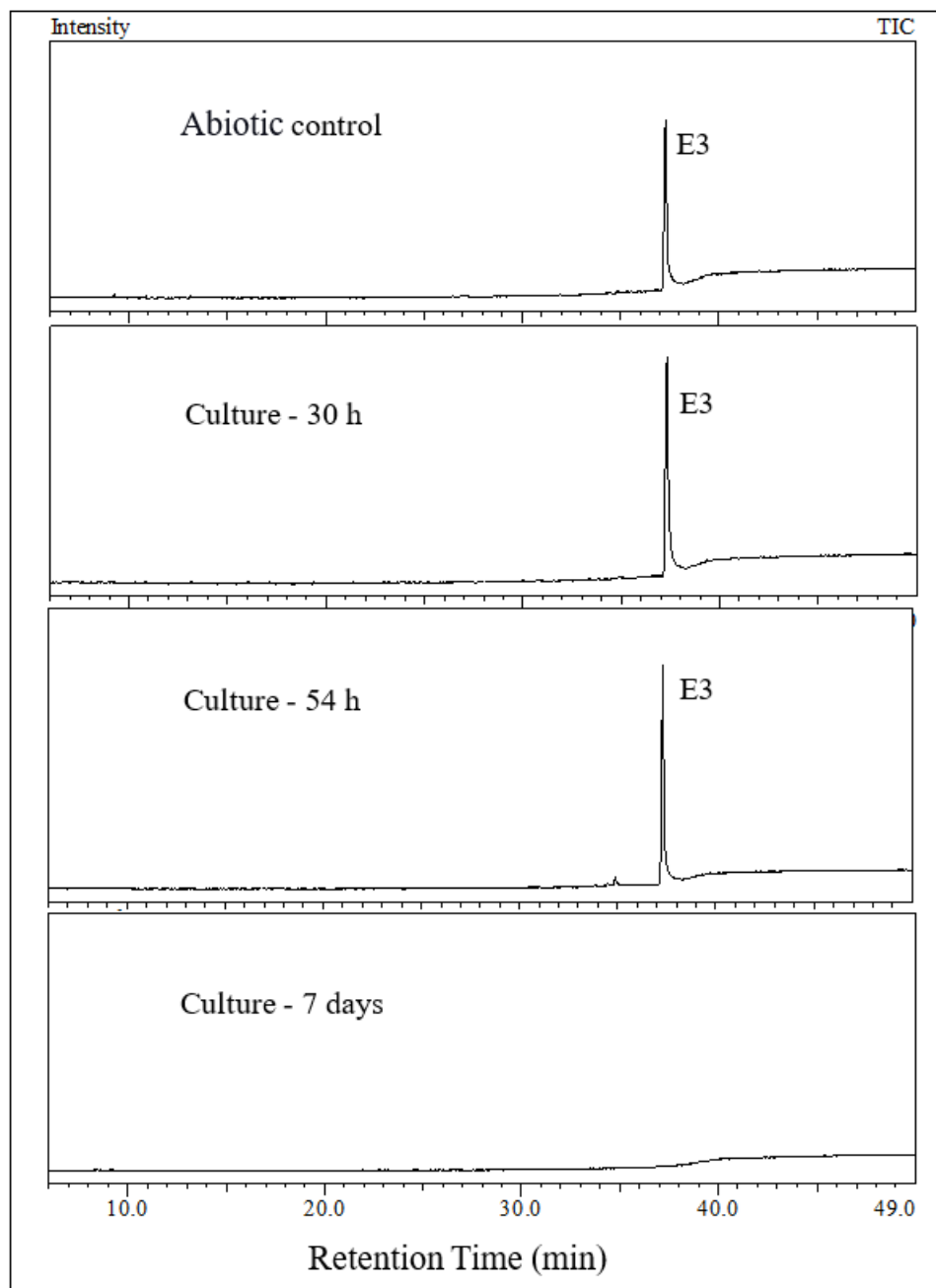


Fig. 4.17: Total ion chromatograms from GC-MS analysis showing degradation of 0.1 mM E3 by a bacterial consortium obtained from inflow wastewater samples. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

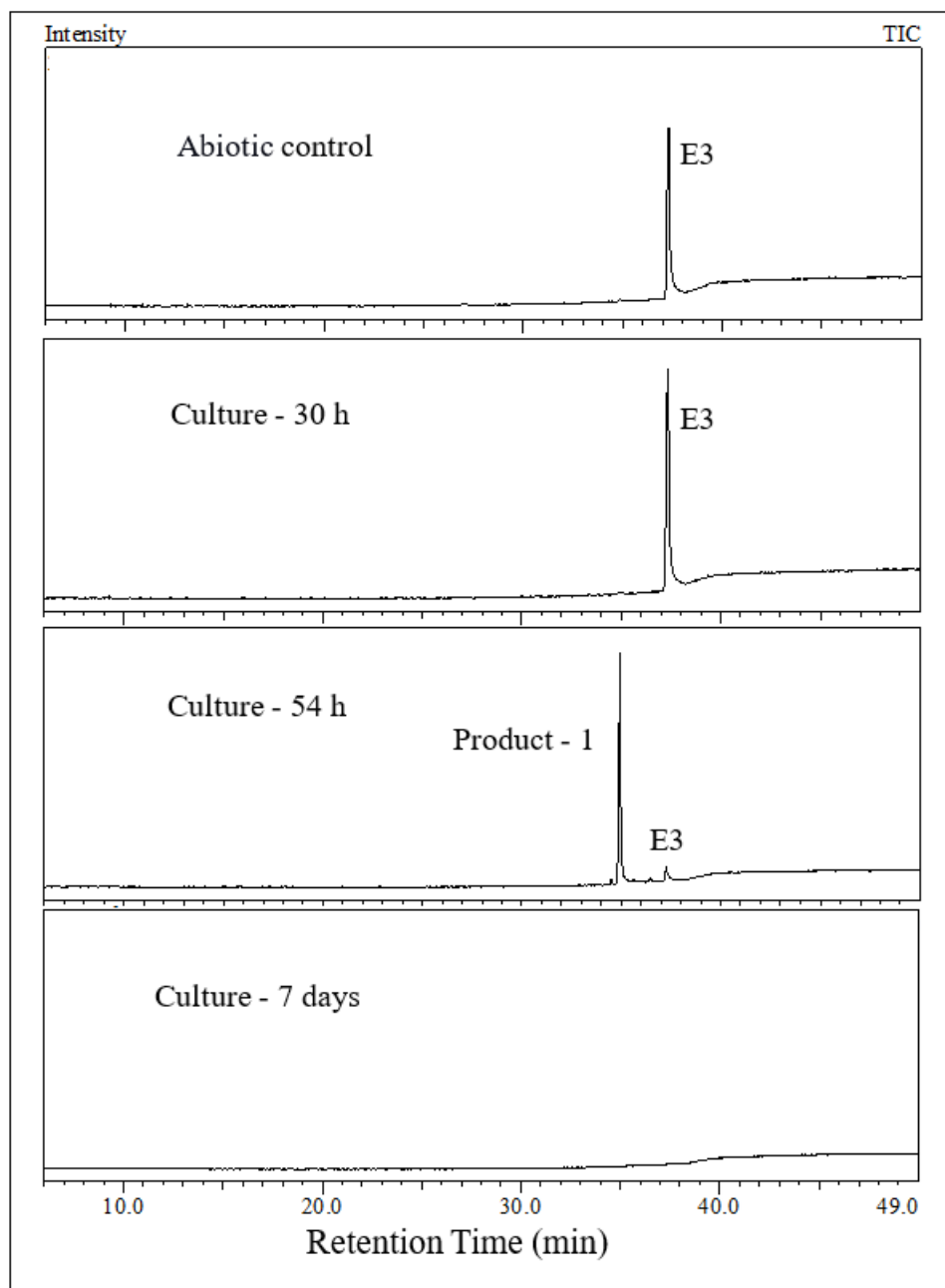


Fig. 4.18: Total ion chromatograms from GC-MS analysis showing degradation of 0.1 mM E3 by a bacterial consortium obtained from thickened sludge samples. The chromatograms show the formation of an unknown product after 54 h of incubation. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

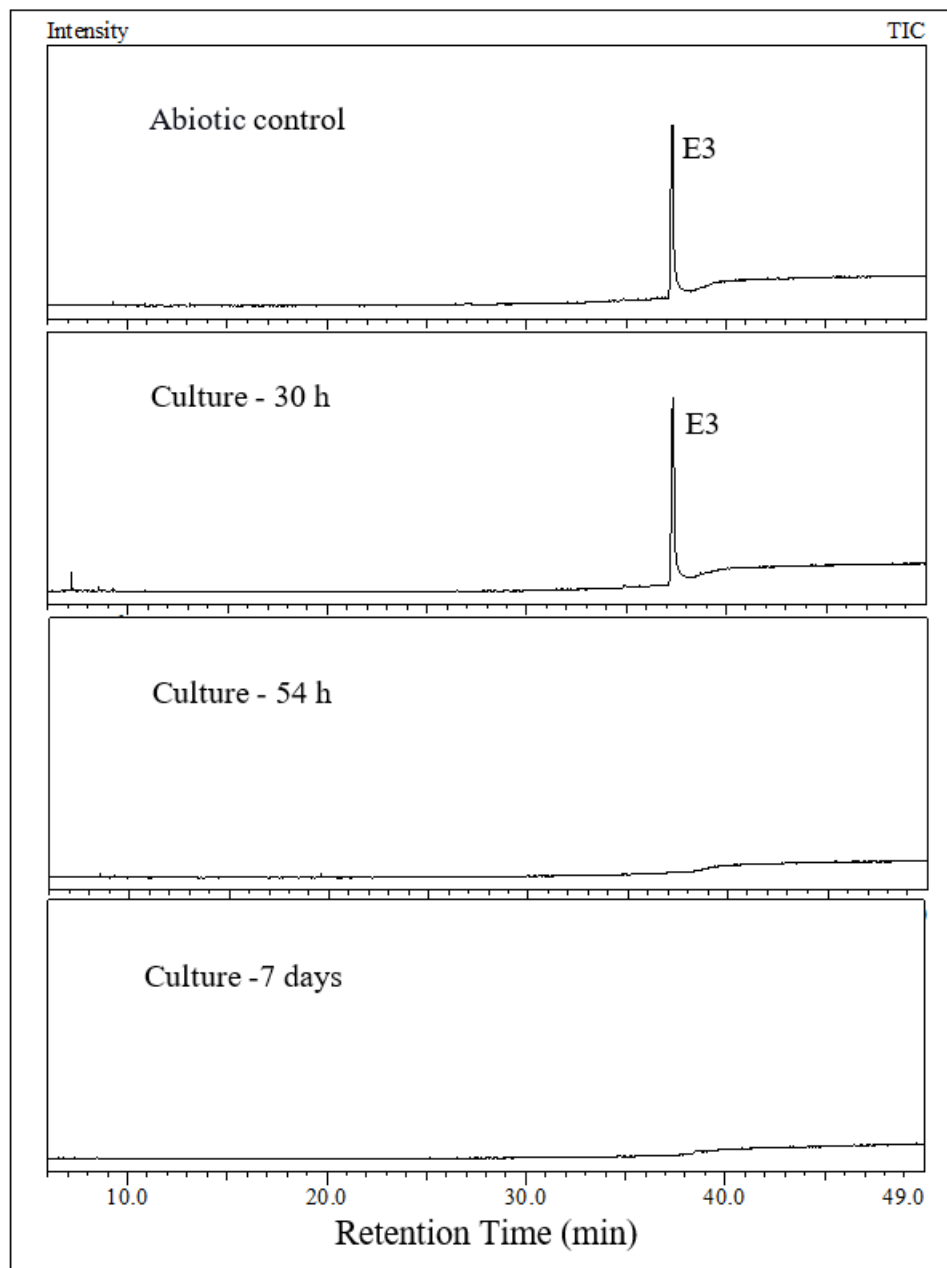
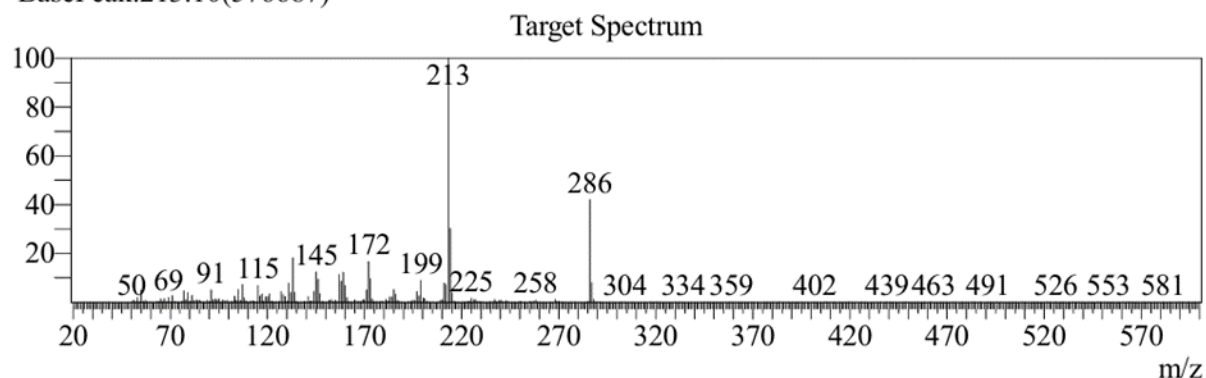


Fig. 4.19: Total ion chromatograms from GC-MS analysis showing degradation of 0.1 mM E3 by a bacterial consortium obtained from activated sludge samples. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

Unknown product - 1

Line#:4 R.Time:34.570(Scan#:5715) MassPeaks:380

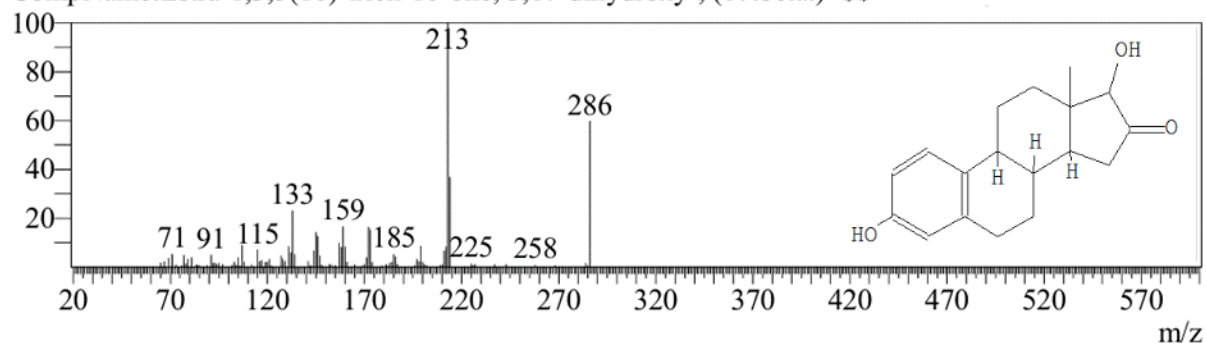
BasePeak:213.10(576687)



Hit#:1 Entry:129753 Library:NIST17-1.lib

SI:93 Formula:C₁₈H₂₂O₃ CAS:566-75-6 MolWeight:286 RetIndex:2341

CompName:Estra-1,3,5(10)-trien-16-one, 3,17-dihydroxy-, (17.β.)- \$\$

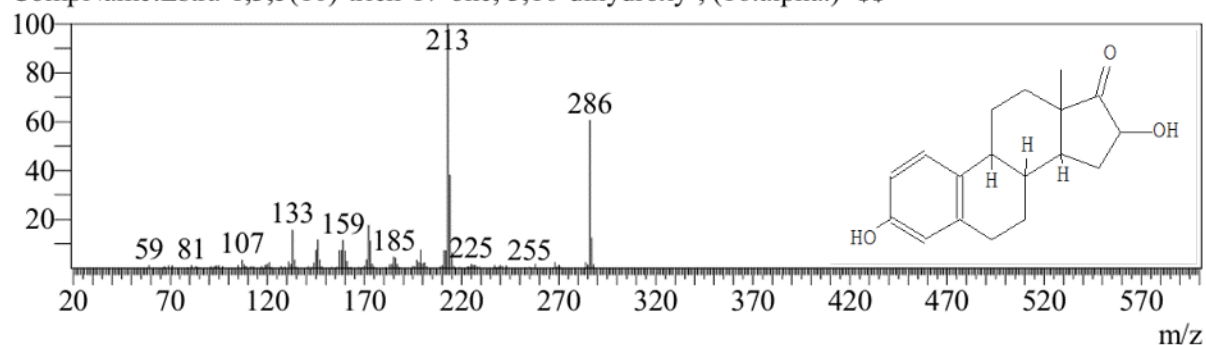


Result Spectrum

Hit#:2 Entry:129754 Library:NIST17-1.lib

SI:86 Formula:C₁₈H₂₂O₃ CAS:566-76-7 MolWeight:286 RetIndex:2341

CompName:Estra-1,3,5(10)-trien-17-one, 3,16-dihydroxy-, (16.α.)- \$\$



Result Spectrum

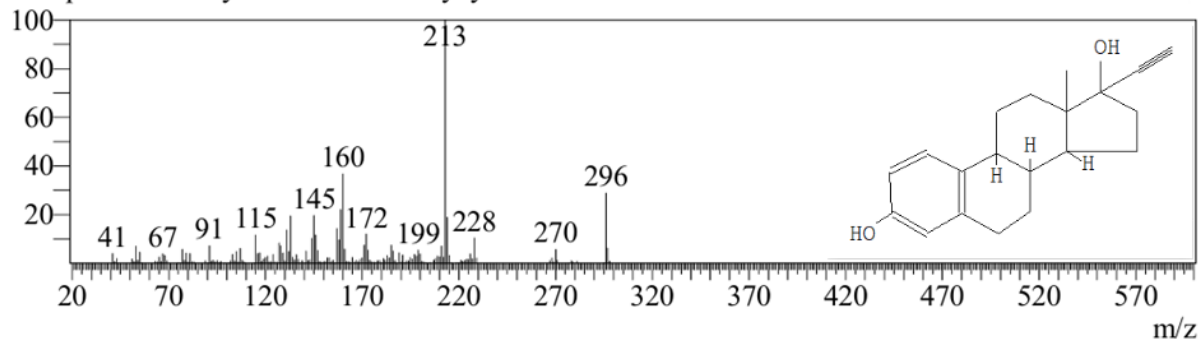
Fig. 4.20: Legend on next page

Unknown product - 1 : Continued

Hit#:3 Entry:32755 Library:NIST17s.lib

SI:76 Formula:C₂₀H₂₄O₂ CAS:57-63-6 MolWeight:296 RetIndex:2290

CompName:Ethinyl Estradiol \$\$ Ethynylestradiol \$\$

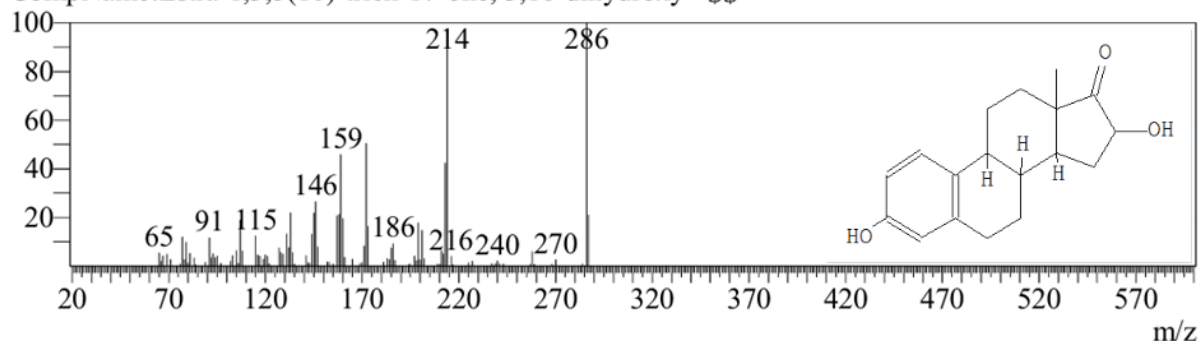


Result Spectrum

Hit#:4 Entry:129766 Library:NIST17-1.lib

SI:76 Formula:C₁₈H₂₂O₃ CAS:18186-49-7 MolWeight:286 RetIndex:2341

CompName:Estra-1,3,5(10)-trien-17-one, 3,16-dihydroxy- \$\$

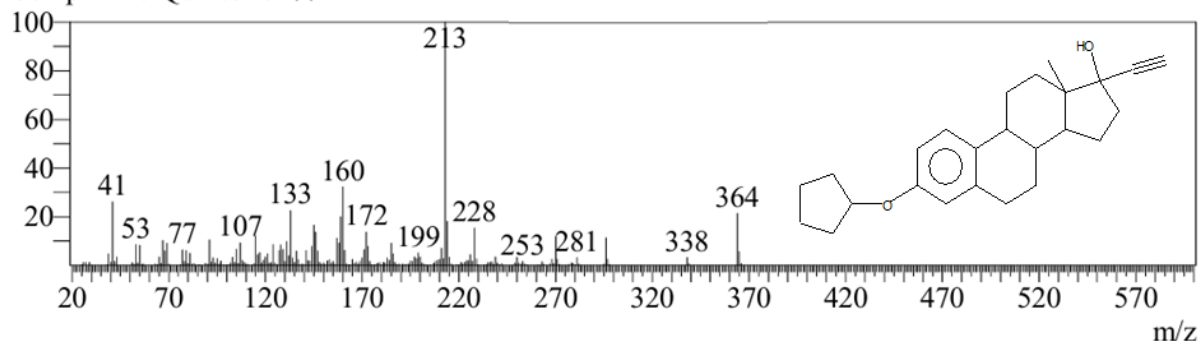


Result Spectrum

Hit#:5 Entry:204206 Library:NIST17-1.lib

SI:74 Formula:C₂₅H₃₂O₂ CAS:152-43-2 MolWeight:364 RetIndex:2699

CompName:Quinestrol \$\$



Result Spectrum

Fig. 4.20: Predicted mass spectrum of product-1 and the matching spectra from the GC-MS spectral library NIST17-1.

4.4. Isolation of E3-transforming bacteria

When serially diluted aliquots from the enriched E3-degrading cultures were spread on LB-agar plates, many morphologically different colonies could be observed. This indicates that the enriched cultures are composed of consortia or mixed bacterial cultures. Those colonies were purified and then screened for E3 degradation. Visual observation of the E3 cultures of the isolates did not reveal temporal changes in the turbidity, suggesting that they cannot grow on E3 or even the associated solvent DMSO as a carbon source. Nonetheless, GC-MS analysis revealed that 5 of those isolates transformed E3 into other products (Figures 4.21 - 4.25). Three of the isolates (TH-11, TH-16, TH-30) belong to the thickened sludge consortium, one isolate (IF-20) belongs to the inflow consortium, and the last one (AS-31) was isolated from the activated sludge consortium. Interestingly cultures of all isolates transformed E3 into product-1, which was identical to the product detected in the E3-grown culture of the thickened sludge consortium. Furthermore, a new product, designated product-2, was detected in the E3 culture of the isolate AS-31 (Figure 4.25).

Product-2 had a retention time of 38.7 min, indicating that it has a molecular mass higher than that of E3. One of the close matches from the mass spectral library had a BasePeak at m/z 330. The core structure of this compound is similar to that of E3, bearing a 3-methoxy group and a hydroxyethyl group at C17 (Figure 4.26). However, the similarity index of the closest match is low, thus rendering the structure prediction not accurate, and further analysis such as NMR are required.

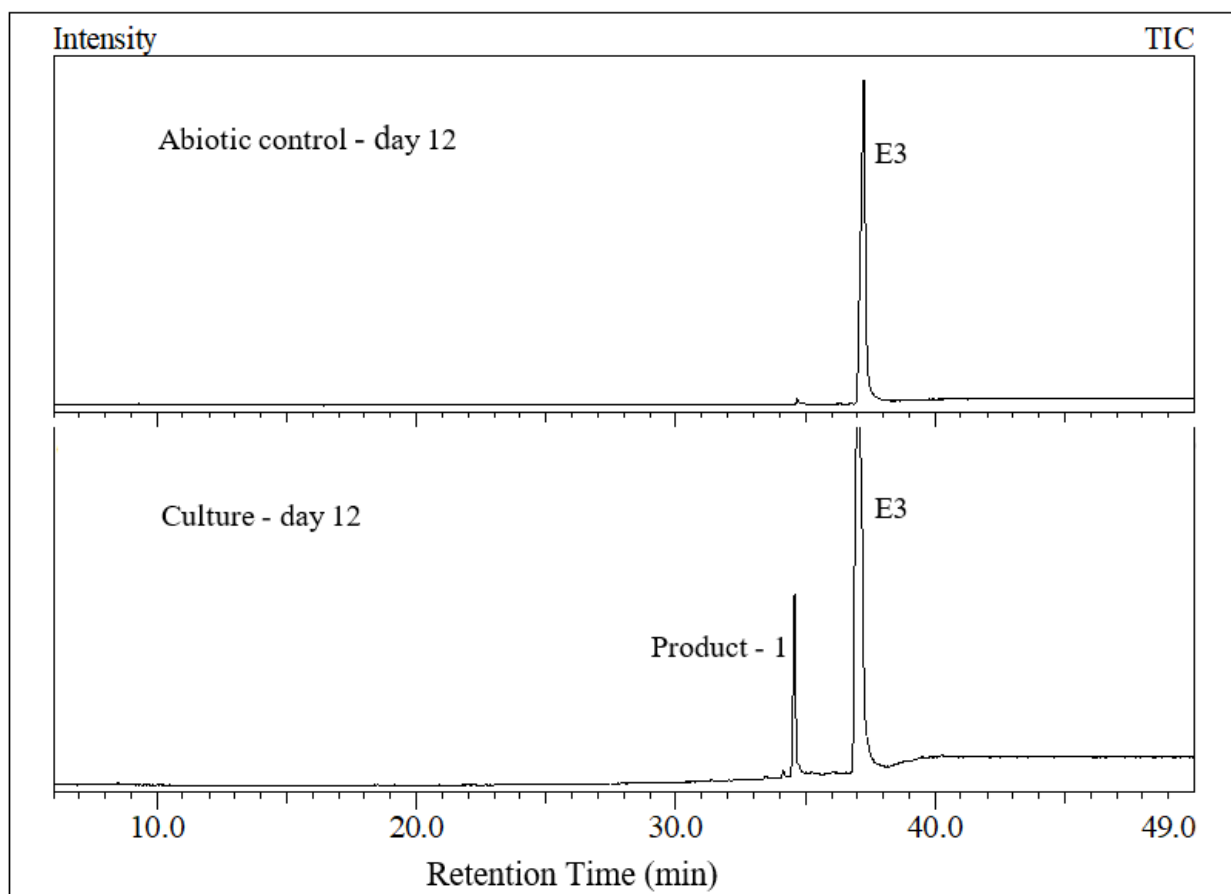


Fig. 4.21: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-11 cultures grown in CDM containing 1 mM E3. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

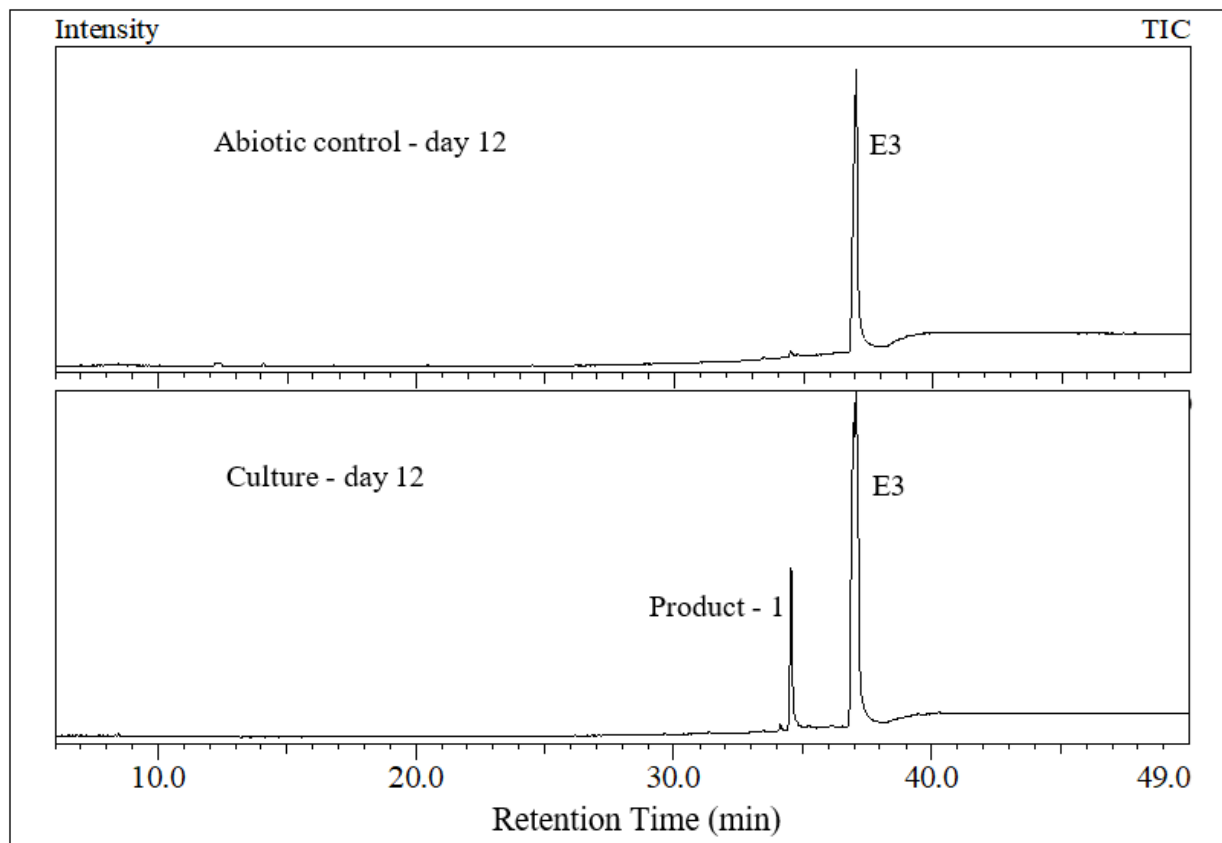


Fig. 4.22: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-16 cultures grown in CDM containing 1 mM E3. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

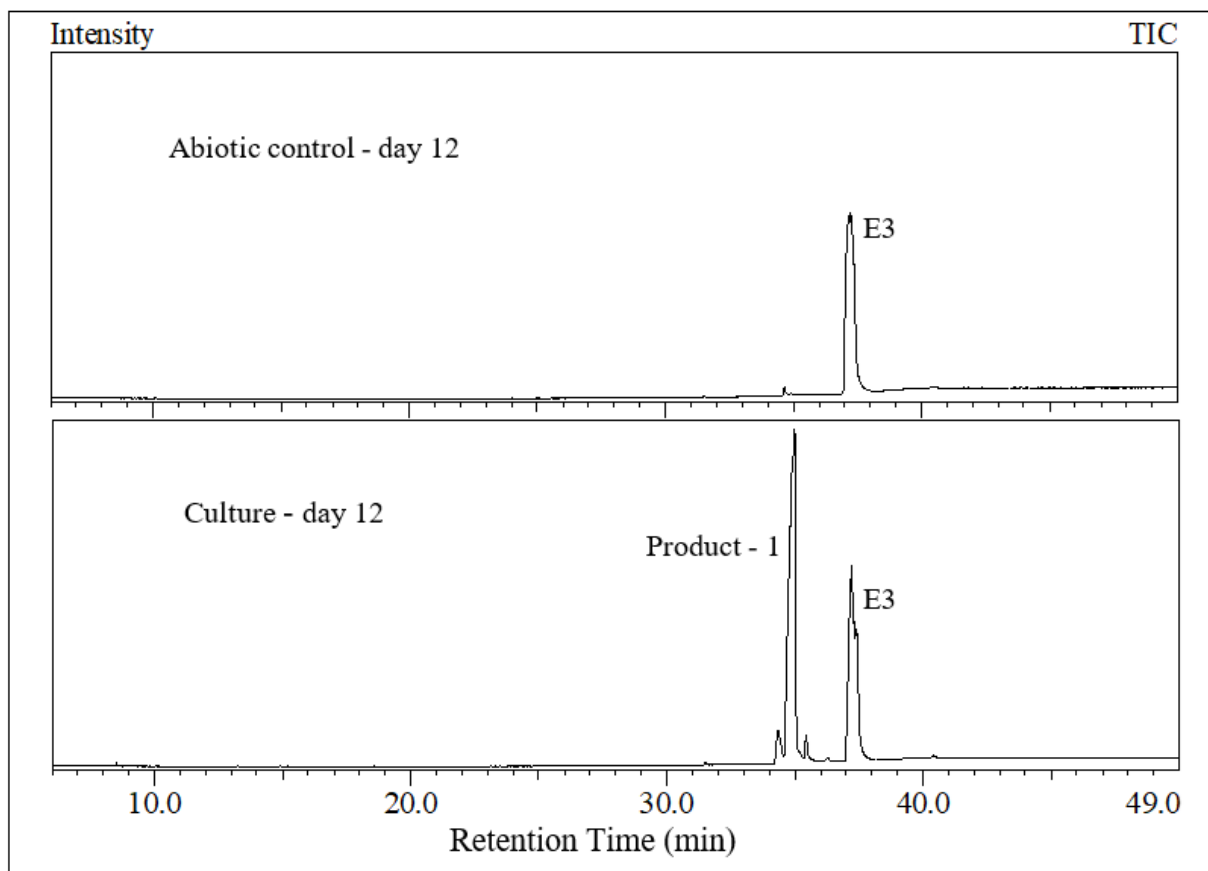


Fig. 4.23: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-30 cultures grown in CDM containing 1 mM E3. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

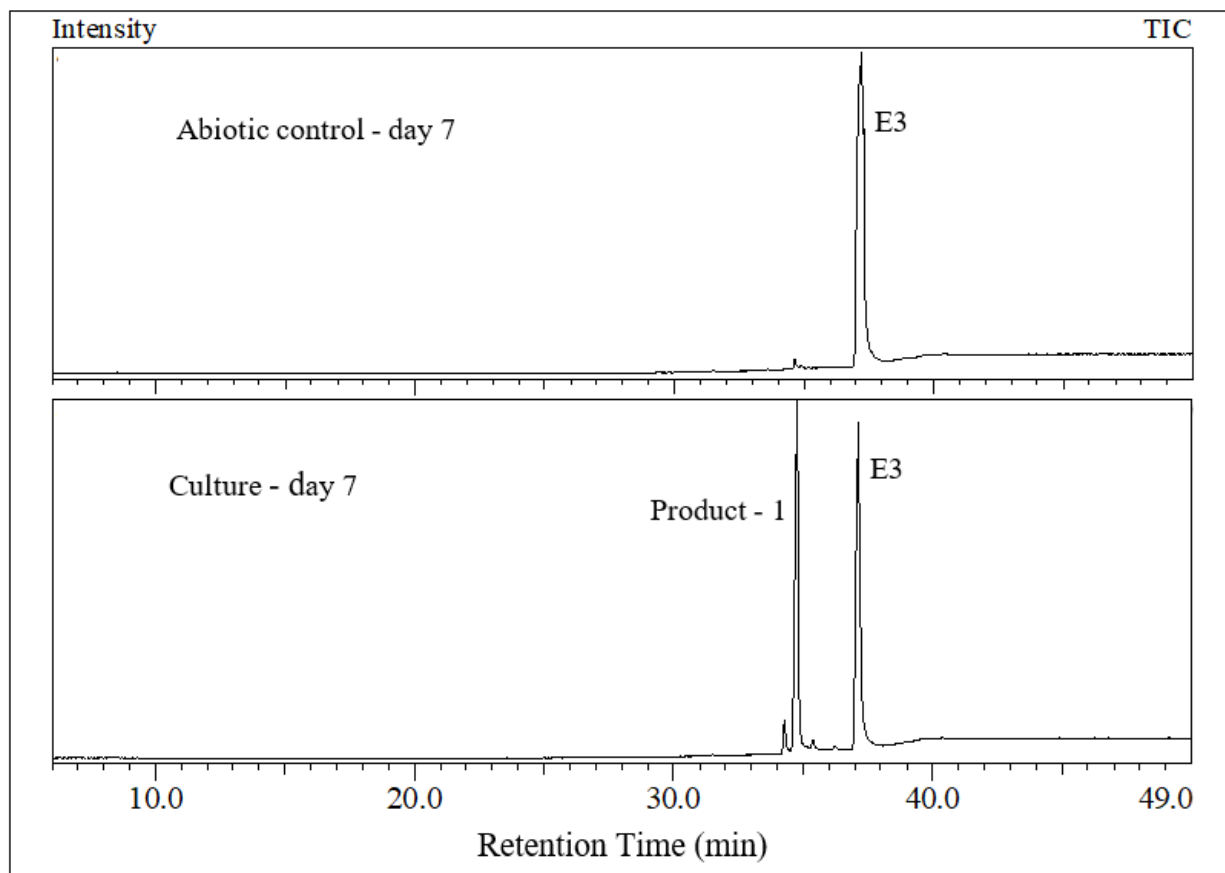


Fig. 4.24: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain IF-20 cultures grown in CDM containing 1 mM E3. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

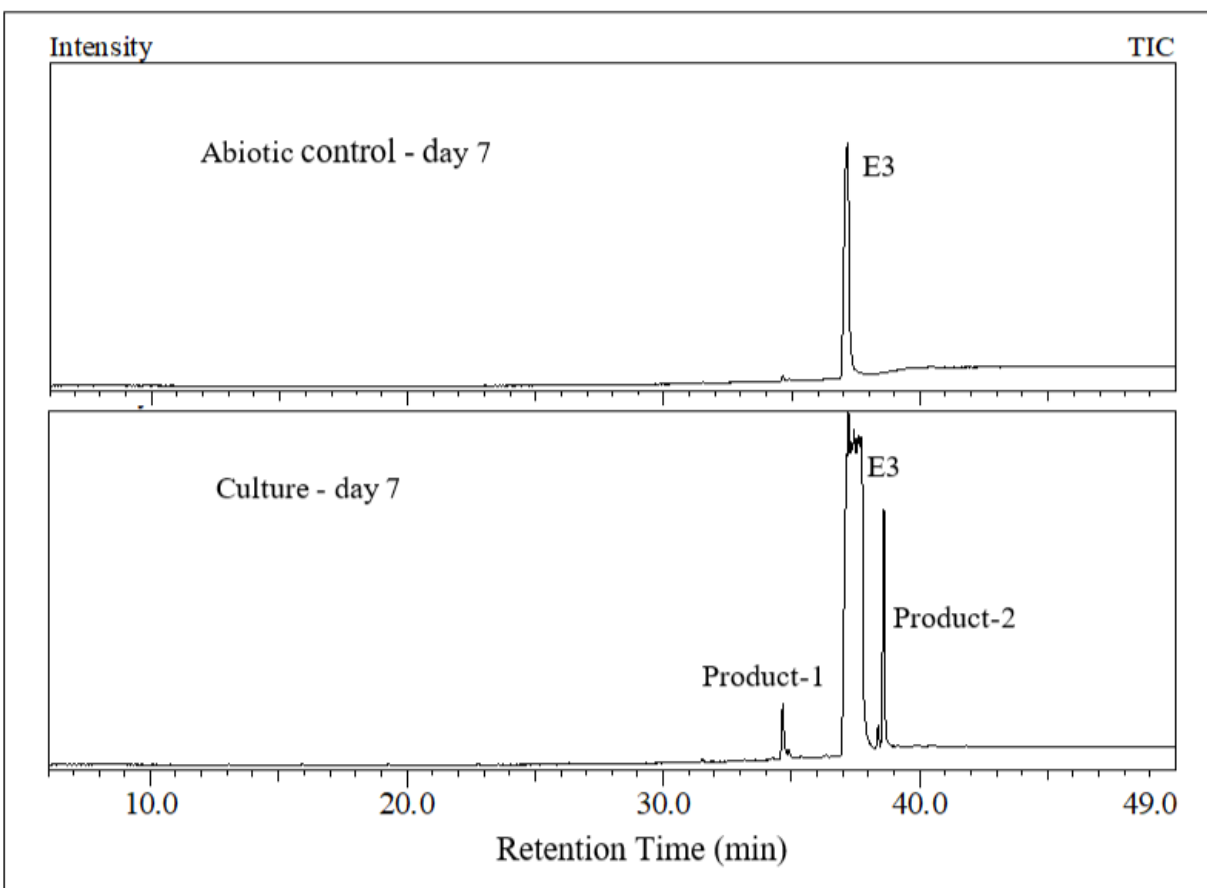
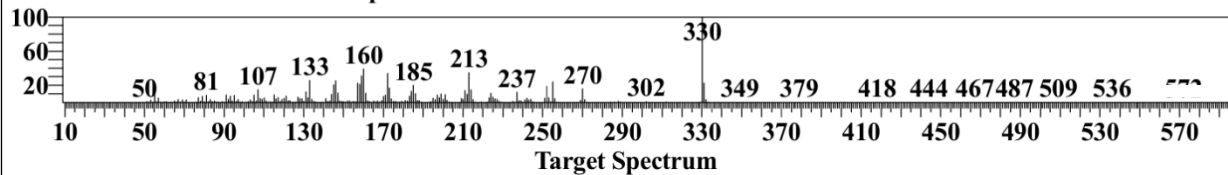


Fig. 4.25: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain AS-31 cultures grown in CDM containing 1 mM E3. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

Unknown product - 2

Line#:3 R.Time:38.610(Scan#:6523) MassPeaks:306
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BG Mode:Calc. from Peak Group 1 - Event 1 Scan



Hit#:10 Entry:174318 Library:NIST17-1.lib
SI:62 Formula:C₂₁H₃₀O₃ CAS:56145-32-5 MolWeight:330 RetIndex:2461
CompName:19-Norpregna-1,3,5(10)-triene-11,20-diol, 3-methoxy-, (11.alpha.,20R)- SS

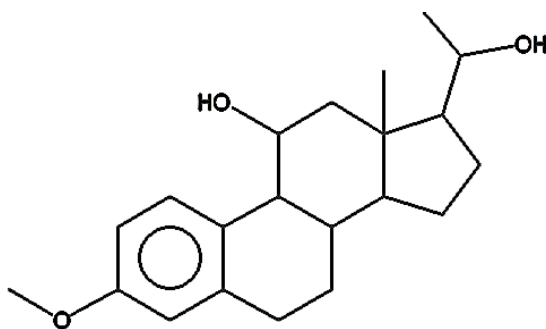
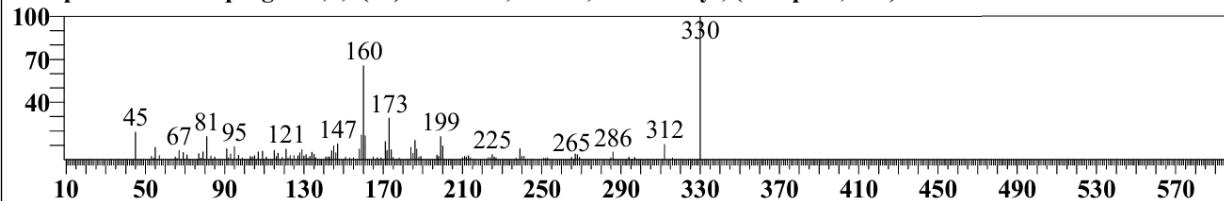


Fig. 4.26: The predicted mass spectrum of product-2 and a matching mass spectrum of from the GC-MS spectral library, NIST17-1.

To further confirm lack of growth on E3, all 5 isolates were cultured again in CDM containing 0.1 mM E3. As shown in Figure 4.27, none of the isolates could grow under these conditions even after 14 days of incubation, confirming that these isolates cannot utilize E3 or even the solvent as a carbon source. Exceptionally, isolate TH-16 exhibited a little increase of the OD₆₀₀ from 0.1 to 0.34 after 7 days, followed by a stationary phase. Samples from those cultures were retrieved at time intervals and analyzed by GC-MS (Figure 4.28-4.32) to unravel whether E3 was transformed. This analysis further confirmed that E3 was transformed into a main product, which is product-1, by all 5 isolates. Uninoculated control cultures did not reveal any changes to E3, indicating that the transformation to product-1 was biologically mediated through the isolates. However, we could not detect in this analysis product-2 which was observed in the preceding analysis in cultures of the isolate AS-31.

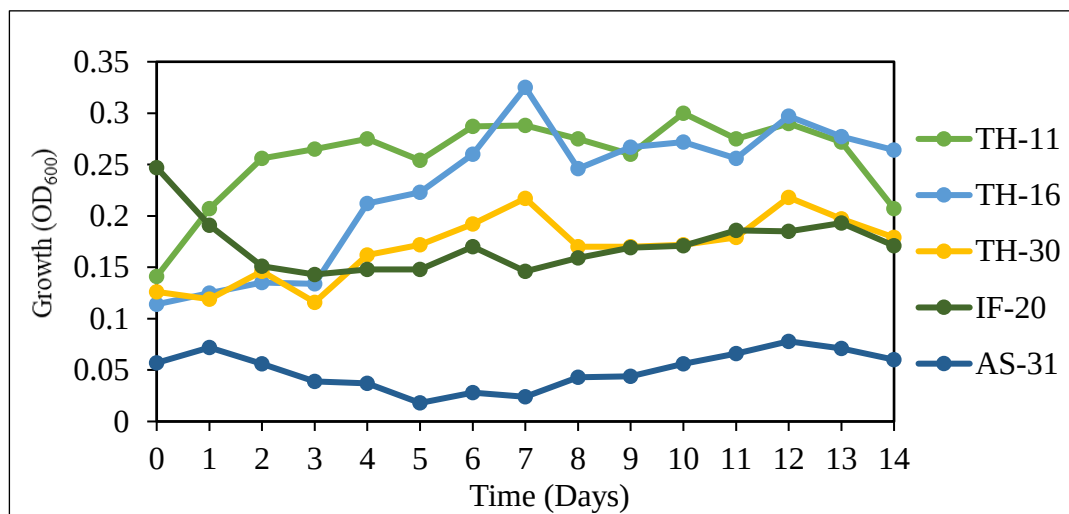
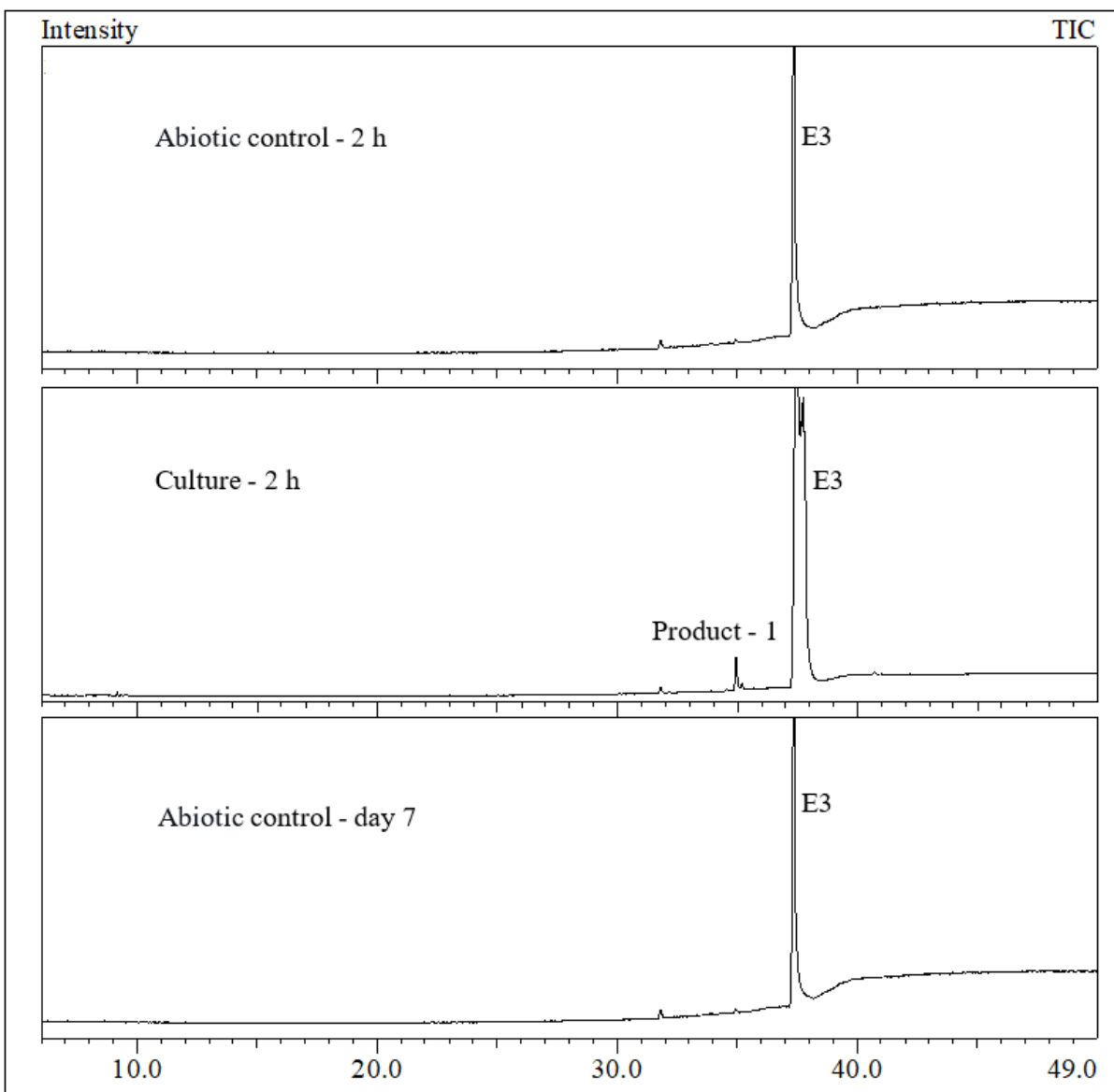


Fig. 4.27: Growth profiles of the isolated strains in CDM containing 0.1 mM E3, which was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).



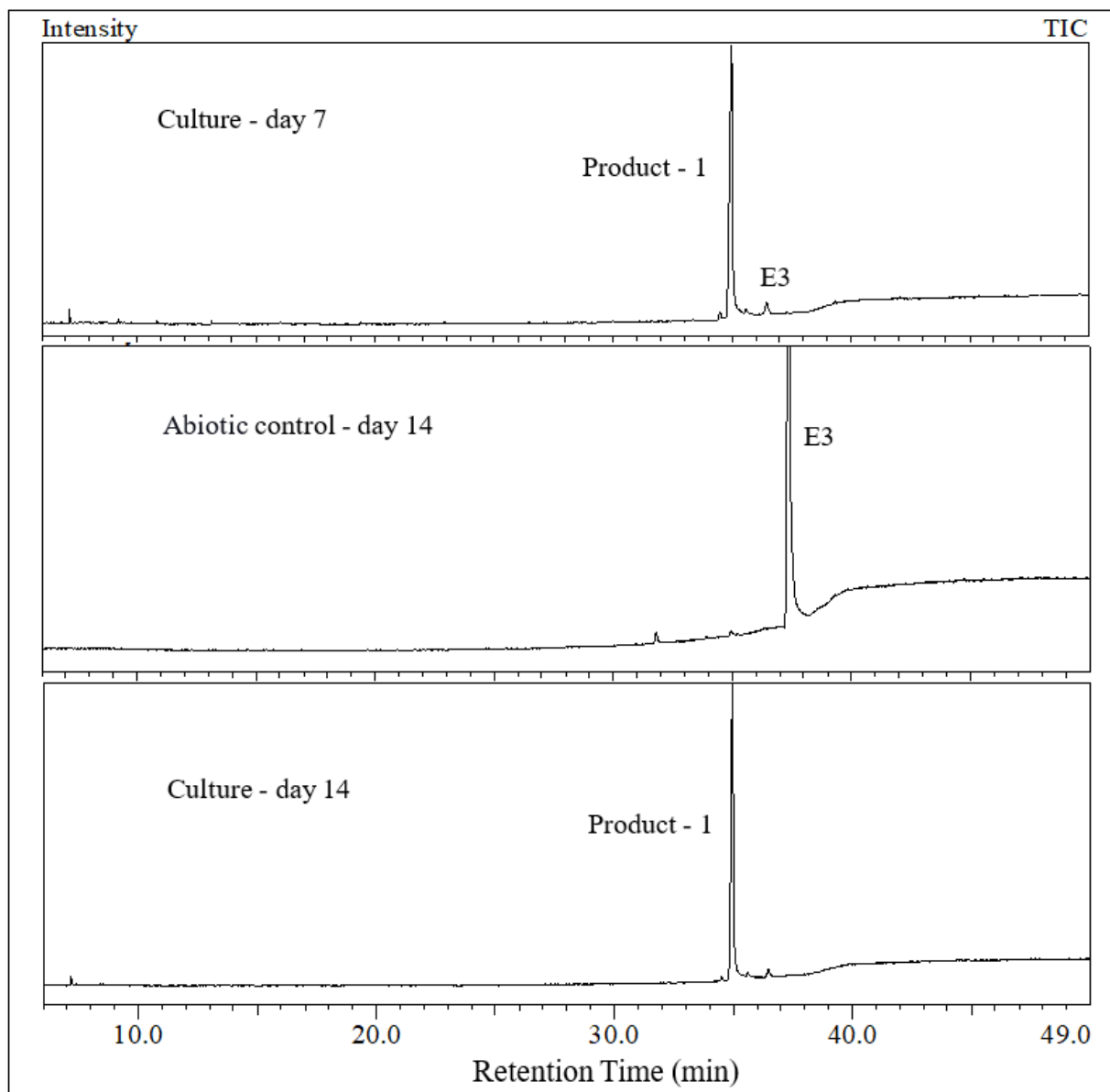
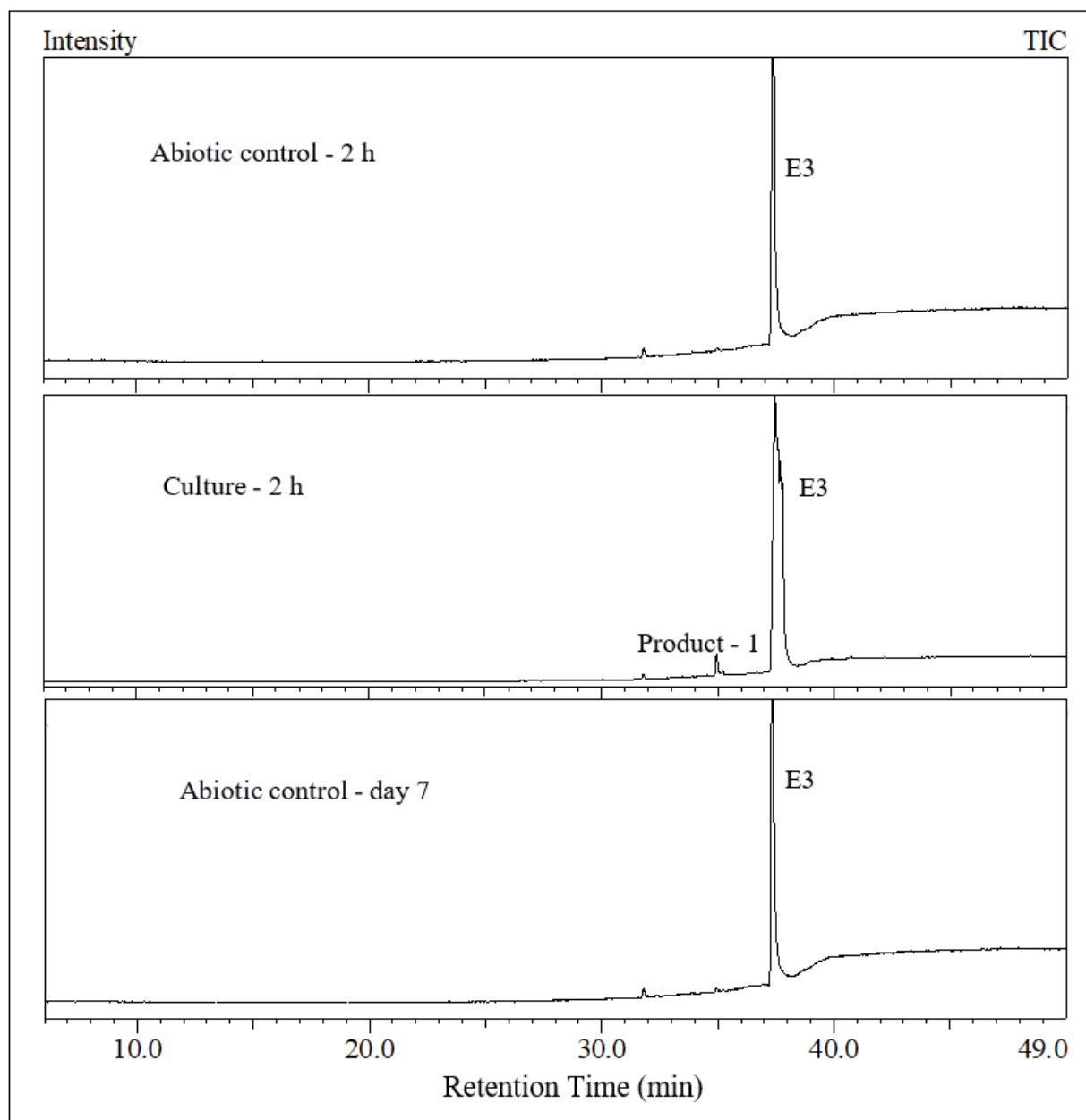


Fig. 4.28: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-11 cultures grown in CDM containing 0.1 mM E3 and incubated for different time intervals. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).



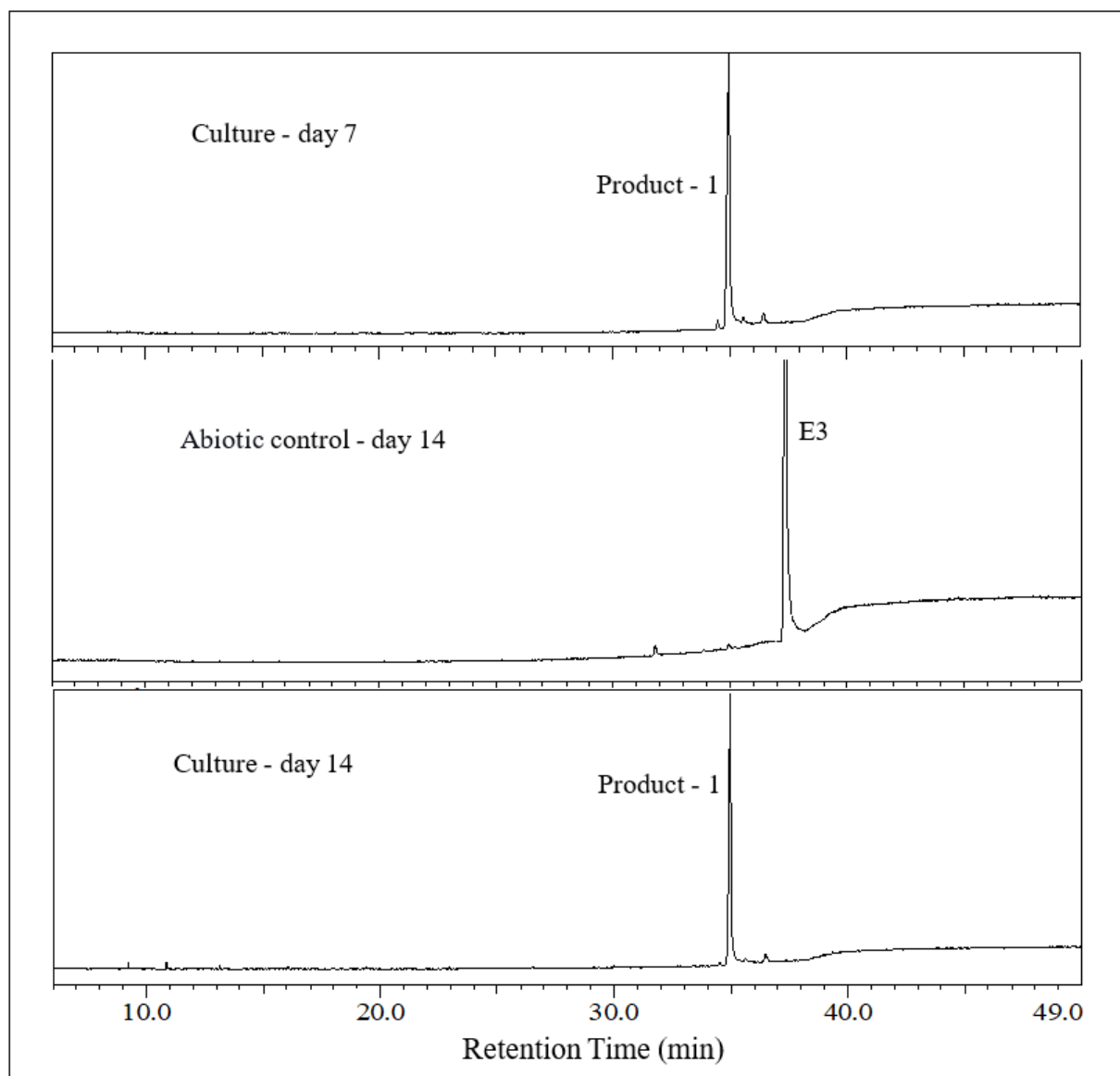
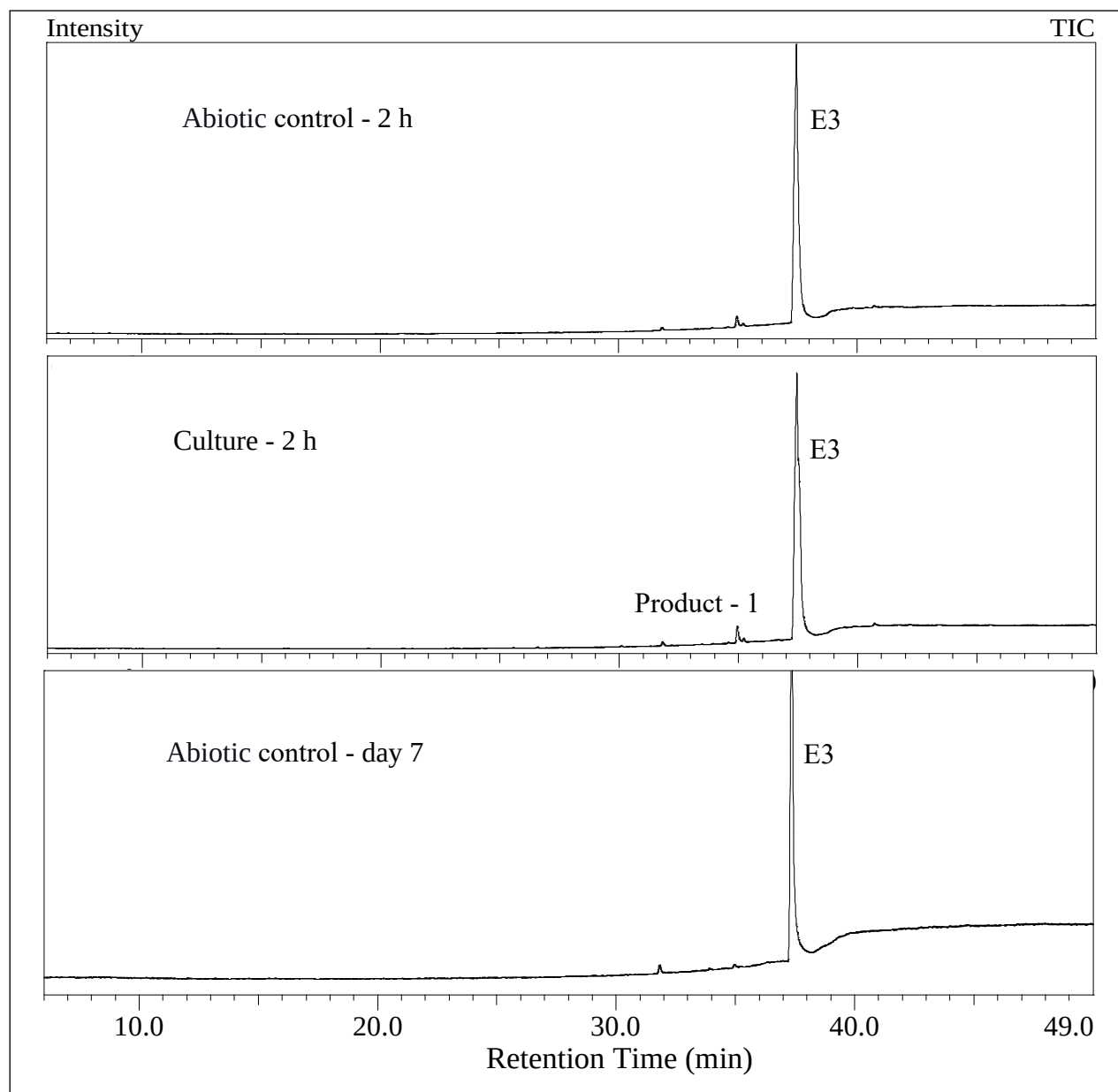


Fig. 4.29: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-16 cultures grown in CDM containing 0.1 mM E3 and incubated for different time intervals. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).



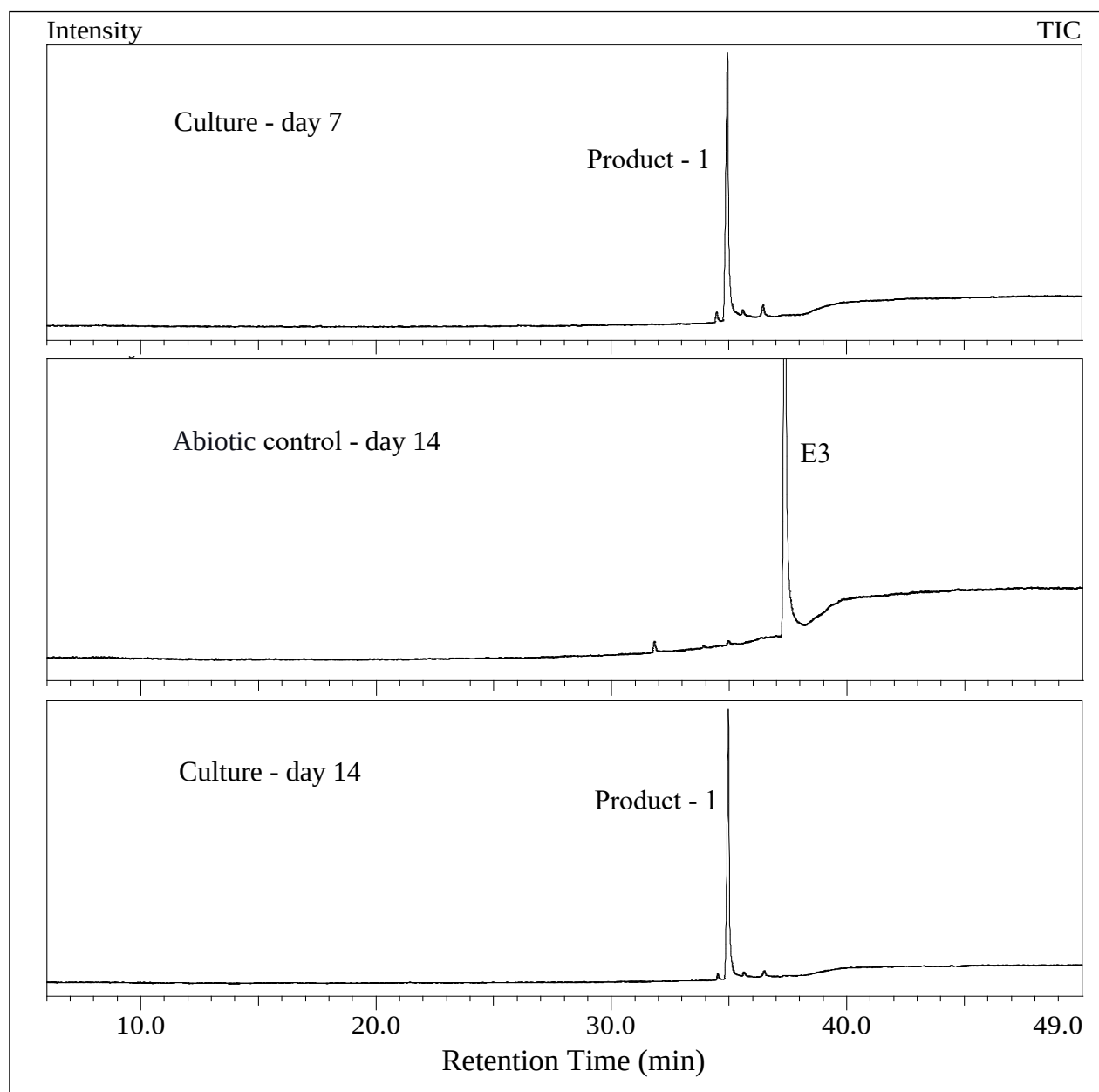
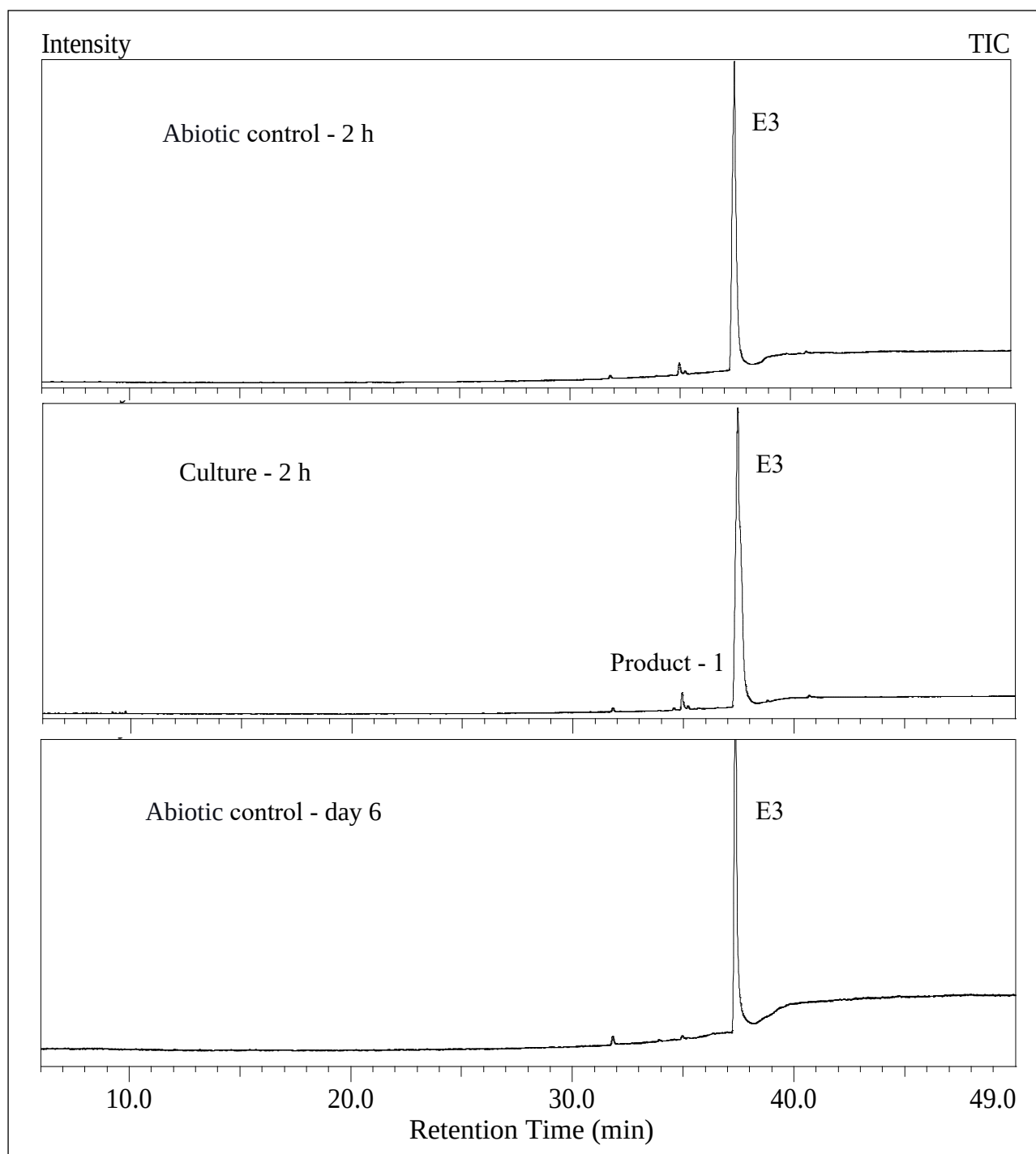


Fig. 4.30: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-30 cultures grown in CDM containing 0.1 mM E3 and incubated for different time intervals. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).



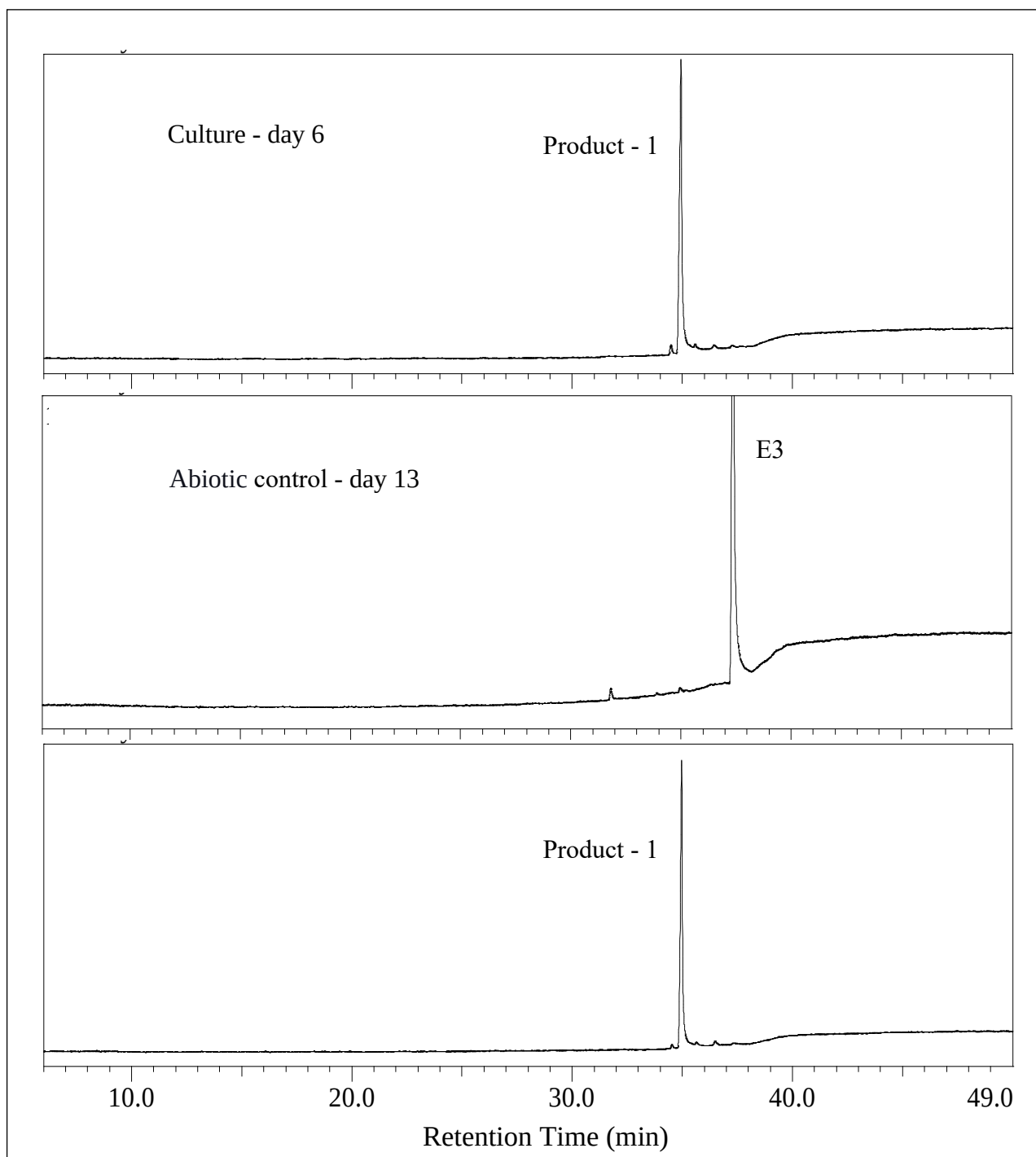
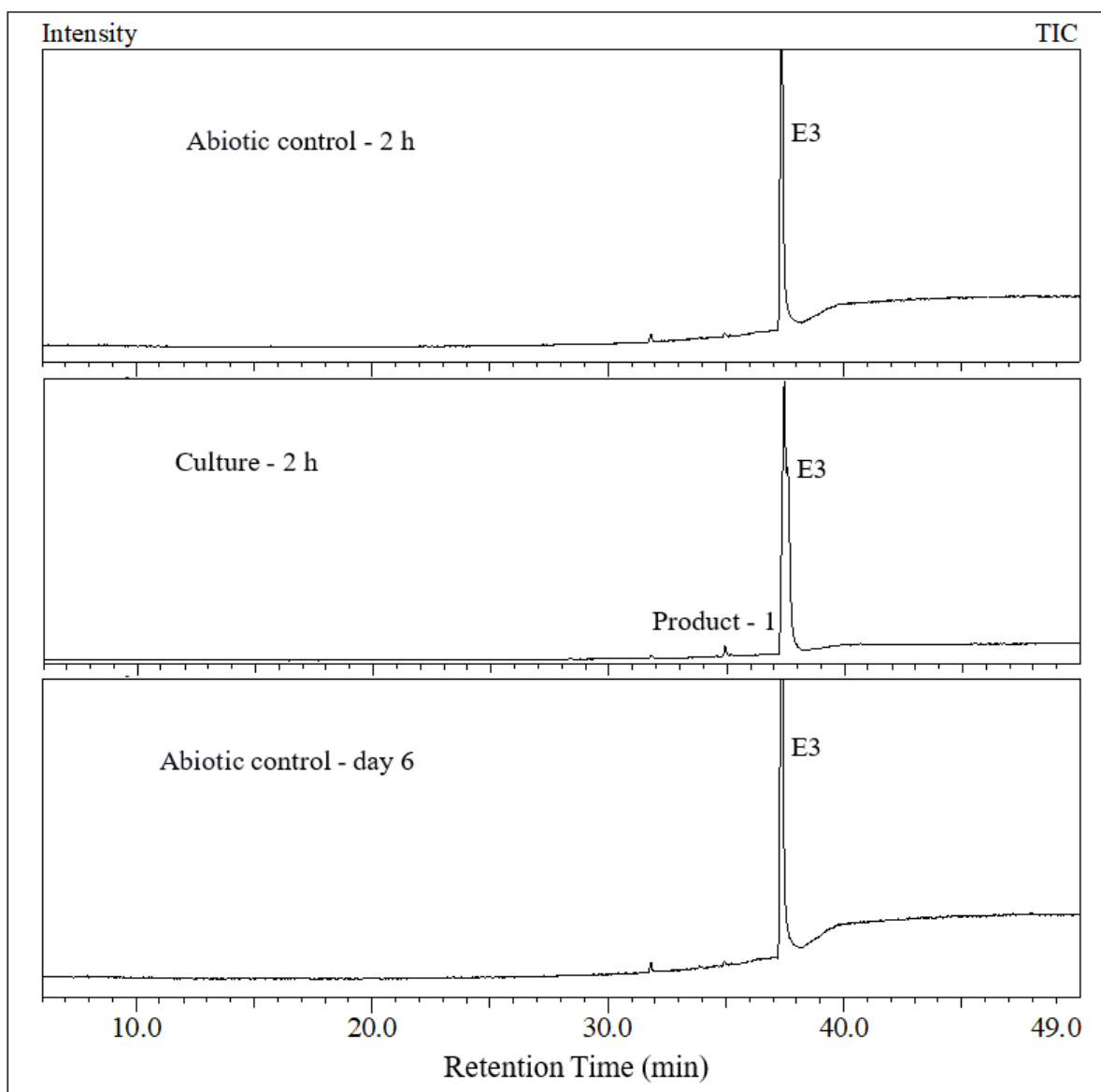


Fig. 4.31: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain IF-20 cultures grown in CDM containing 0.1 mM E3 and incubated for different time intervals. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).



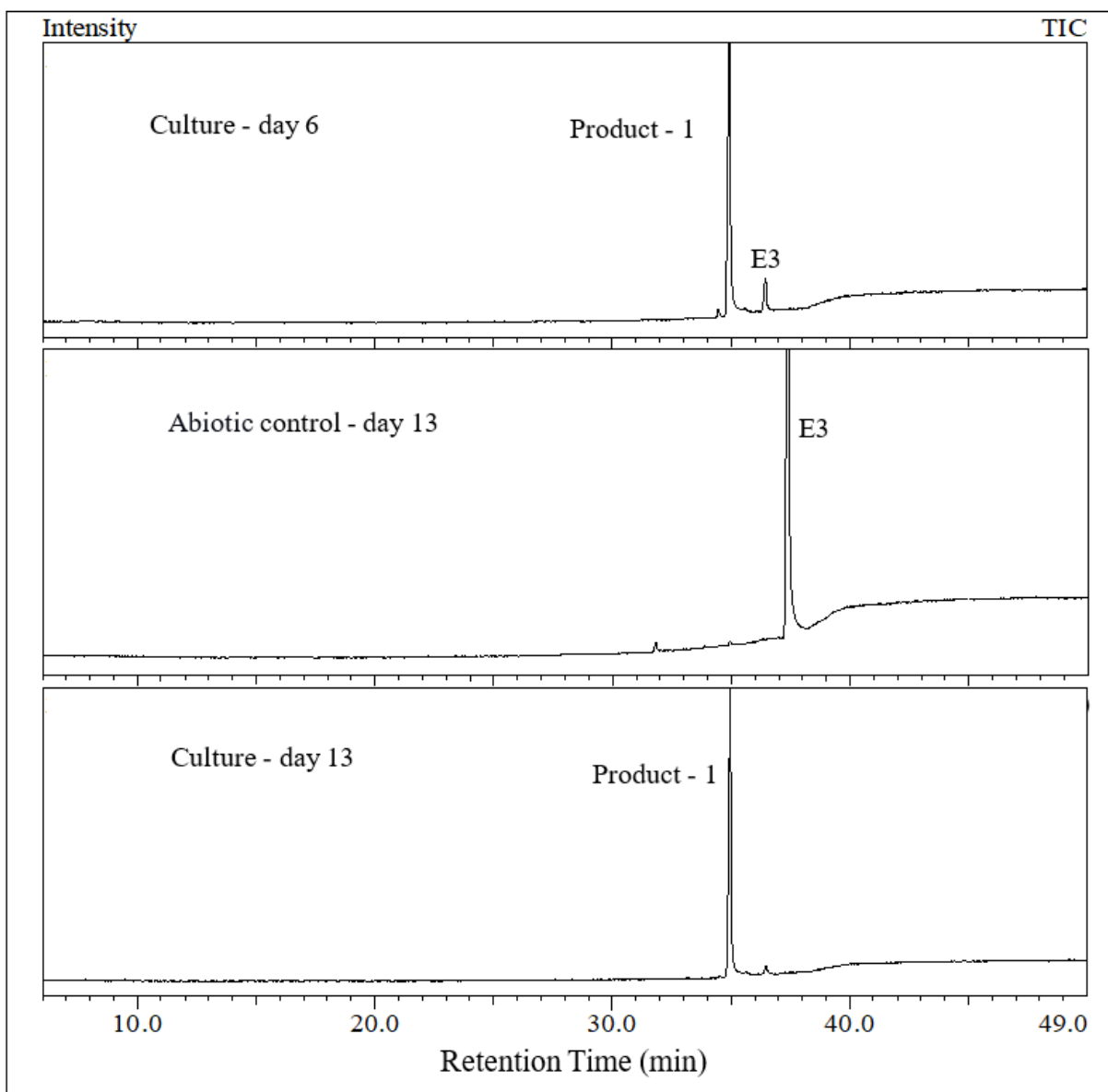


Fig. 4.32: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain AS-31 cultures grown in CDM containing 0.1 mM E3 and incubated for different time intervals. E3 was added from a stock solution in DMSO. All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

Assuming that DMSO could have an inhibitory effect on the growth of the isolates, they were cultured again in CDM supplemented with E3 as solid crystals (without DMSO). As shown in Figure 4.33, the growth profiles were similar to those observed in cultures containing E3/DMSO. In both cases, only one isolate exhibited minor increase in the OD₆₀₀. Furthermore, all cultures transformed E3 into product - 1 as observed before. However, the kinetics of product-1- formation were different among the isolates (Figures 4.34-4.38).

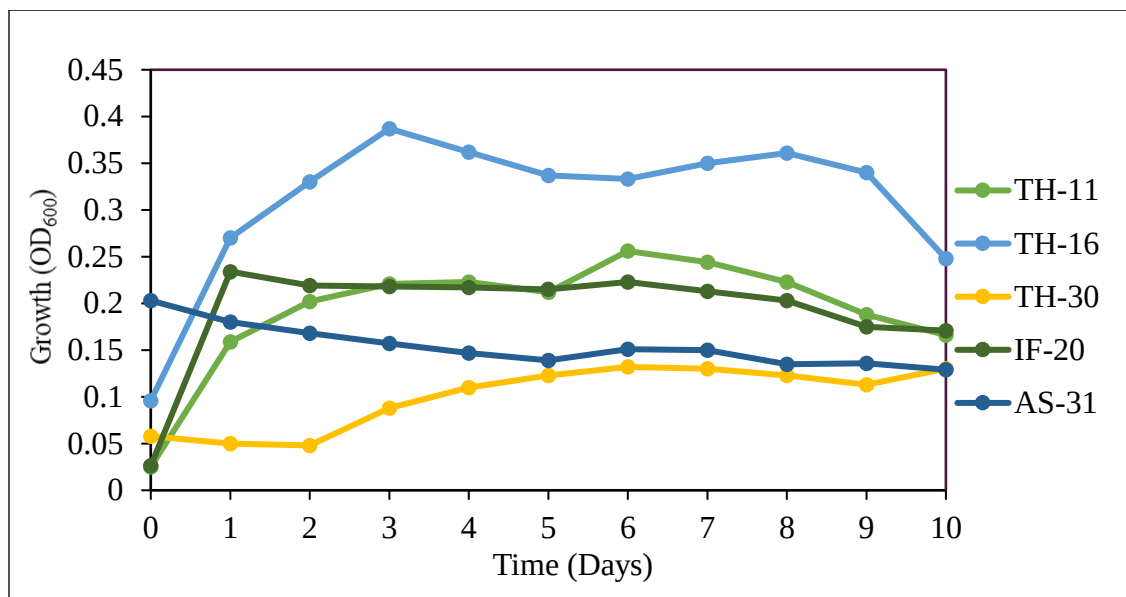


Fig. 4.33: Growth profiles of the isolated strains in CDM containing E3 crystals (50 mg/L). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

In cultures of the isolates TH-11, TH-16, and TH-30, product -1 was detectable after 5 h of incubation, while in cultures of IF-20 and AS-31 it appeared in culture samples after 12 days. Product -2 was not detected in the AS-31 cultures. Instead a new product designated product-3, was detected after 12 days of incubation. This product appeared at retention time 36.5 min, after product -1 and has a BasePeak at m/z 214.05 and predicted molecular mass of 284. The closest matches obtained from

the spectral library have the core estrogen ring structure. In addition, one of the matches reveals cleavage of the ring structure. In all cases, the similarity index was relatively low (67-77). From the mass spectrum of product-3 it can be envisaged that it is 4 mass units smaller than E3 and could be another oxidation/dehydrogenation product (Figure 4.39).

Eventually, all isolates were cultured in CDM containing 1% DMSO to check whether they can utilize it as a sole carbon source. None of the tested isolates could grow under these conditions, with the exception of minor increase in OD₆₀₀ observed in the TH-11 culture (Figure 4.40). Altogether, these findings indicate that the tested 5 bacterial isolates don't grow on E3 as a sole carbon source. Nonetheless, they can transform it into one main product (product - 1) and two other minor products.

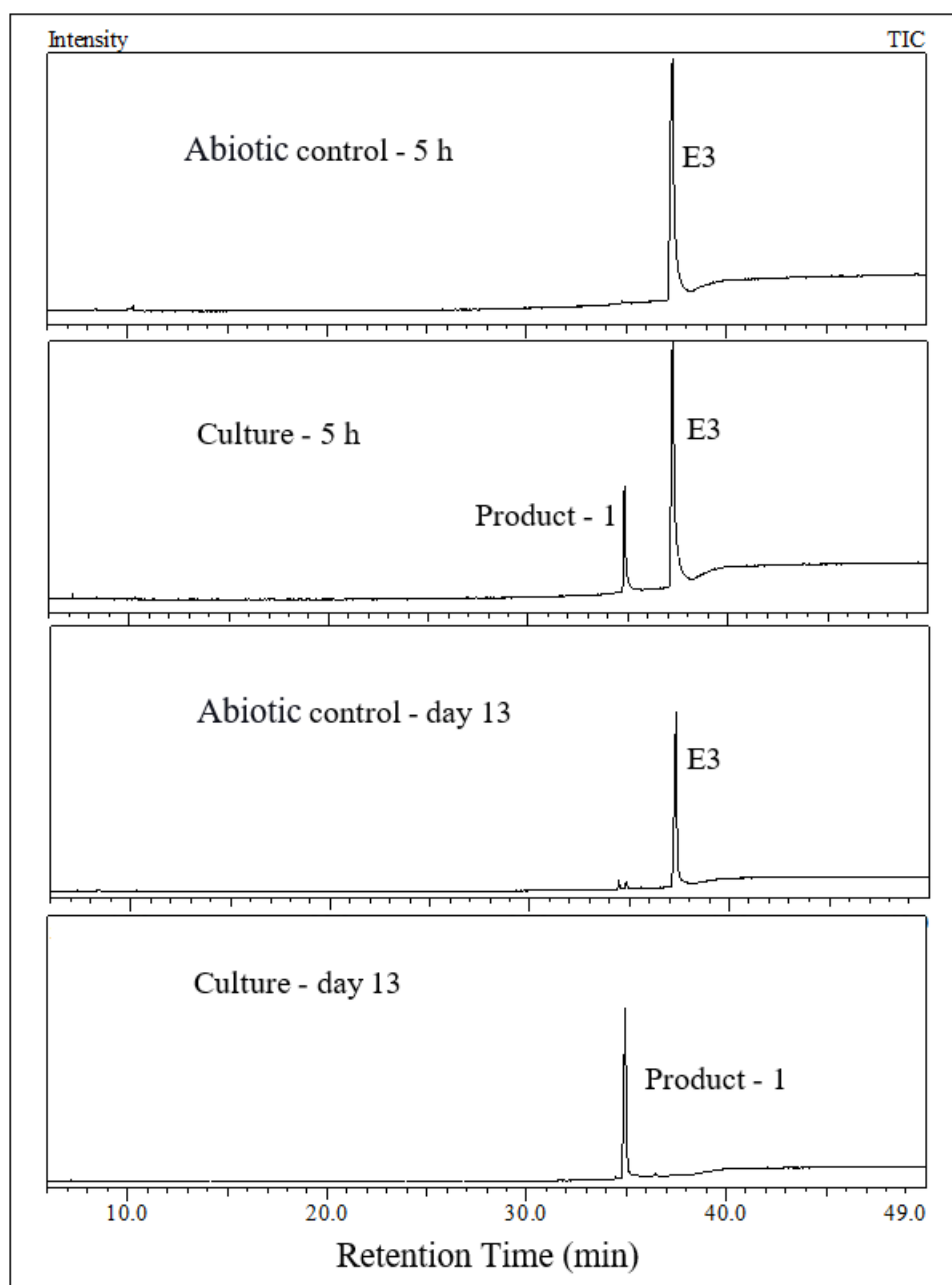


Fig. 4.34: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-11 cultures grown in CDM containing 50 mg/L E3 and incubated for different time intervals. E3 was added as crystals (without DMSO). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

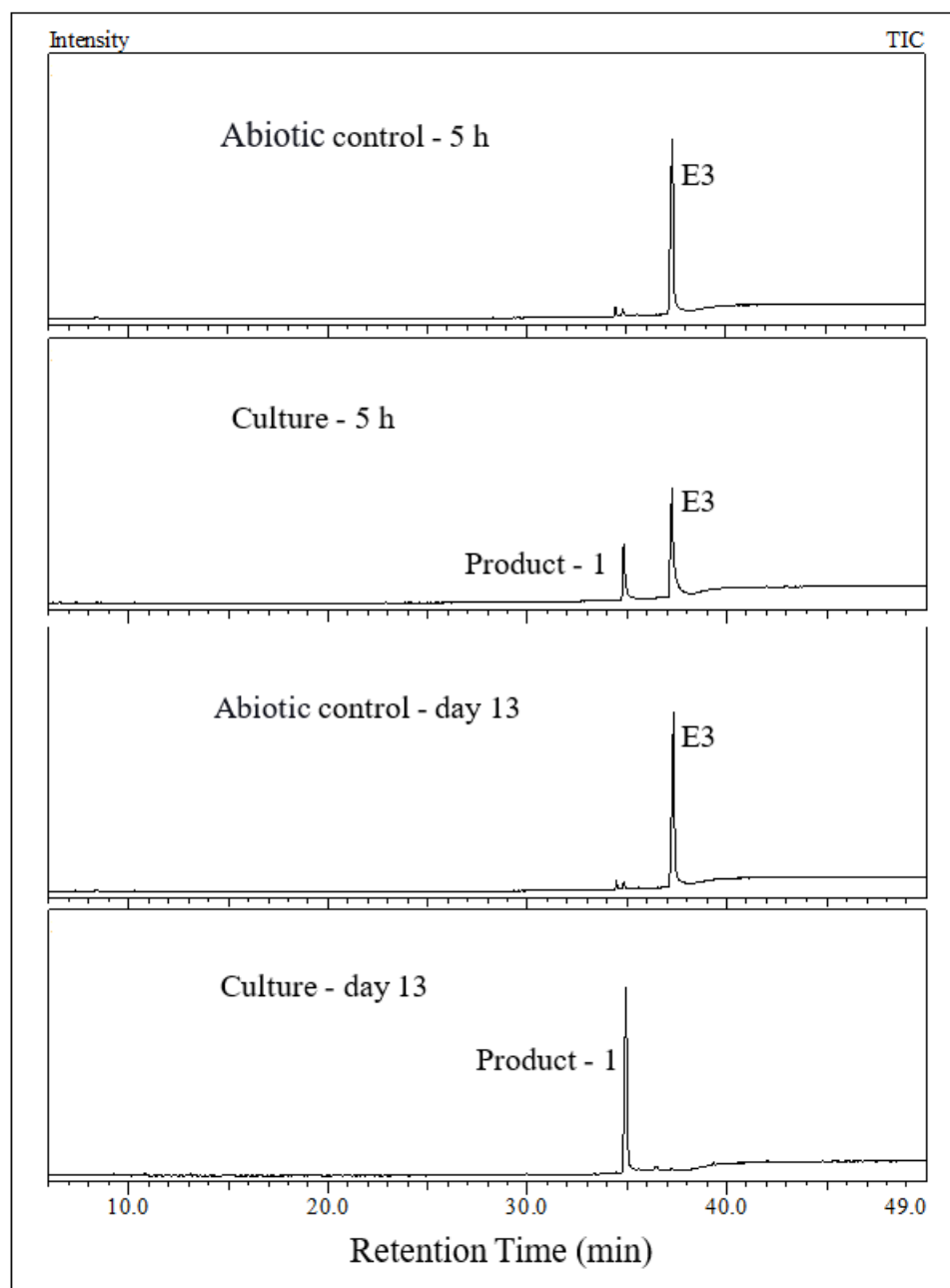


Fig. 4.35: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-16 cultures grown in CDM containing 50 mg/L E3 and incubated for different time intervals. E3 was added as crystals (without DMSO). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

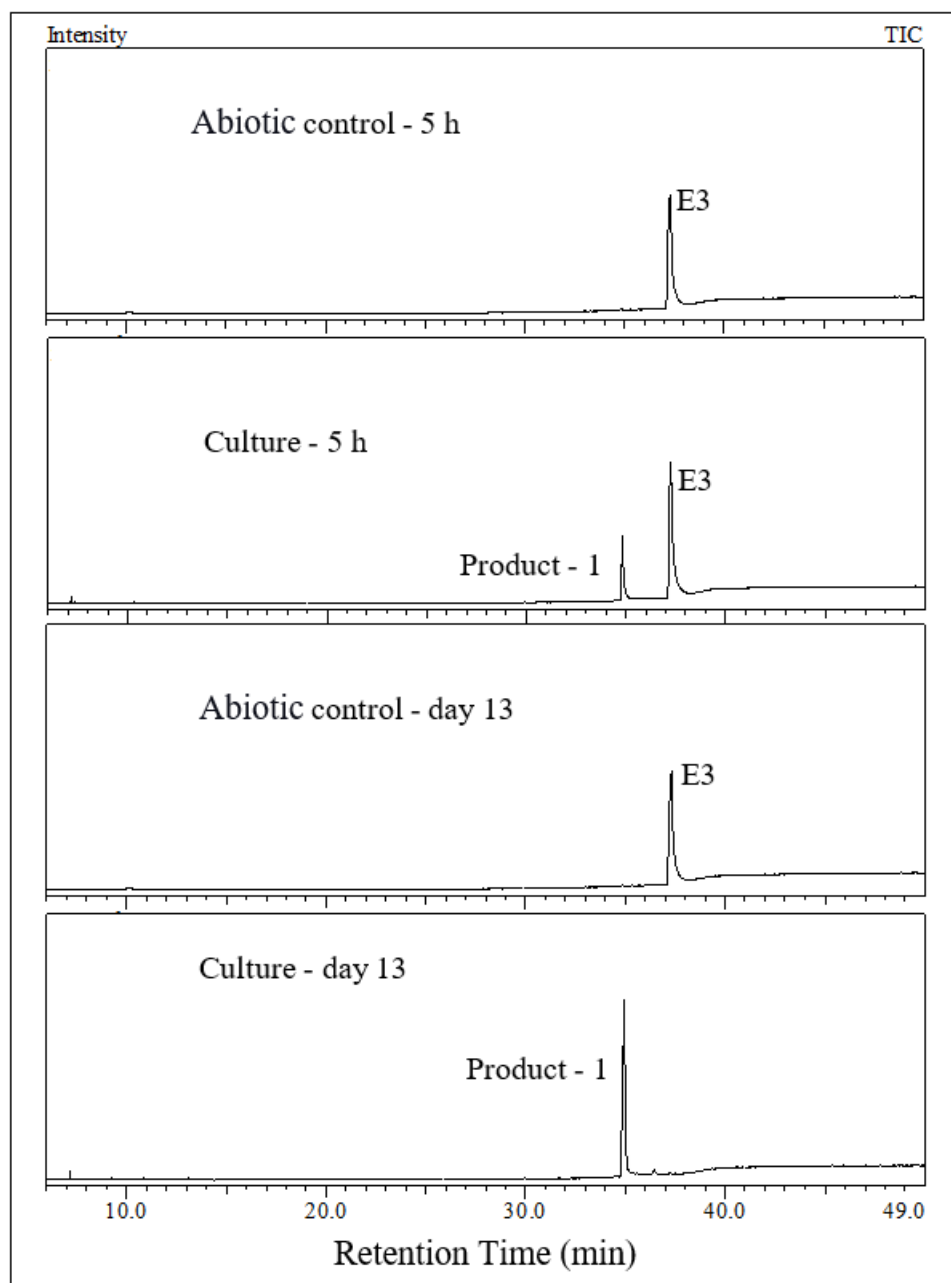


Fig. 4.36: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain TH-30 cultures grown in CDM containing 50 mg/L E3 and incubated for different time intervals. E3 was added as crystals (without DMSO). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

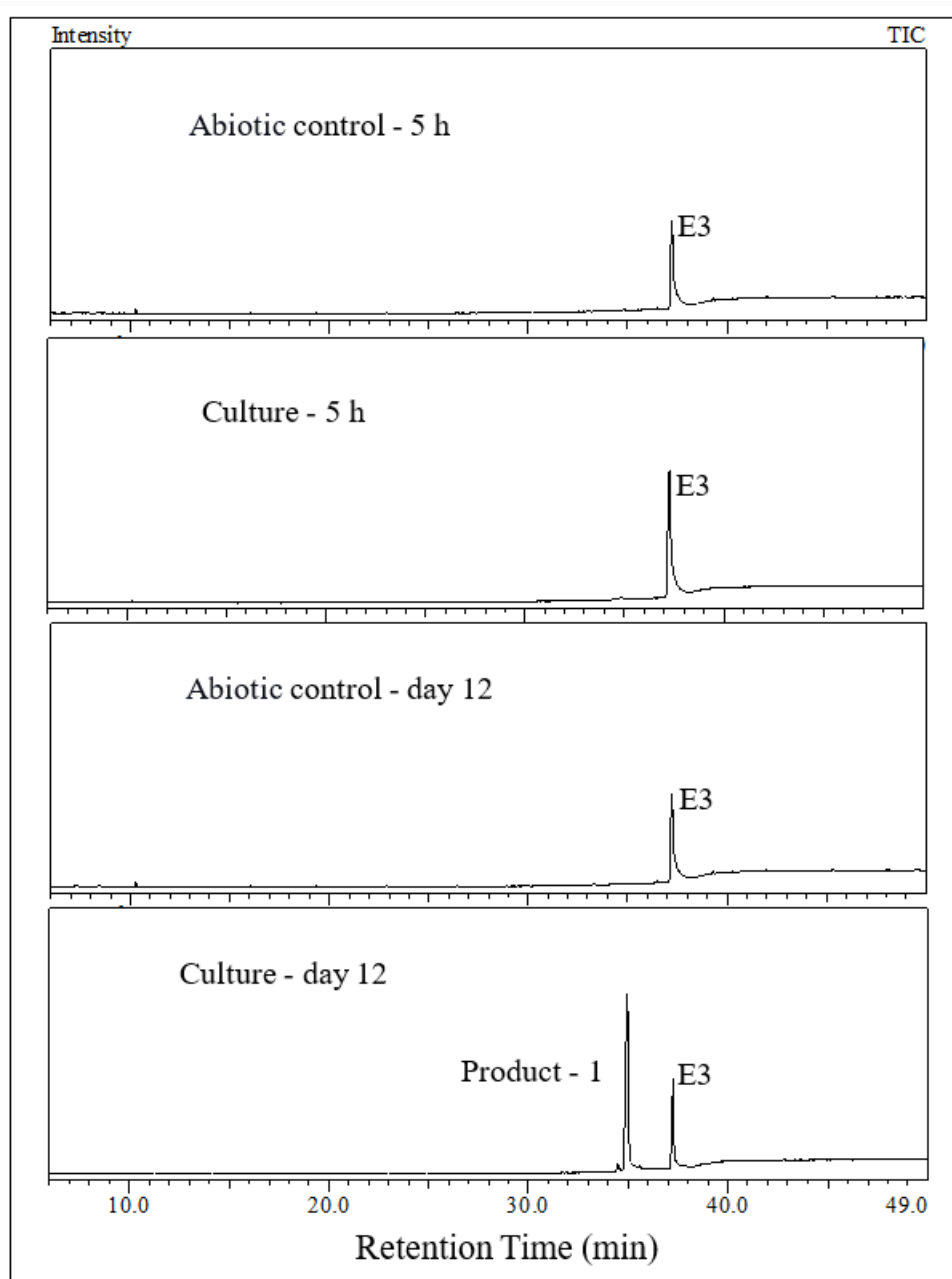


Fig. 4.37: Total ion chromatograms from GC-MS analysis showing transformation of E3 to unknown products by strain IF-20 cultures grown in CDM containing 50 mg/L E3 and incubated for different time intervals. E3 was added as crystals (without DMSO). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

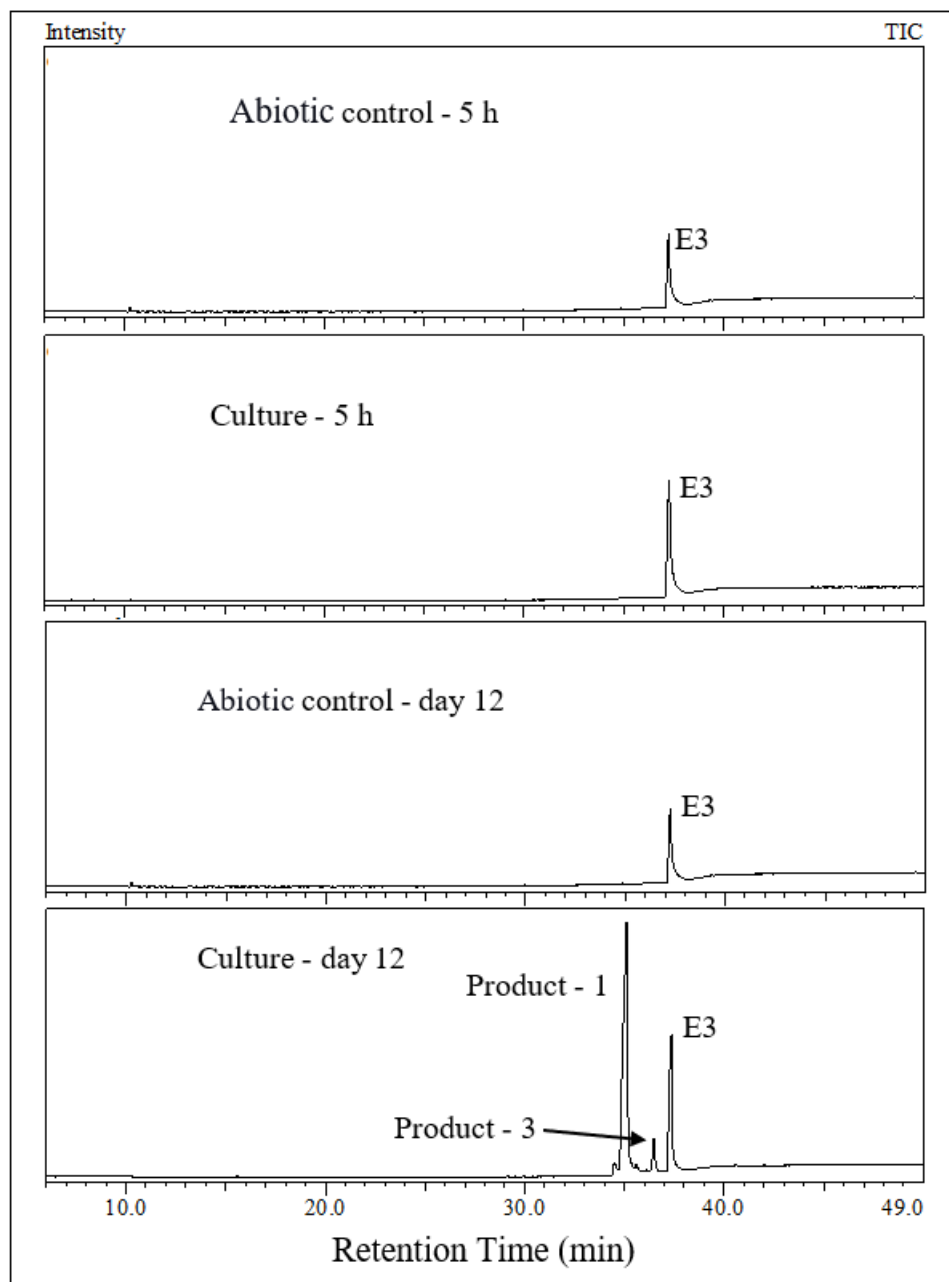


Fig. 4.38: Total ion chromatograms from GC-MS analysis showing the transformation of E3 to unknown product - 1 and an additional product, product - 2, by strain AS-31. Cultures grown in CDM containing 50 mg/L E3 and incubated for different time intervals. E3 was added as crystals (without DMSO). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

Unknown product - 3

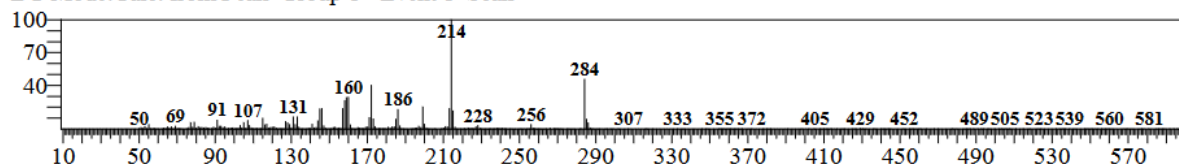
Library

<< Target >>

Line#:4 R.Time:36.475(Scan#:6096) MassPeaks:357

RawMode:Averaged 36.470-36.480(6095-6097) BasePeak:214.05(106875)

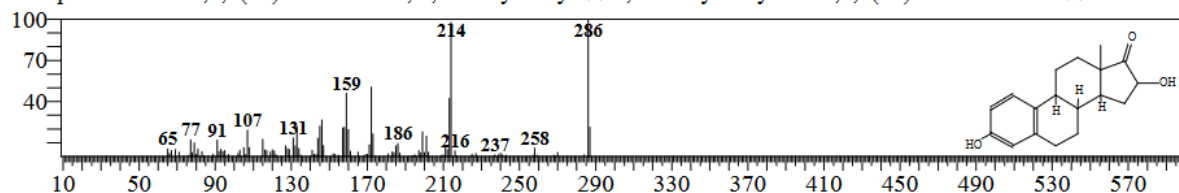
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



Hit#:1 Entry:129766 Library:NIST17-1.lib

SI:77 Formula:C₁₈H₂₂O₃ CAS:18186-49-7 MolWeight:286 RetIndex:2341

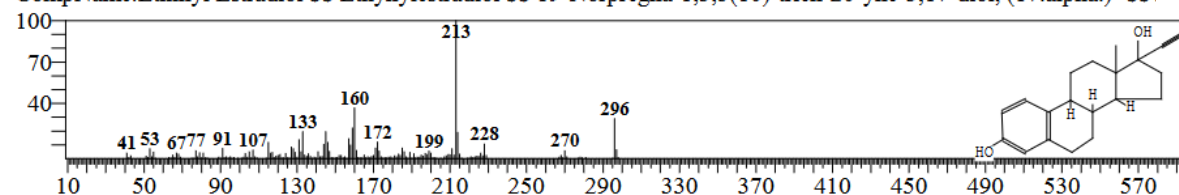
CompName:Estra-1,3,5(10)-trien-17-one, 3,16-dihydroxy- SS 3,16-Dihydroxyestra-1,3,5(10)-trien-17-one # SS



Hit#:3 Entry:32755 Library:NIST17s.lib

SI:71 Formula:C₂₀H₂₄O₂ CAS:57-63-6 MolWeight:296 RetIndex:2290

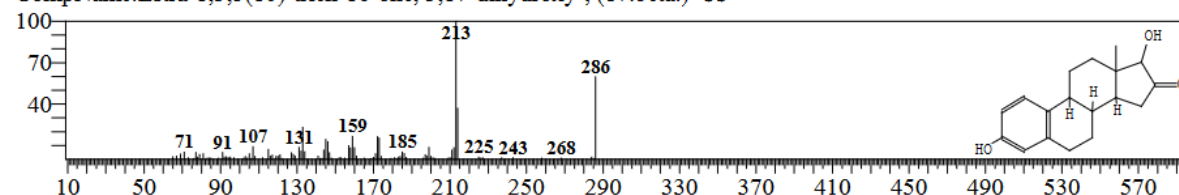
CompName:Ethinyl Estradiol SS Ethynylestradiol SS 19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol, (17.alpha.)- SS



Hit#:4 Entry:129753 Library:NIST17-1.lib

SI:70 Formula:C₁₈H₂₂O₃ CAS:566-75-6 MolWeight:286 RetIndex:2341

CompName:Estra-1,3,5(10)-trien-16-one, 3,17-dihydroxy-, (17.beta.)- SS



Hit#:9 Entry:127473 Library:NIST17-1.lib

SI:67 Formula:C₁₇H₁₆O₄ CAS:0-00-0 MolWeight:284 RetIndex:2334

CompName:.alpha.-Pyrone, 4-butanoyloxy-6-(2-phenylethenyl)- S

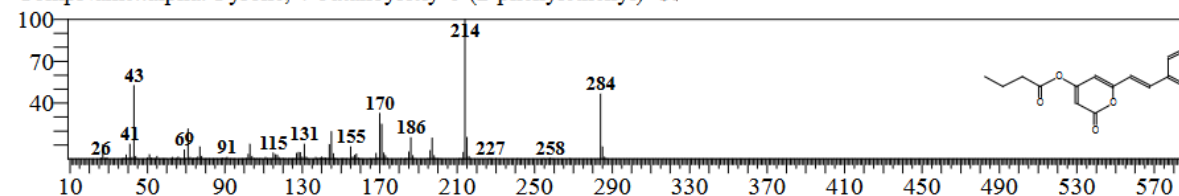


Fig. 4.39: Predicted mass spectrum of product-3 and matching mass spectra retrieved from the GC-MS spectral library, NIST17-1.

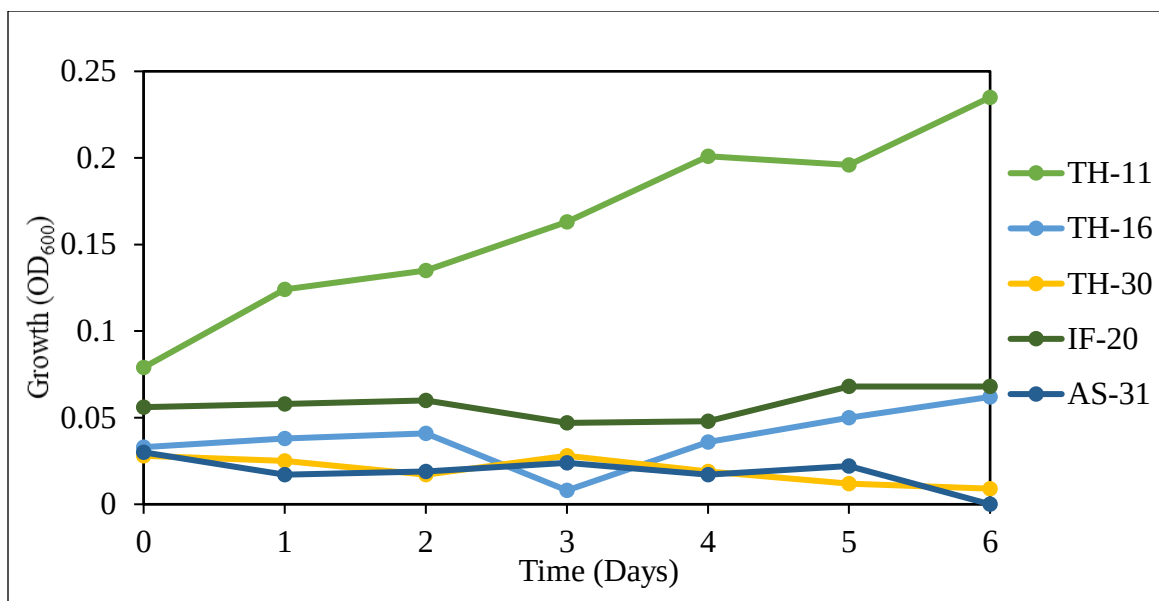


Fig. 4.40: Growth profiles of the isolated strains on 1% (v/v) DMSO in CDM.

4.5. Identification of the E3-transforming isolates

Genomic DNA was extracted from the five E3- transforming isolates and used as a template to amplify the 16S rRNA gene (Figure 4.41, Table 4.1). The amplicons were then purified, sequenced and the sequences were analyzed for identification of the isolates. The sequence and closest match for each isolate are shown in the appendix. Phylogenetic analysis further confirmed the identity of isolates (Figures 4.42-4.44). The IF-20 isolate was most closely related to *Microbacterium* spp. with the highest % identity to *Microbacterium schleiferi* DSM 20489, whereas the AS-31 isolate was 98.48% identical to *Hydrogenophaga intermedia* DSMZ 5680. On the other hand, all three isolates obtained from the thickened sludge were affiliated with the genus *Gordonia* and specifically to *G. cholesterolivorans* and *G. sehwensis*. However, the % identity to the closest matches was different with the isolate TH-16 exhibiting the lowest (97.29- 97.43 %).

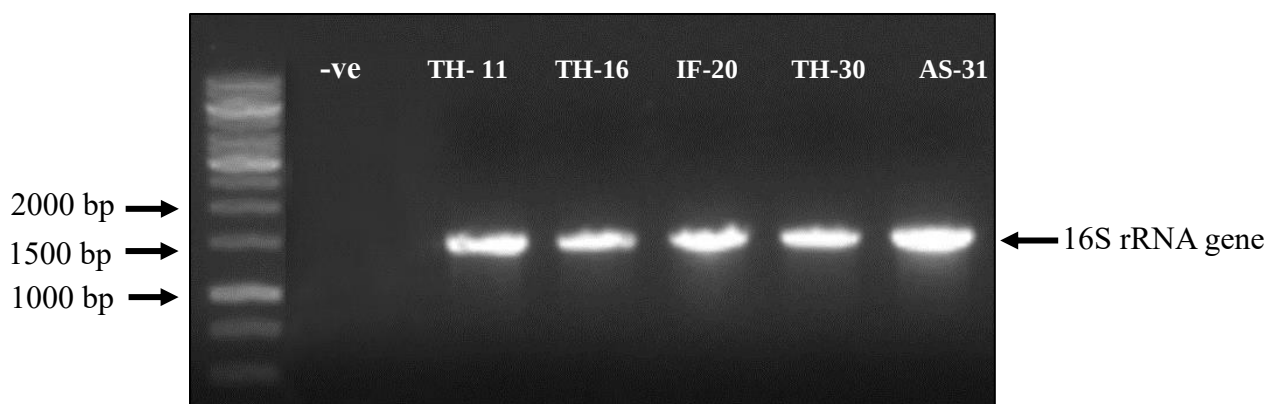


Fig. 4.41: Agarose gel (1%) showing 16S rRNA genes amplified by PCR with universal primers from the genomic DNA extracted from the E3-transforming strains the strains TH-11, TH-16, IF-20, TH-30, and AS-31. -ve: a negative (no-template) control.

Table (4.1): Quantification of the 16S rRNA gene products amplified from the isolated strains.

Isolate	Nanodrop				Fluorometer
	Conc. (ng/ μ L)	A ₂₆₀	A ₂₆₀ /A ₂₃₀	A ₂₆₀ /A ₂₈₀	Conc. (ng/ μ L)
Strain TH-11	83.08	1.662	2.13	1.90	80.8
Strain TH-16	64.31	1.286	2.10	1.92	63.6
Strain IF-20	102.50	2.050	2.11	1.87	98.0
Strain TH-30	66.55	1.331	2.14	1.92	66.0
Strain AS-31	115.01	2.300	2.05	1.87	116

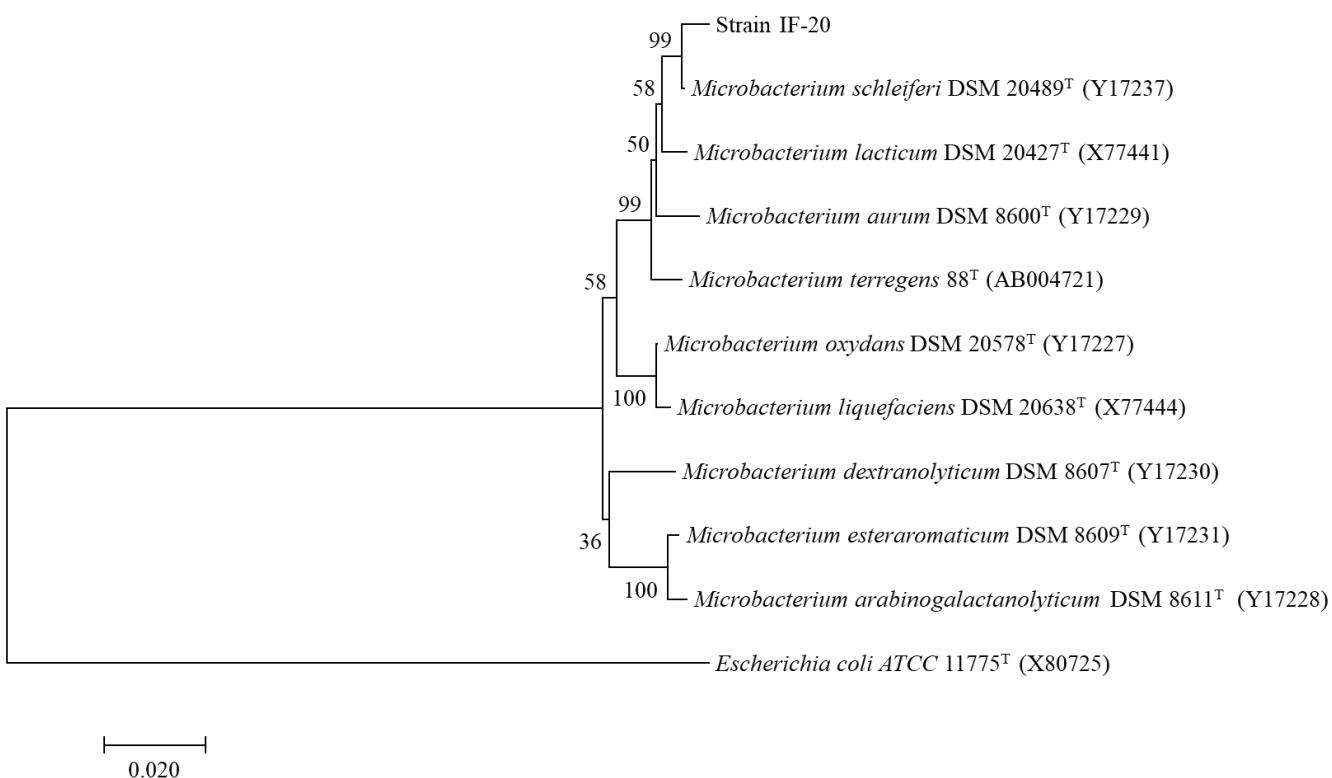


Fig. 4.42: Phylogenetic tree based on 1431 nucleotides of the 16S rRNA gene sequences indicating the position of strain IF-20 among related members of the genus *Microbacterium*. Reference type strains are included, and GenBank sequence accession numbers are given between parentheses. *Escherichia coli* was used as an out group. Bootstrap values, expressed as percentage of 1000 replications, are given at the nodes. Bar: two substitutions per 100 nucleotides.

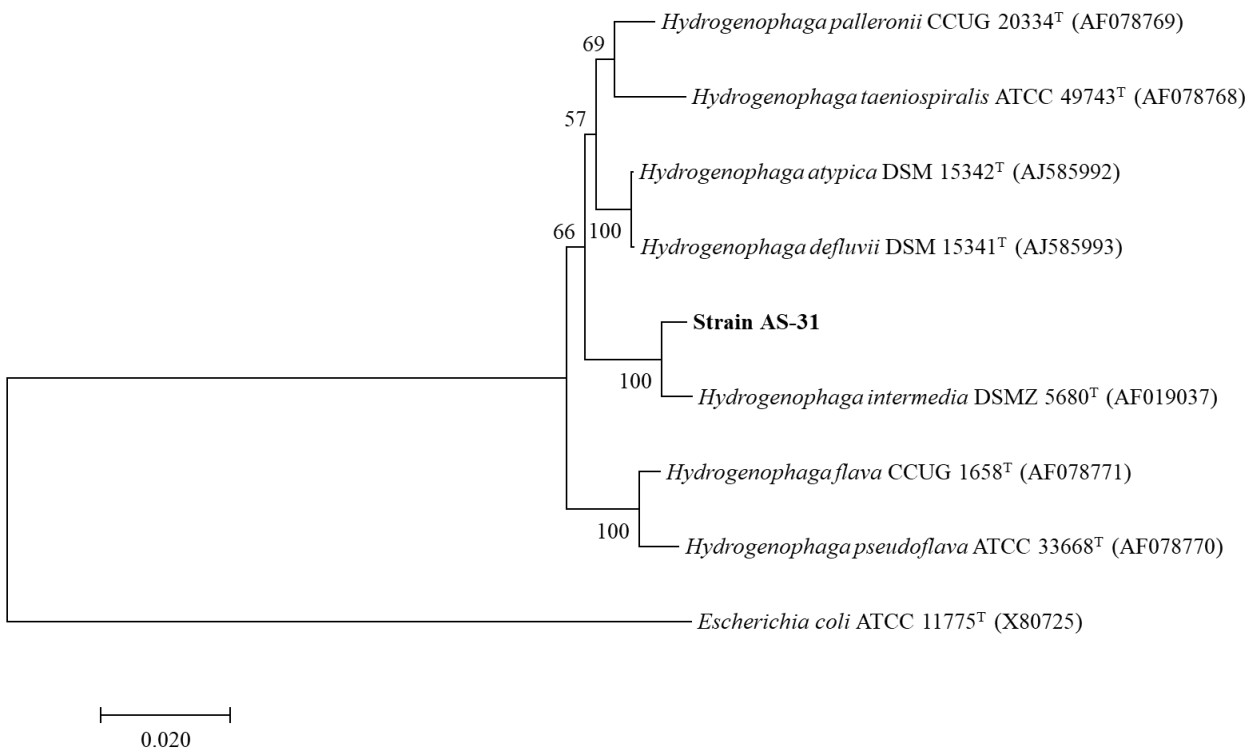


Fig. 4.43: Phylogenetic tree based on 1448 nucleotides of the 16S rRNA gene sequences indicating the position of strain AS-31 among related members of the genus *Hydrogenophaga*. Reference type strains are included, and GenBank sequence accession numbers are given between parentheses. *Escherichia coli* was used as an out group. Bootstrap values, expressed as percentage of 1000 replications, are given at the nodes. Bar: two substitutions per 100 nucleotides.

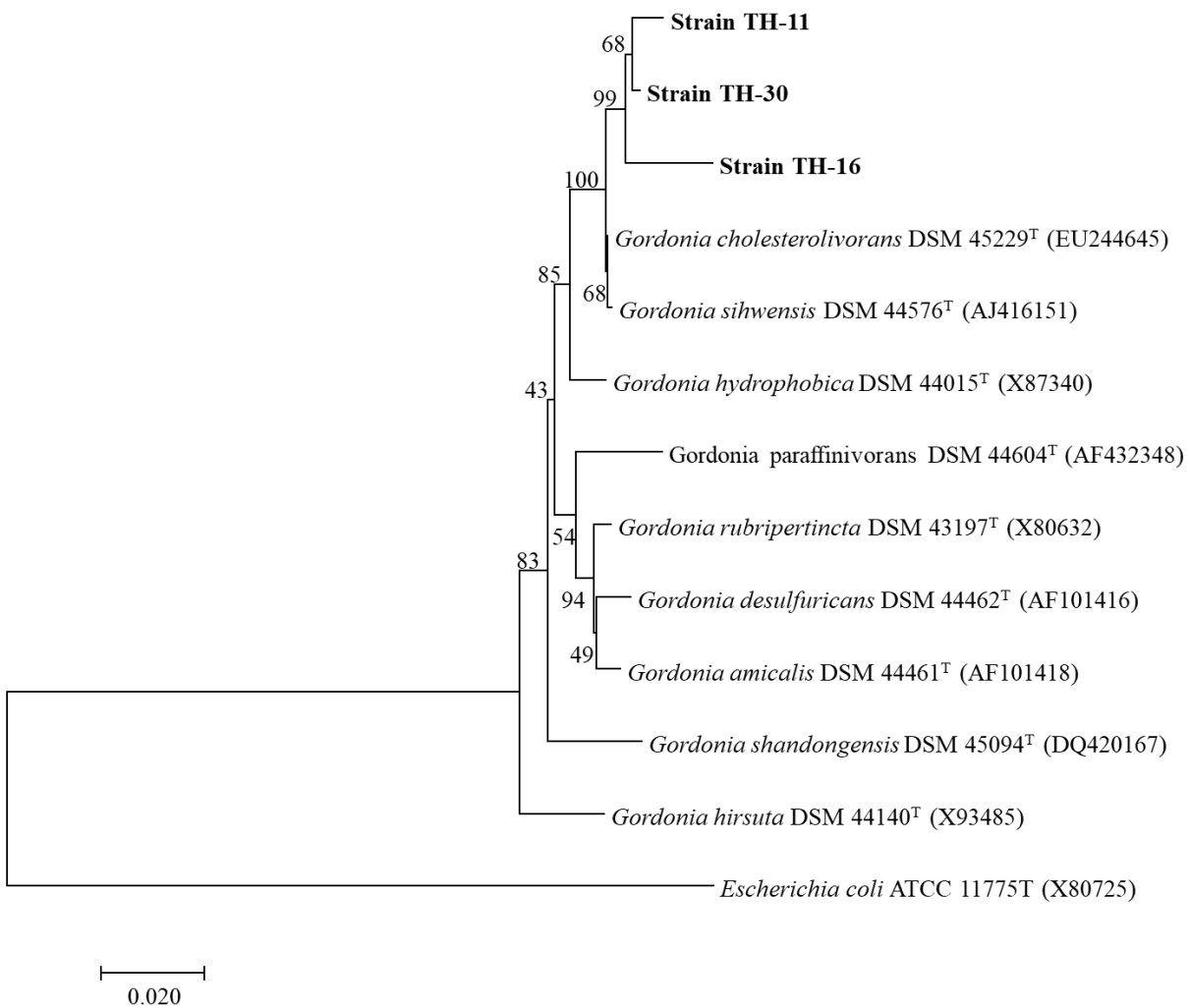


Fig. 4.44: Phylogenetic tree based on 1437, 1446 and 1445 nucleotides of the 16S rRNA gene sequences indicating the position of strains TH-11, TH-16 and TH-30, respectively, among related members of the genus *Gordonia*. Reference type strains are included, and GenBank sequence accession numbers are given between parentheses. *Escherichia coli* was used as an out group. Bootstrap values, expressed as percentage of 1000 replications, are given at the nodes. Bar: two substitutions per 100 nucleotides.

4.6. Growth of the E3- degrading consortia on glycerol

The finding that the isolated five bacterial strains cannot grow on E3 as a sole carbon source, raised questions on the growth profiles of the consortia in CDM containing E3 (Figure 4.16). Despite the observation that E3 in those cultures disappeared with time, this could be due to biotransformation and the observed growth can be on other carbon sources in the medium. The culture medium contains DMSO (solvent of E3) and glycerol (carry over in the inoculum from the frozen stocks). To find out whether the observed growth of the consortia was on glycerol, the consortia were cultured in CDM without the addition of any exogenous carbon sources. This approach made glycerol carry over from the inocula as the sole carbon source. As shown in Figure 4.45, the three consortia grew well in these cultures, indicating that they can utilize glycerol as a carbon and energy source. The growth profiles appeared very similar to those of cultures contains E3/ DMSO (Figure 4.16), in addition to glycerol. Based on these findings, it can be hypothesized that the bacterial consortia transform E3 but cannot grow and utilize it as a carbon and energy source. In this case, growth observed in the cultures of the consortia is due to glycerol utilization. Alternatively, the consortia may have bacterial species that utilize E3 and others that can utilize glycerol simultaneously. To test those hypotheses, the three consortia were cultured in CDM containing E3/DMSO after exclusion of glycerol carry over from the inoculum.

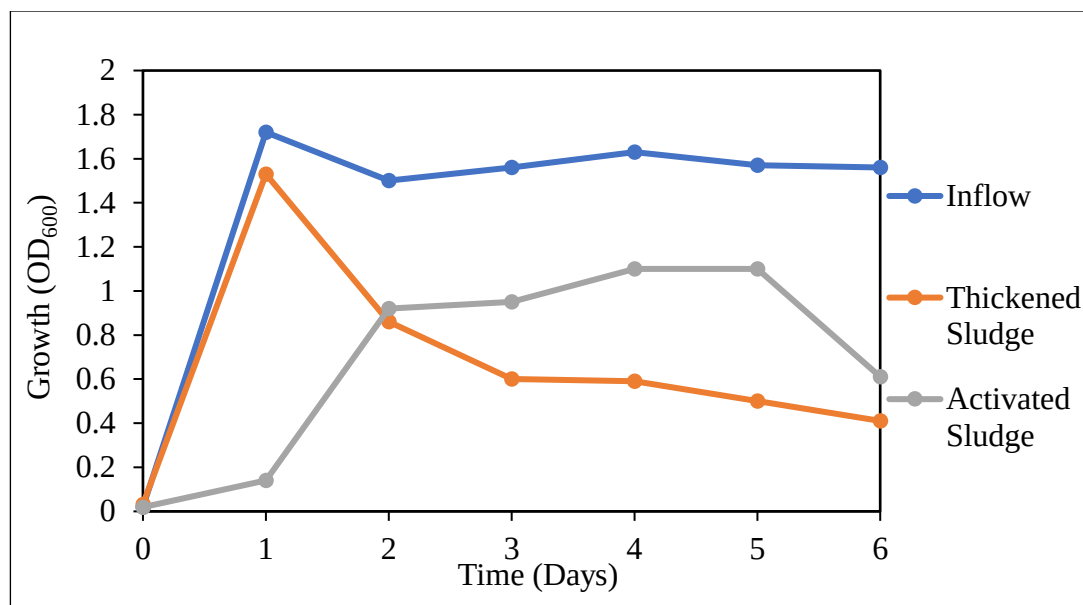


Fig. 4.45: Growth curves of the E3-degrading consortia on 0.125% glycerol (v/v). All cultures were in 100 mL CDM and were incubated at 30 °C in an orbital shaker (180 rpm).

4.7. Growth profiles of the E3-degrading consortia after glycerol exclusion

As shown in Figures 4.46, 4.48, and 4.50, all three consortia grew in glycerol-free CDM containing E3/DMSO. However, the maximum OD₆₀₀ attained by each culture decreased by ca 50%, compared to the maximum OD₆₀₀ attained in glycerol-containing cultures. Moreover, the growth profiles in glycerol-free and glycerol-containing cultures were different. Lag phases were longer in glycerol-free cultures. Nonetheless, the activated sludge consortium attained the lowest maximum OD₆₀₀ among the three consortia, resembling the trend in the glycerol-containing cultures. Based on these findings, it can be proposed that in the presence of glycerol, the three consortia grew by utilizing glycerol as a carbon source, and E3 was either only transformed or mineralized simultaneously by a different sub-population of the

consortium. To find out whether E3 was consumed in the glycerol-free cultures, culture samples were retrieved at intervals and analyzed by GC-MS. Interestingly, GC-MS analysis revealed that E3 was utilized with time in the glycerol-free cultures (Figures 4.47, 4.49, and 4.51), and product-1 was detected in the cultures of the thickened sludge consortium, similar to the glycerol-containing cultures. The absence of further specific transformation products is in favor of the postulation that E3 is utilized by the consortia as a carbon source, regardless of the presence of glycerol. This is further backed by the notion that the three consortia were enriched from the wastewater samples and utilized E3 in the absence of glycerol.

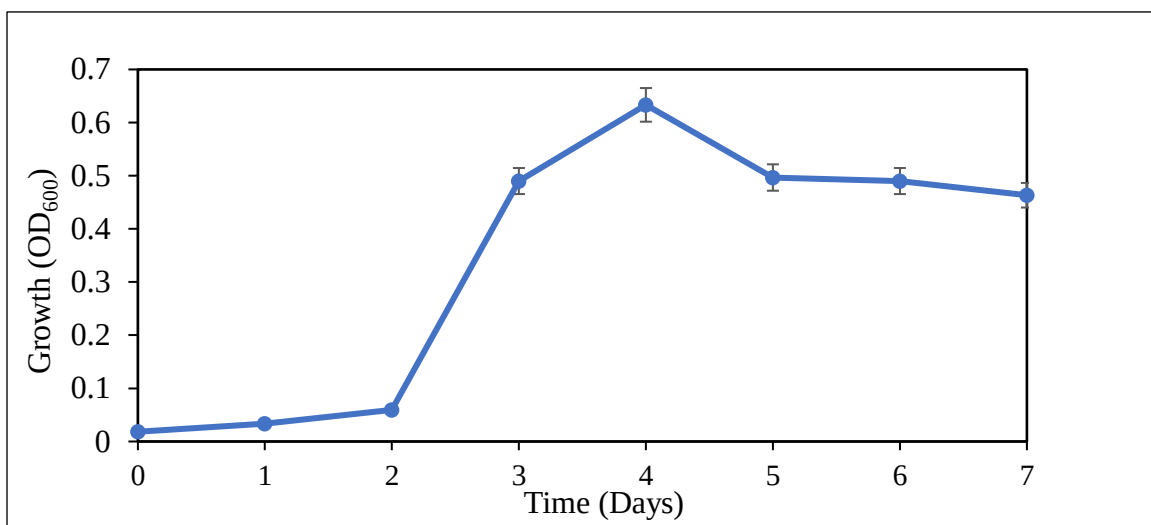


Fig. 4.46: Growth curves of the inflow consortium in 100 mL CDM containing 0.1 mM E3 at 30 °C with shaking at 180 rpm. Results are average of triplicate measurements and the error bars represent standard error. E3 was added from a stock solution in DMSO, and the inoculum was prepared from precultures grown without glycerol.

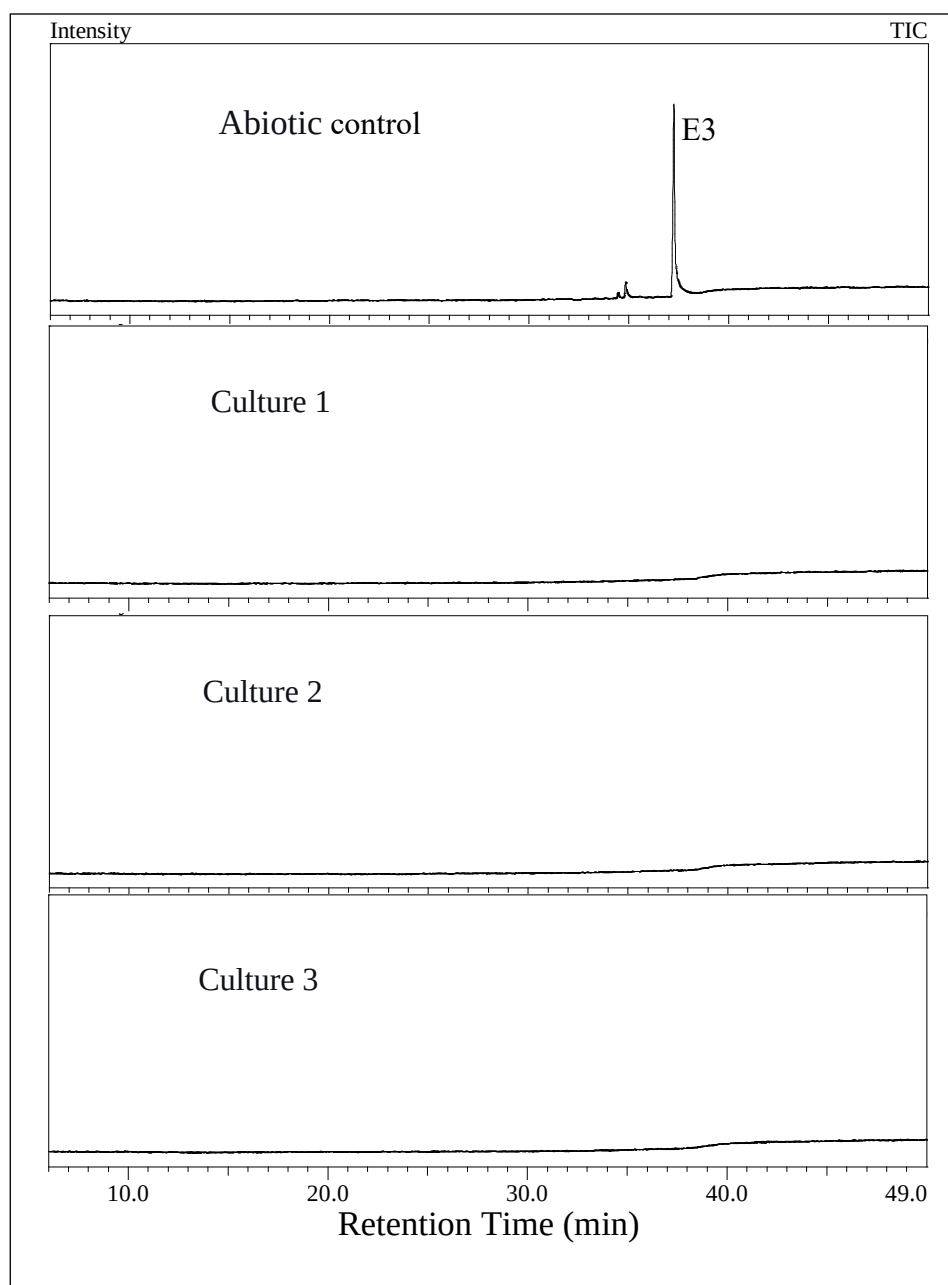


Fig. 4.47: Total ion chromatograms from GC-MS analysis showing degradation of E3 by cultures of the inflow consortium in CDM containing 0.1 mM E3, which was added from a DMSO stock solution. Results from 3 biological replicates are shown. Culture samples were retrieved after 4 days of incubation, extracted with ethyl acetate and analyzed by GC-MS.

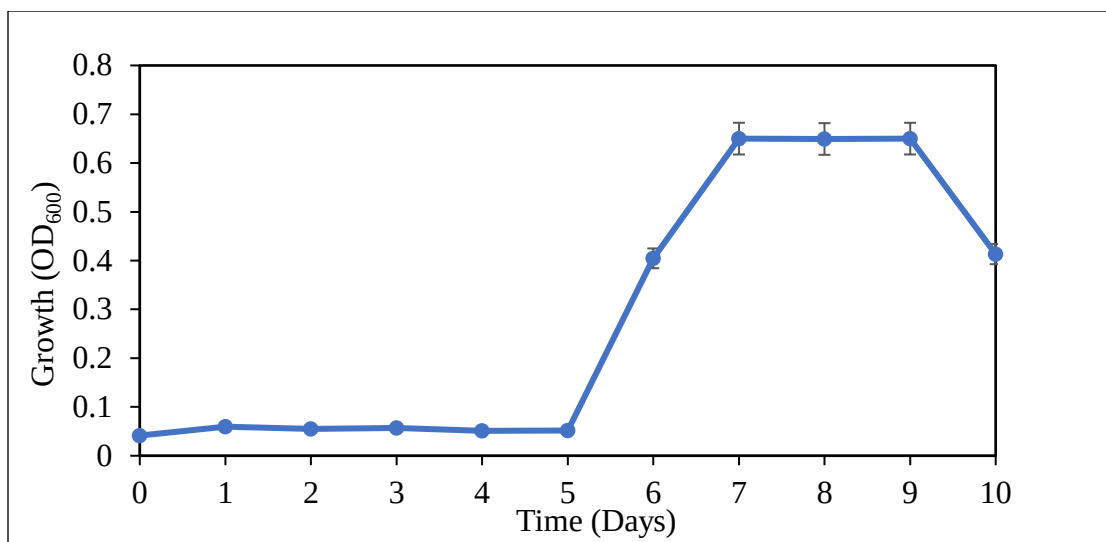


Fig. 4.48: Growth curves of the thickened sludge consortium in 100 mL CDM containing 0.1 mM E3 at 30 °C with shaking at 180 rpm. Results are average of triplicate measurements and the error bars represent standard error. E3 was added from a stock solution in DMSO, and the inoculum was prepared from precultures grown without glycerol.

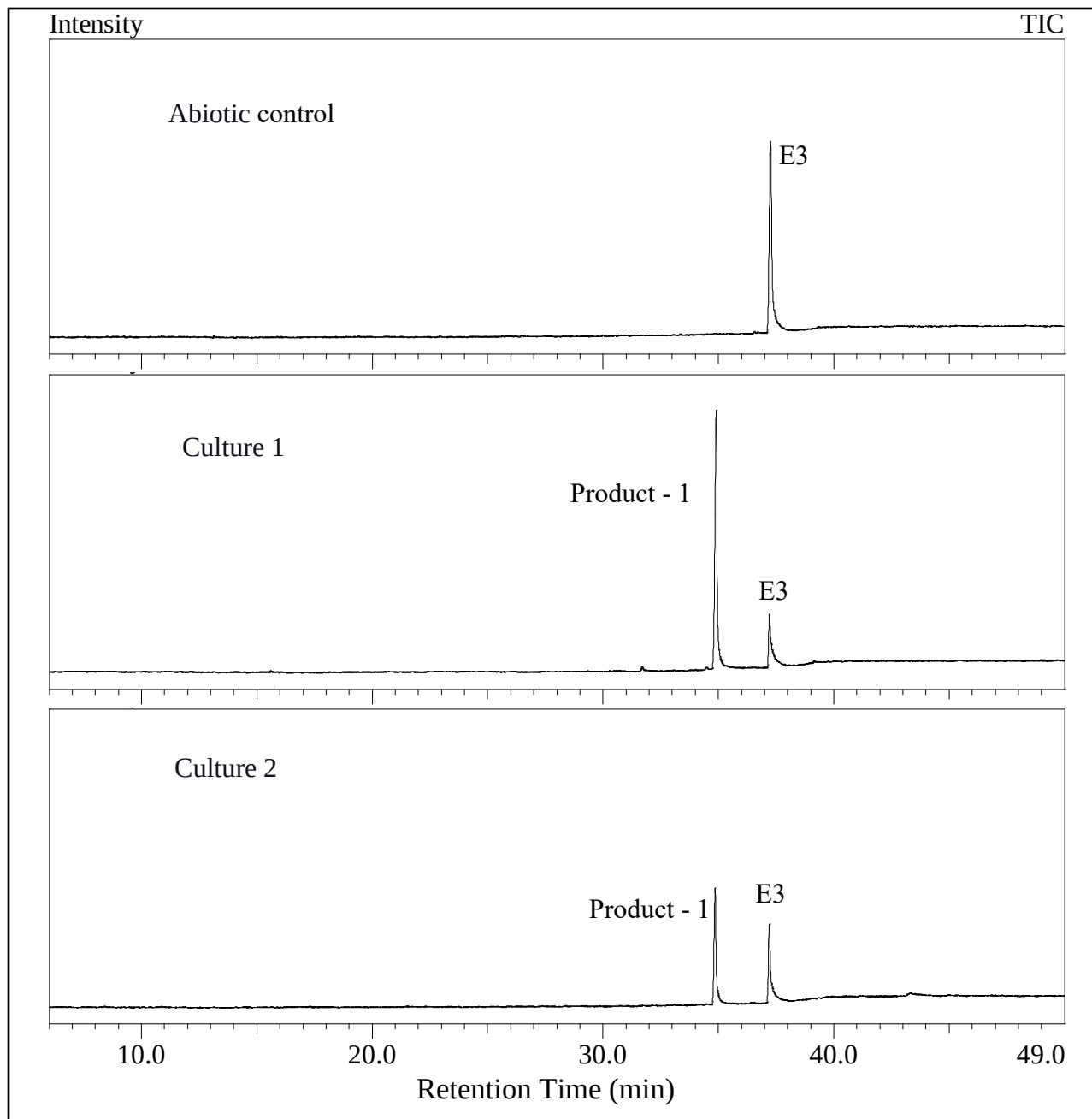


Fig. 4.49: Total ion chromatograms from GC-MS analysis showing degradation of E3 by cultures of the thickened sludge consortium in CDM containing 0.1 mM E3, which was added from a DMSO stock solution. Results from two biological triplicates are shown. Culture samples were retrieved after 5 days of incubation, extracted with ethyl acetate and analyzed by GC-MS.

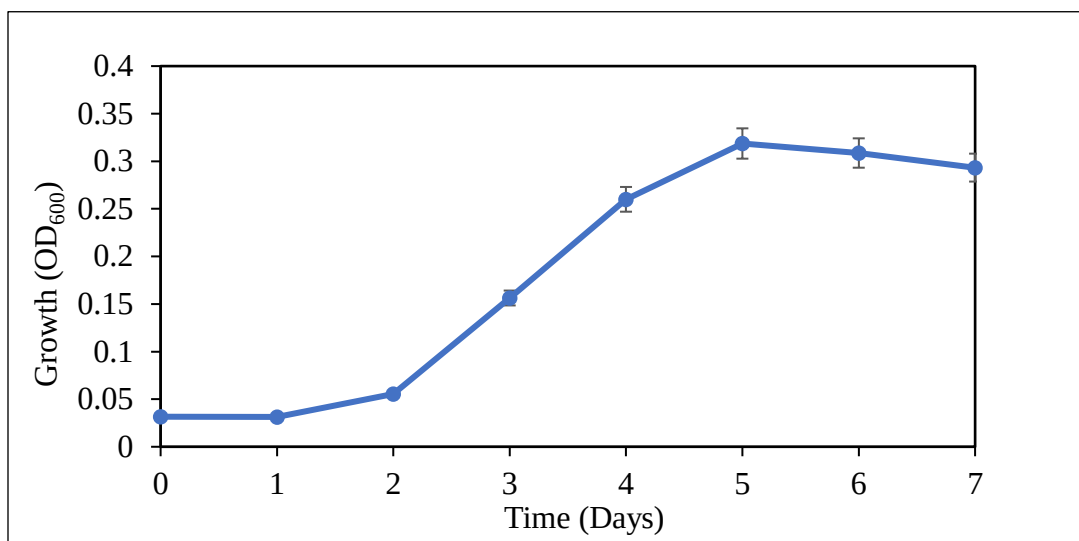


Fig. 4.50: Growth curves of the activated sludge consortium in 100 mL CDM containing 0.1 mM E3 at 30 °C with shaking at 180 rpm. Results are average of triplicate measurements and the error bars represent standard error. E3 was added from a stock solution in DMSO, and the inoculum was prepared from precultures grown without glycerol.

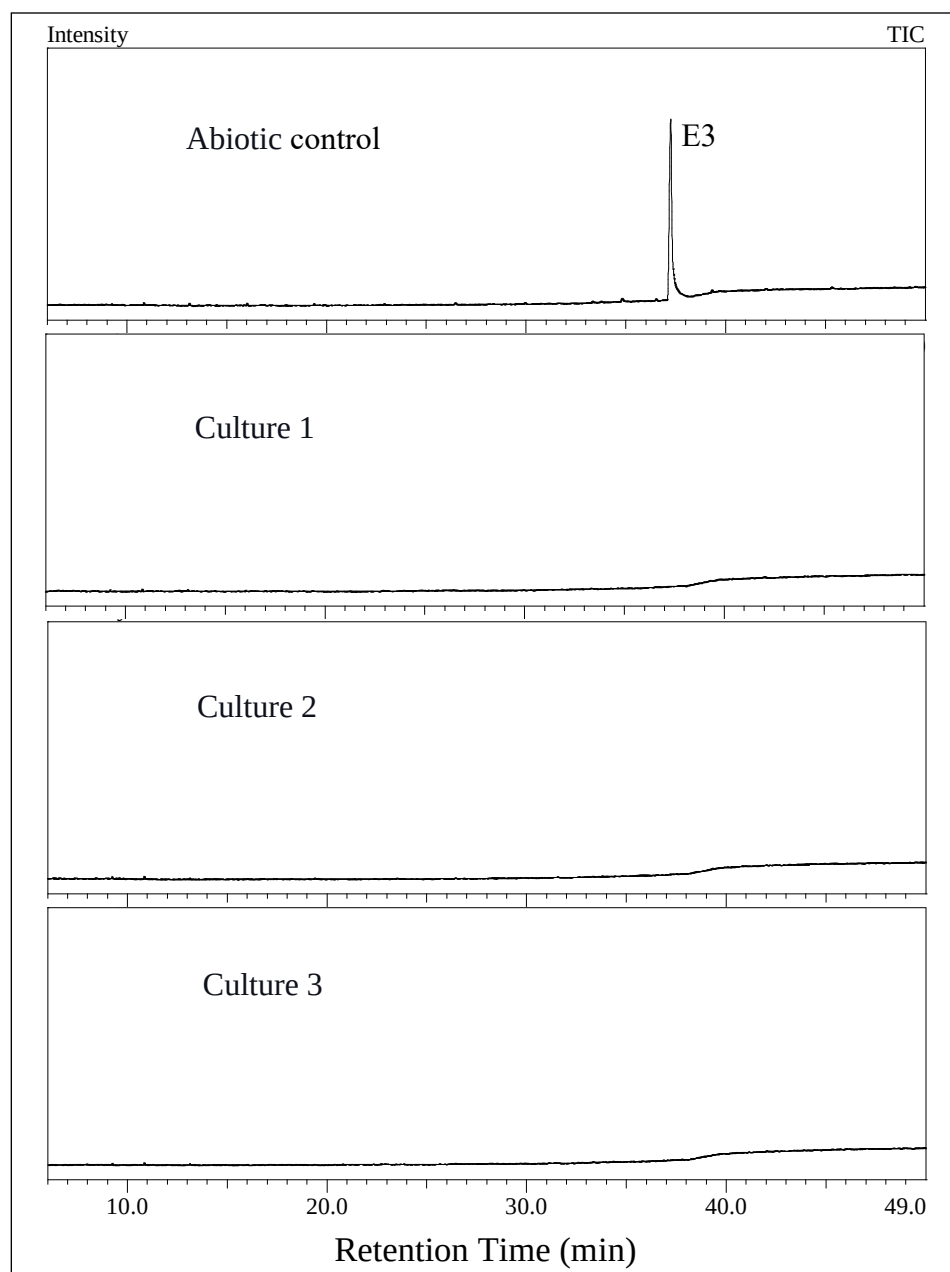


Fig. 4.51: Total ion chromatograms from GC-MS analysis showing degradation of E3 by cultures of the activated sludge consortium in CDM containing 0.1 mM E3, which was added from a DMSO stock solution. Results from three biological replicates are shown. Culture samples were retrieved after 4 days of incubation, extracted with ethyl acetate and analyzed by GC-MS.

4.8. Substrate spectrum of the inflow consortium

To reveal whether the inflow consortium can utilize carbon sources other than E3, it was cultured in CDM with different carbon sources. The inflow consortium grew in CDM containing either glucose, E1, E2, a mixture of E1, E2, and E3, a mixture of E1, E2, E3, EE2, and or DMSO as a sole carbon source (Figures 4.52, 4.53).

The ability of the inflow consortium to grow on DMSO as a sole carbon source raises the question whether the tested estrogens were utilized. GC-MS analysis confirmed the utilization of E1, E2, and E3 (as shown before) when added as a mixture (Figure 4.54) or individually and E2 was transformed to E1 to be further metabolized via the 4,5-*seco* pathway (Chen et al., 2017) (Figures 4.55, 4.57). On the contrary, EE2 was not consumed even after 60 days of incubation (Figure 4.58), indicating that growth observed in the EE2 cultures was due to DMSO utilization. In contrast, when the 4 estrogens were added as a mixture, only E2 and E3 were fully consumed, and product-1 was detected. However, E1 and EE2 persisted up to 60 days of incubation (Figure 4.55).

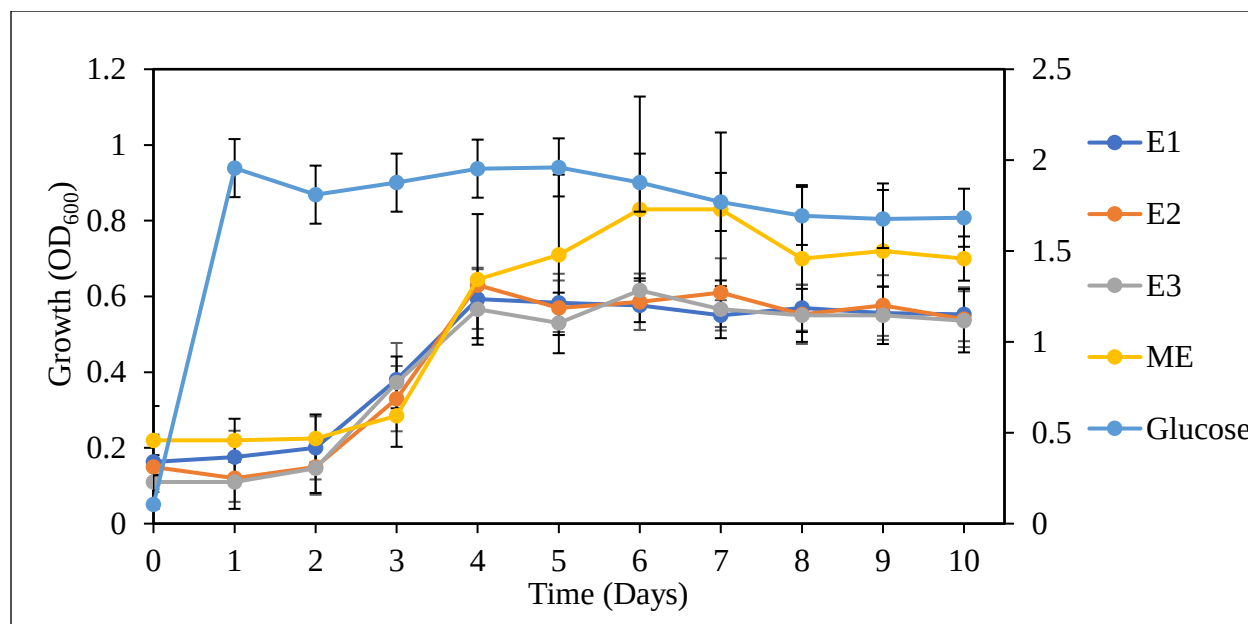


Fig. 4.52: Growth curves of the IF-consortium in 100 mL CDM containing glucose (10 mM) or estrogens (0.1 mM) individually and as a mixture (ME) at 30 °C with shaking at 180 rpm. Results are average of triplicate measurements and the error bars represent standard error. Estrogens were added from stock solutions in DMSO, and the inoculum was prepared from precultures grown without glycerol.

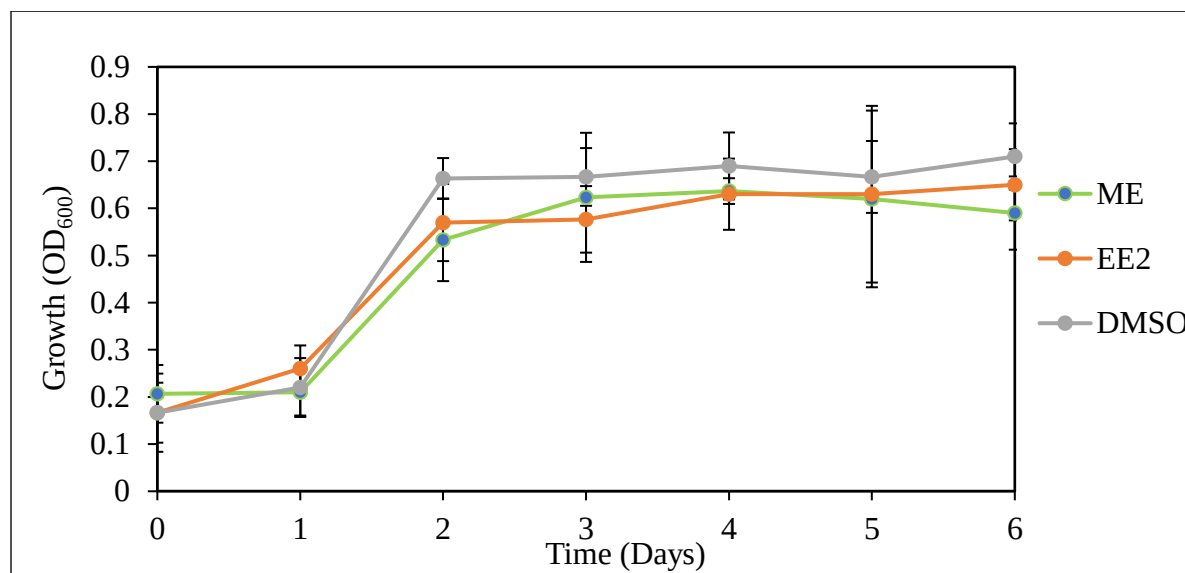


Fig. 4.53: Growth curves of the IF-consortium in 100 mL CDM containing DMSO (1% v/v), EE2 (0.1 mM), and a mixture of four estrogens (ME, 0.1 mM each) at 30 °C with shaking at 180 rpm. Results are average of triplicate measurements and the error bars represent standard error. Estrogens were added from stock solutions in DMSO, and the inoculum was prepared from precultures grown without glycerol.

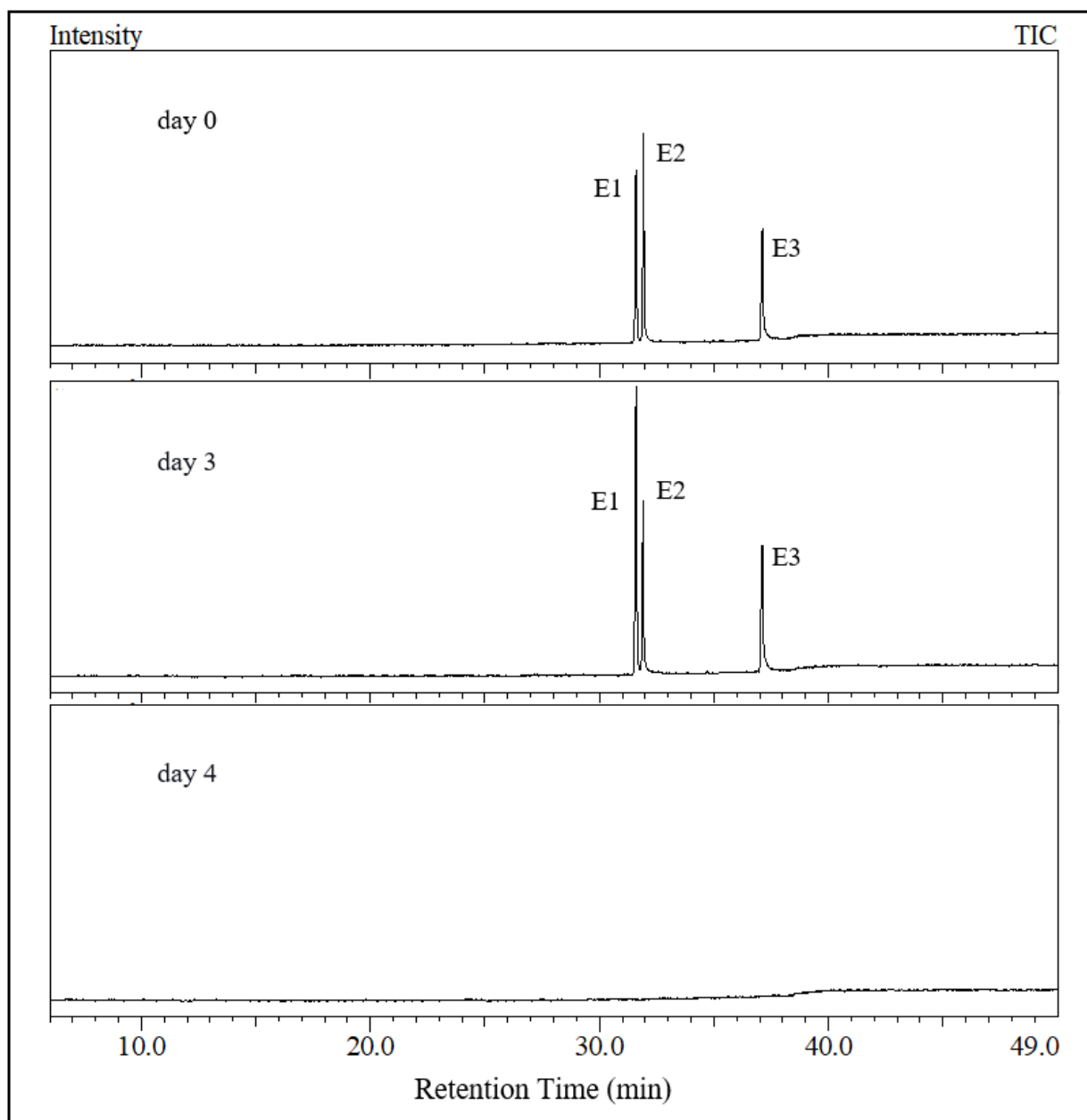


Fig. 4.54: Total ion chromatograms from GC-MS analysis of cultures and comparison of the cultures at various time points of the IF-consortium in CDM containing a mixture of three estrogens (0.1 mM each), which were added from DMSO stock solutions.

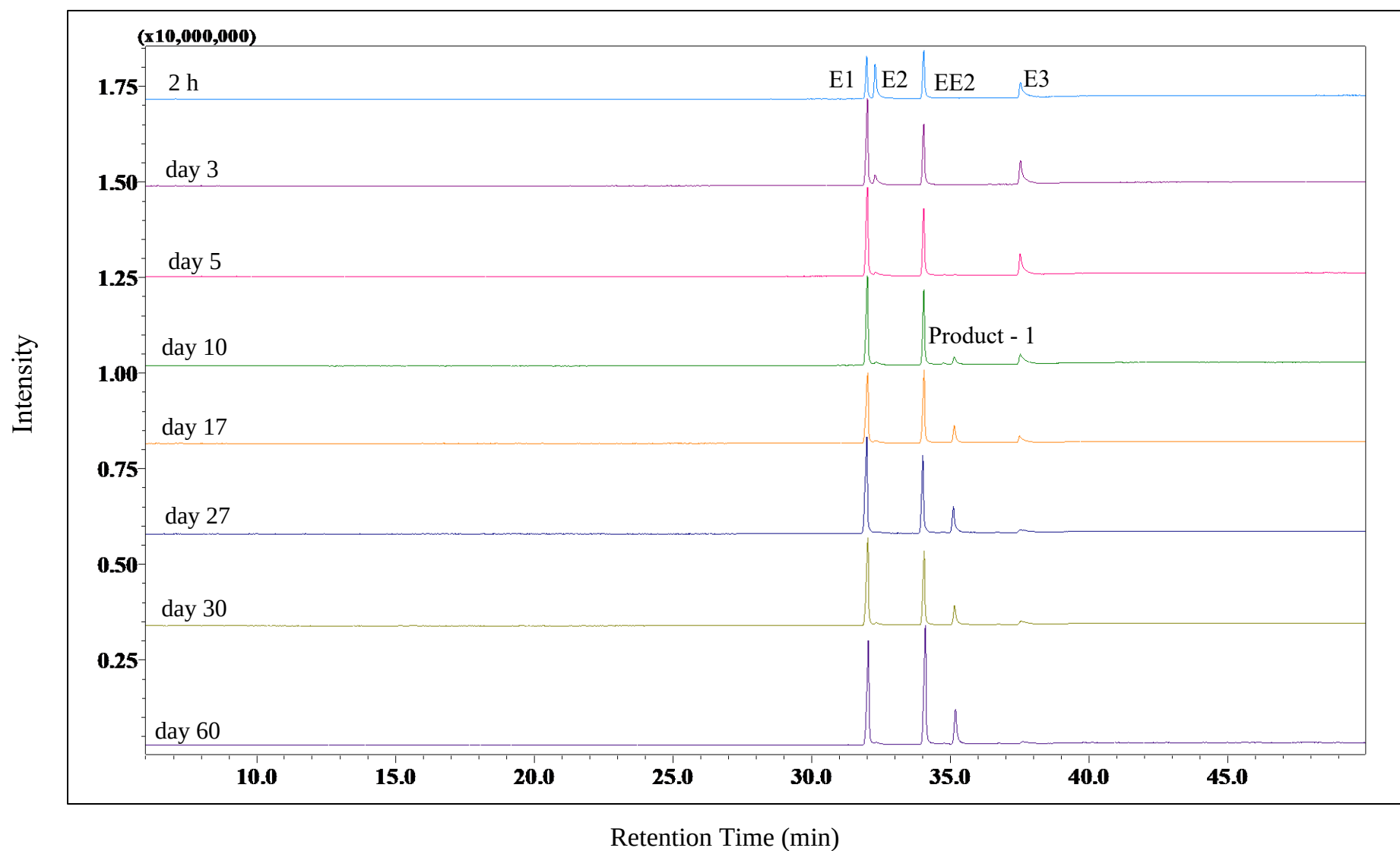


Fig. 4.55: Total ion chromatograms from GC-MS analysis of cultures of the IF-consortium in CDM containing a mixture of four estrogens (0.1 mM each), which were added from DMSO stock solutions.

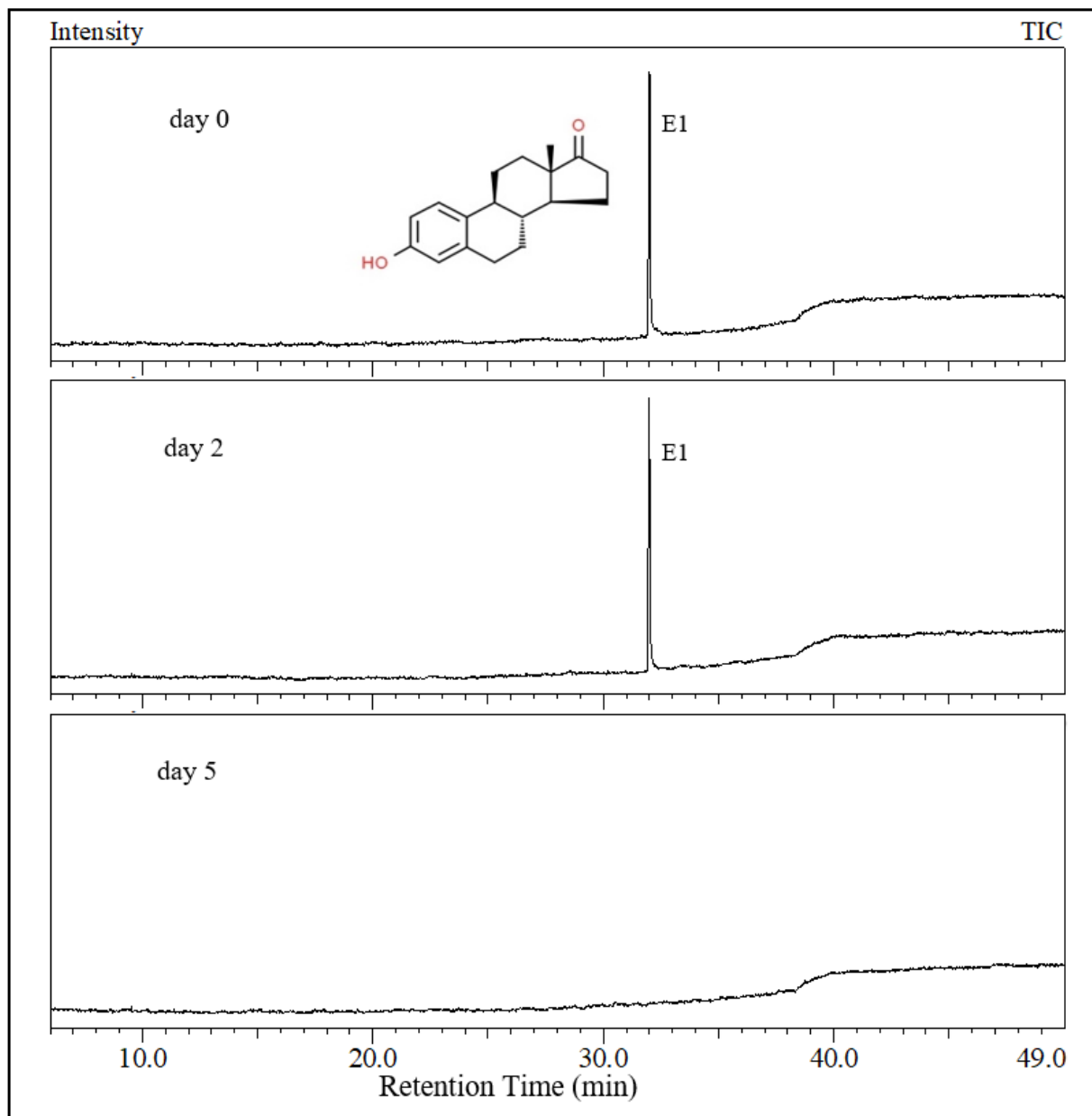


Fig. 4.56: Total ion chromatograms from GC-MS analysis of cultures of the IF-consortium grown in CDM containing E1 (0.1 mM) as a carbon source. E1 was added from DMSO stock solutions.

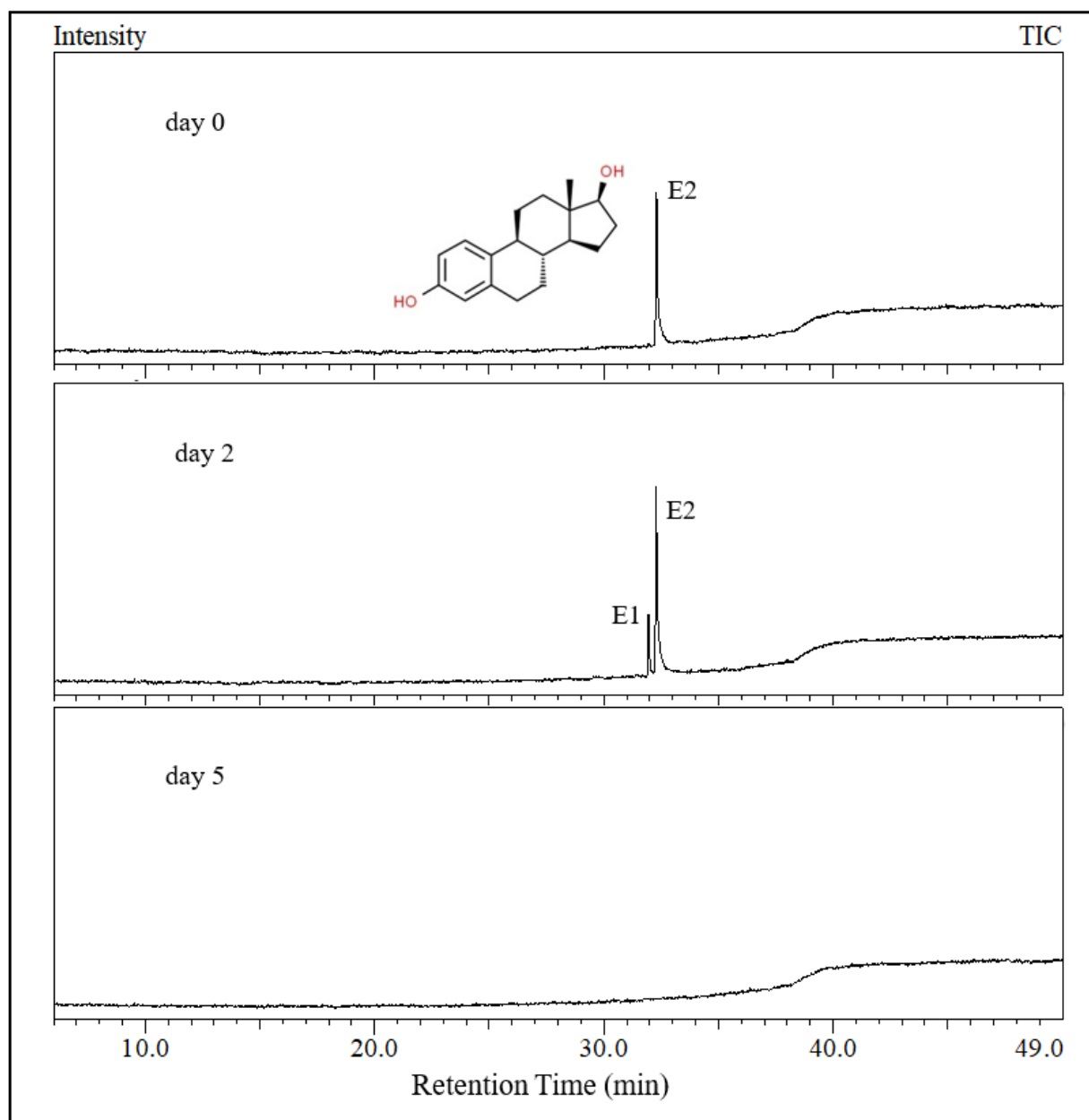


Fig. 4.57: Total ion chromatograms from GC-MS analysis of cultures of the IF-consortium grown in CDM containing E2 (0.1 mM) as a carbon source. E2 were added from DMSO stock solutions.

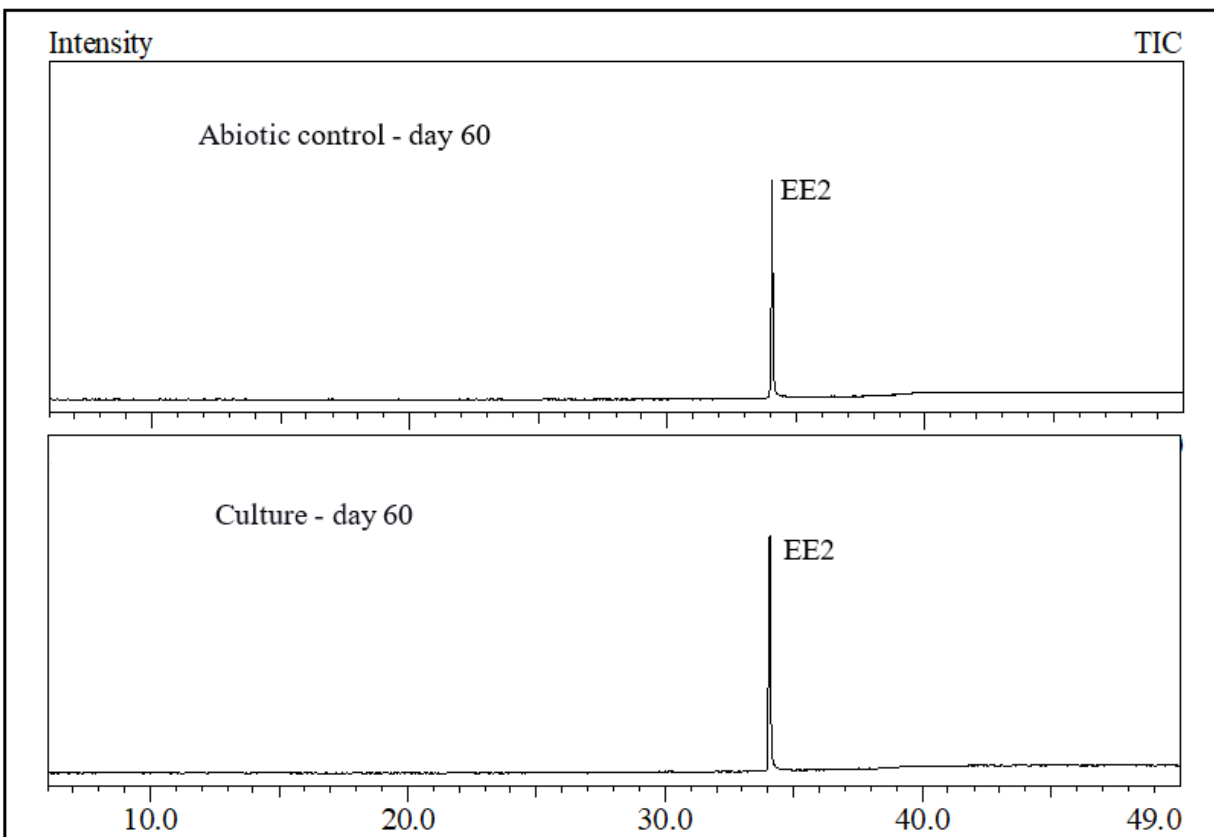


Fig. 4.58: Total ion chromatograms from GC-MS analysis of cultures of the IF-consortium grown in CDM containing EE2 (0.1 mM) as a carbon source. EE2 was added from a DMSO stock solution.

4.9. The E3 biodegradation pathway

To elucidate the E3 degradation pathway and quantify degradation metabolites, the inflow consortium was cultured in CDM with 1 mM E3, and culture samples were retrieved at time intervals and analyzed by UPLC and HRMS. As a control and for comparative analysis, E1 cultures were prepared and run under the same conditions. As shown in Figure 4.59, four estrogen degradation metabolites were detected in the E3 cultures, namely E1, 4-OH-E1, pyridinestrone acid (PEA), and HIP.

The concentration of E3 decreased with time and by day 14 almost 80% of the added E3 was consumed (Figure 4.60). The drop in the E3 concentration was accompanied by an increase of E1 concentration, which was detected after 6 days of incubation at a concentration of 0.01 mM, increasing to 0.61 mM at day 14. All the other intermediates were detected in much lower concentrations only starting from day 10 (Figure 4.60). In the E1 cultures, the E1 concentration decreased with time and by day 14, 50% of the initial E1 concentration was consumed (Figure 4.61).

This drop in E1 concentration was accompanied by formation of 4-OH-E1, which was detected after 4 days of incubation at 0.01 mM, increasing with time to reach 0.11 mM after 14 days of incubation. PEA was detected at day 6 and reached a concentration of 0.05 mM after 14 days. The next intermediate, HIP, was detected in the E1 cultures after 8 days and its maximum concentration was 0.03 mM attained after 14 days (Figure 4.61).

These findings indicate that E3, similar to E1, is degraded by the inflow consortium via the 4,5-seco pathway (Figure 4.62). Moreover, it can be postulated that the initial step of E3 degradation proceeds by transforming E3 to E1, probably

via dehydroxylation/dehydration at C16 to produce E2, which is then oxidized to E1. Alternatively, E3 could be first oxidized to 16 α -OH-E1 followed by removal of the OH group at C16 to produce E1. In either case, a dehydroxylase/dehydratase and dehydrogenase could be involved. It is worth noting that none of the degradation intermediates were detected in the abiotic controls, indicating formation of these products via biotic or biological activity in the inoculated cultures.

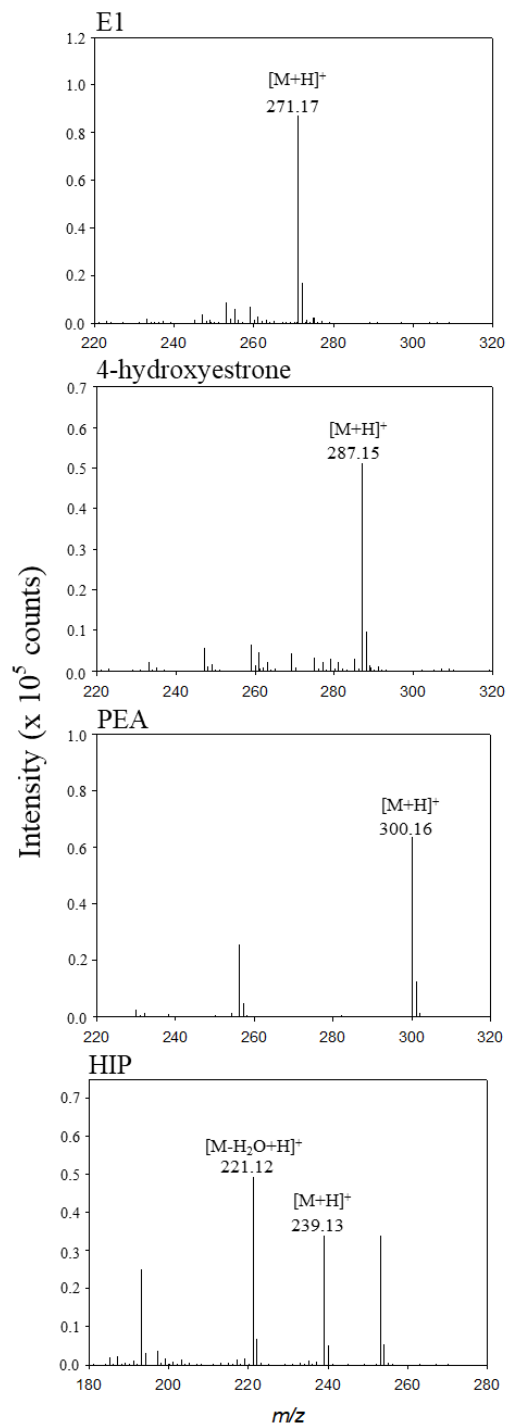


Fig. 4.59: UPLC-ESI-HRMS analysis showing mass spectra of metabolites detected in E3 cultures of the inflow consortium. The samples were extracted using ethyl acetate, and the estrogen metabolites were analyzed through UPLC-ESI-HRMS. The predicted elemental composition of individual metabolite ions was calculated using MassLynx Mass Spectrometry software (Waters).

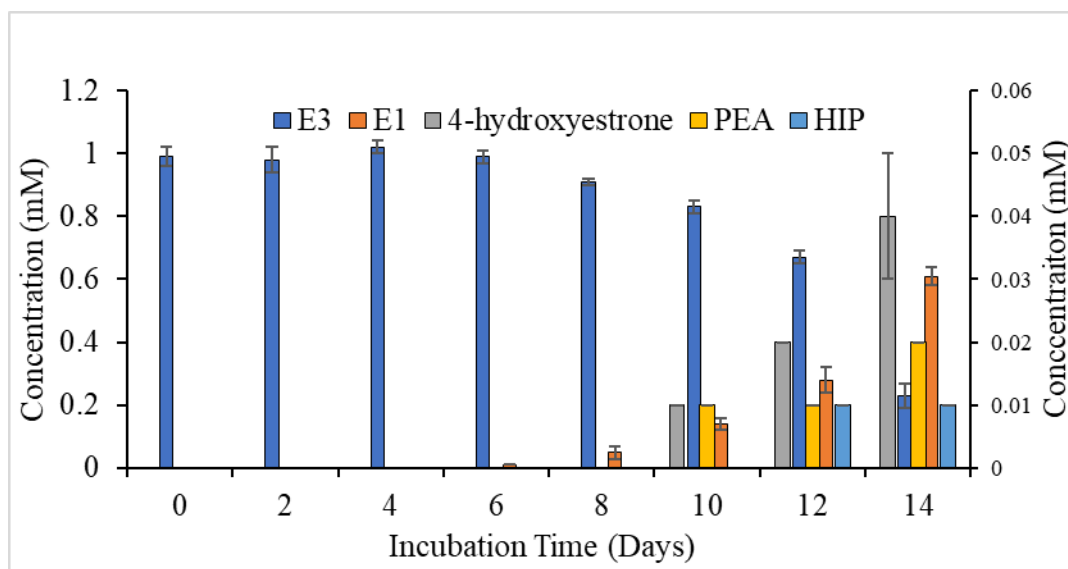


Fig. 4.60: Quantification of temporal change in the concentration of E3 and the detected degradation metabolites detected in E3 (1 mM) cultures of the inflow consortium in CDM. Bars represent average of three biological replicates and error bars represent standard deviation.

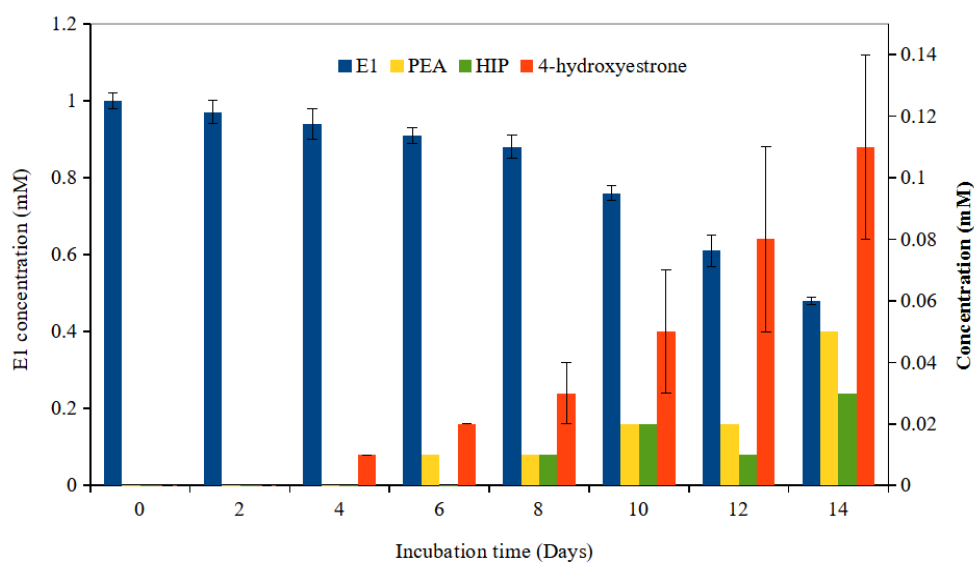


Fig. 4.61: Quantification of temporal change in the concentration of E1 and the detected degradation metabolites detected in E1 (1 mM) cultures of the inflow consortium in CDM. Bars represent average of three biological replicates and error bars represent standard deviation.

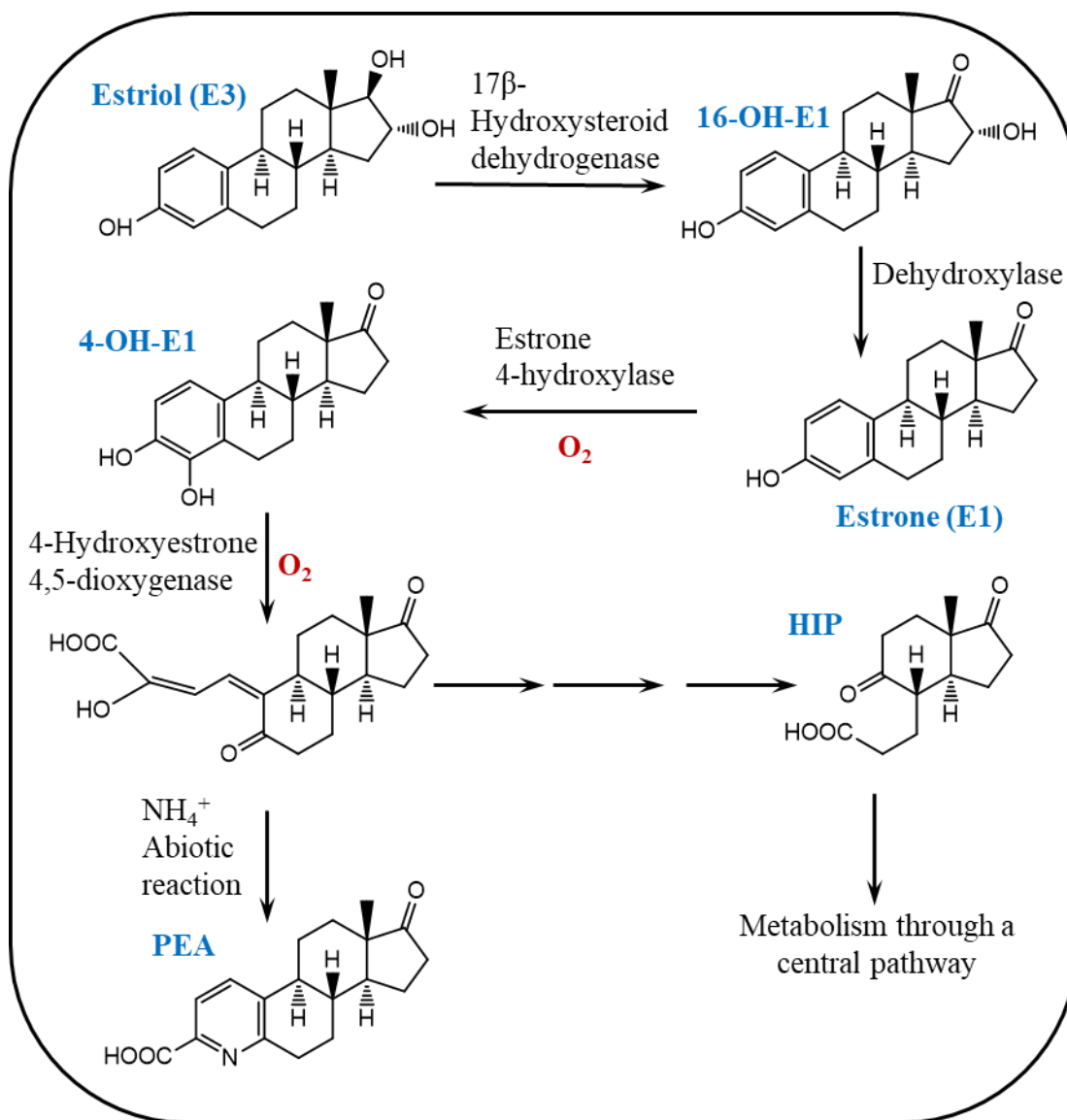


Fig. 4.62: Proposed pathway for E3 biodegradation by the inflow consortium.

4.10. Analysis of the inflow consortium community structure during E3 biodegradation

Considering that all the enriched E3-degrading consortia could consist of many bacterial strains, it is tempting to hypothesize that only some of these bacteria could be directly involved in E3 biodegradation. Therefore, to identify, at least tentatively, key E3-degrading bacteria of the inflow consortium, samples from the inflow consortium cultures on E3 and E1, which were used to identify the degradation metabolites, were collected and analyzed at time intervals to study how the composition of the consortium changes with time and if the type of the estrogen substrate can affect the community structure. Furthermore, glucose cultures of the inflow consortium were run in parallel and analyzed to reveal the community involved in the utilization of non-estrogen substrate, which may facilitate more specific fingerprinting of the estrogen-related community.

Total genomic DNA was isolated from all culture samples. DNA could be isolated from the glucose cultures across all samples except samples of day 0. However, in the estrogen cultures DNA could be isolated only starting from day 10 for the E1 cultures and day 4 in the E3 cultures (Figures 4.63-4.66). Results of the quantification of the DNA samples are shown in Tables 4.2-4.4.

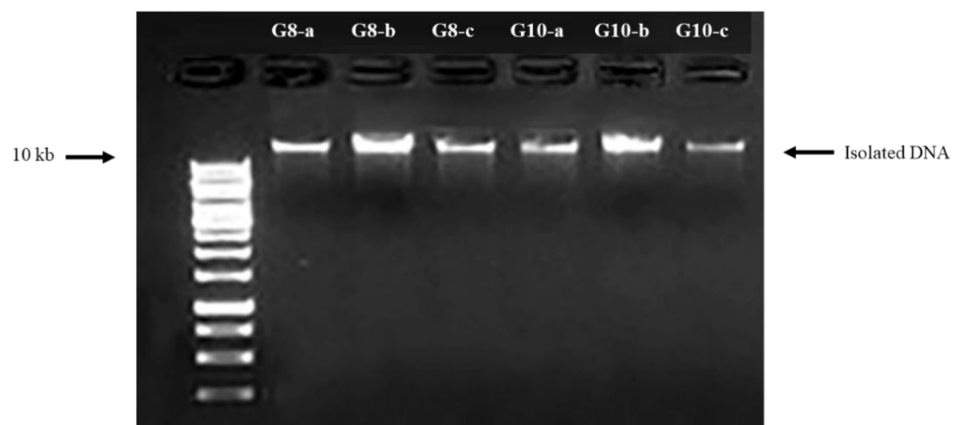


Fig. 4.63: Agarose gel (1%) showing total community DNA isolated from cultures of the IF-consortium after time intervals. G: glucose. (see Materials and Methods for sample description). All cultures were run in biological triplicates.

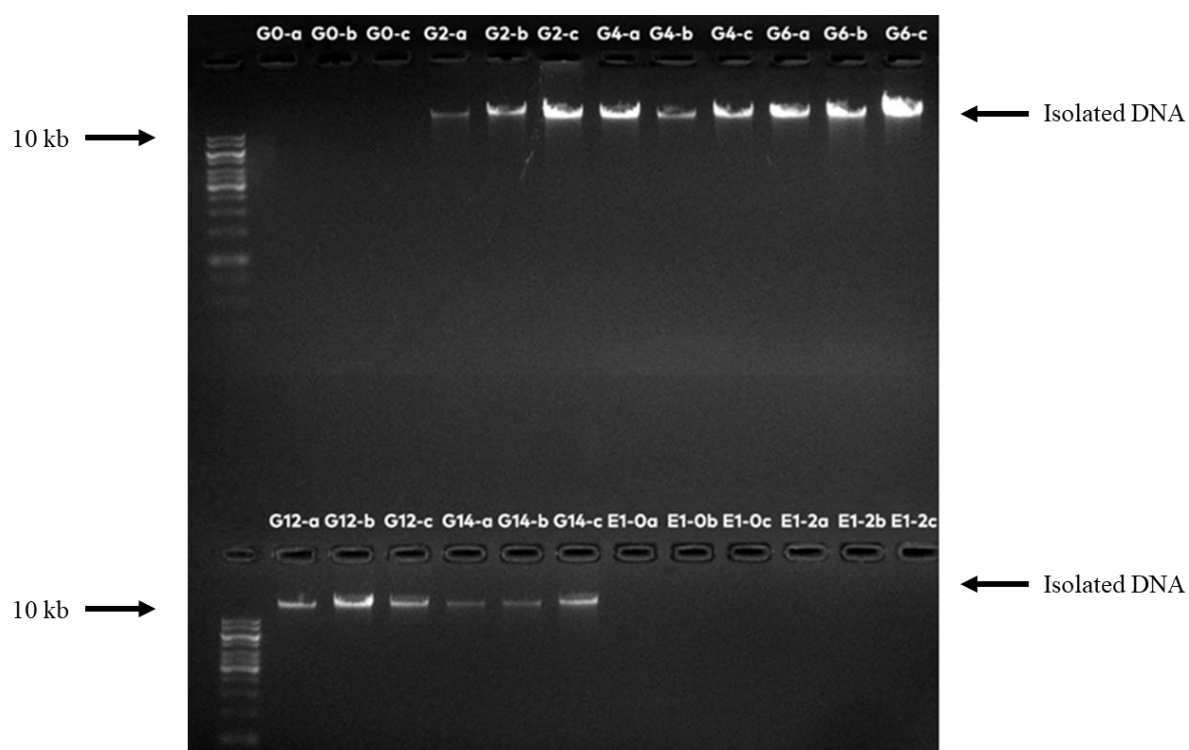


Fig. 4.64: Agarose gel (1%) showing total community DNA isolated from cultures of the IF-consortium after time intervals. G: glucose. (see Materials and Methods for sample description). All cultures were run in biological triplicates.

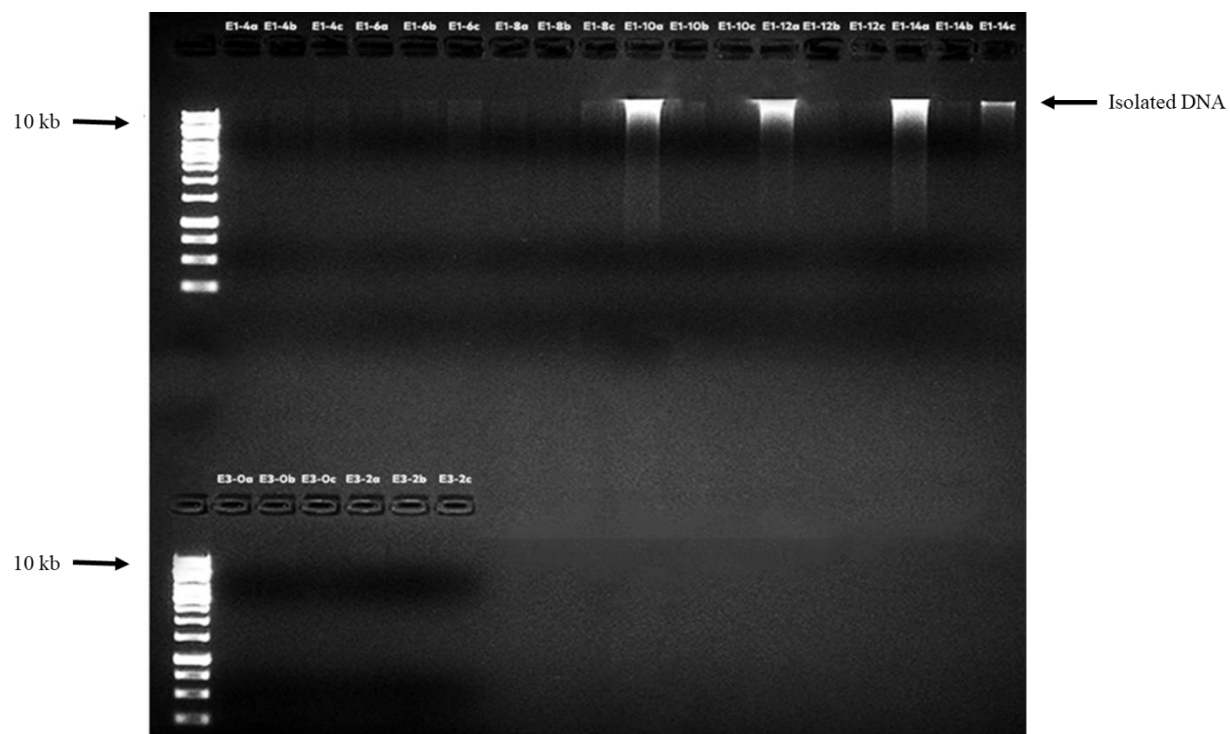


Fig. 4.65: Agarose gel (1%) showing total community DNA isolated from cultures of the IF-consortium after time intervals. G: glucose. (see Materials and Methods for sample description). All cultures were run in biological triplicates.

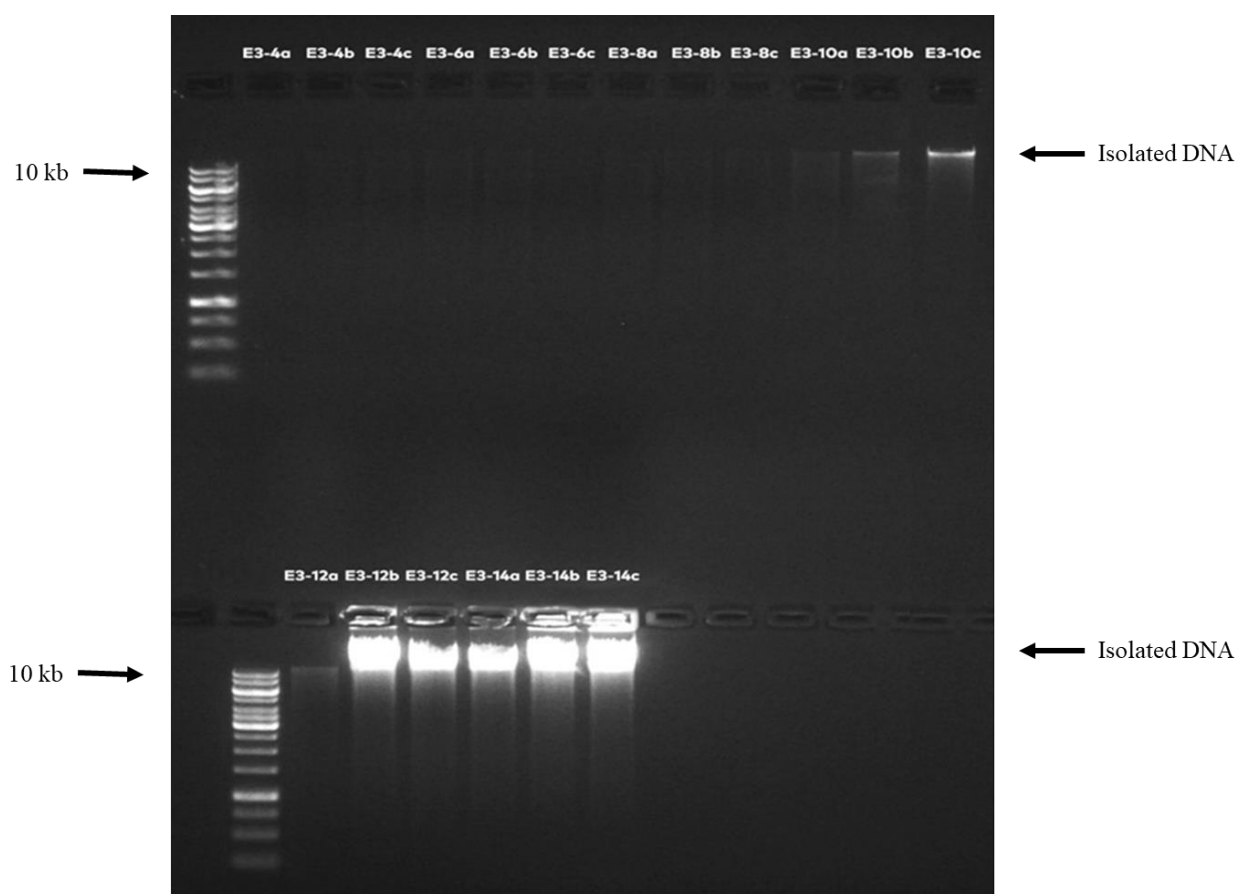


Fig. 4.66: Agarose gel (1%) showing total community DNA isolated from cultures of the IF-consortium after time intervals. G: glucose. (see Materials and Methods for sample description). All cultures were run in biological triplicates.

Table 4.2: Quantification of the total DNA isolated from the glucose cultures of the inflow consortium.

Sample code	Description	A: 260/280	DNA concentration (ng/ μ L)	Total DNA (ng)
G2-a	Day 2	1.66	17.6	1548
G2-b	Day 2	1.89	34.3	3018
G2-c	Day 2	1.78	55.2	4857
G4-a	Day 4	1.85	46.8	4118
G4-b	Day 4	1.82	29.8	2622
G4-c	Day 4	1.81	46.4	4083
G6-a	Day 6	1.78	84.8	7462
G6-b	Day 6	1.79	62.4	5491
G6-c	Day 6	1.78	79.2	6969
G8-a	Day 8	1.31	11.4	1003
G8-b	Day 8	1.51	26	2288
G8-c	Day 8	1.47	11.1	976
G10-a	Day 10	1.20	10.8	950
G10-b	Day 10	1.59	14	1232
G10-c	Day 10	1.50	5.84	513
G12-a	Day 12	1.80	37.0	3256
G12-b	Day 12	1.79	42.8	3766
G12-c	Day 12	1.82	27.3	2402
G14-a	Day 14	1.52	9.24	813
G14-b	Day 14	1.66	10.9	959
G14-c	Day 14	1.63	24.0	2112

Table 4.3: Quantification of the total DNA isolated from E1 cultures of the inflow consortium.

Sample code	Description	A: 260/280	DNA concentration (ng/μl)	Total DNA (ng)
E1-10a	Day 10	1.78	148	13000
E1-10b	Day 10	1.54	2.21	194
E1-12a	Day 12	1.56	107	9416
E1-14a	Day 14	1.18	40.8	3590
E1-14c	Day 14	1.15	7.08	623

Table 4.4: Quantification of the total DNA isolated from E3 cultures of the inflow consortium.

Sample code	Description	A: 260/280	DNA concentration (ng/ μ L)	Total DNA (ng)
E3-4a	Day 4	3.74	2.66	234
E3-4b	Day 4	1.45	3.16	278
E3-4c	Day 4	1.59	3.70	325
E3-6a	Day 6	1.53	4.32	380
E3-6b	Day 6	2.48	4.2	369
E3-6c	Day 6	1.21	3.04	267
E3-8a	Day 8	1.50	3.83	337
E3-8b	Day 8	1.68	4.52	397
E3-8c	Day 8	1.44	3.47	305
E3-10a	Day 10	1.5	6	500
E3-10b	Day 10	1.7	9	800
E3-10c	Day 10	1.7	20	1600
E3-12a	Day 12	1.7	11	900
E3-12b	Day 12	1.79	260	20000
E3-12c	Day 12	1.77	228	20064
E3-14a	Day 14	1.85	200	17600
E3-14b	Day 14	1.74	308	27104
E3-14c	Day 14	1.78	276	24288

4.10.1. Relative abundance of bacterial species

The distribution of species was clearly different between the cultures grown in presence of glucose, E1, or E3 (Figures 4.67-4.70). Most species belonged to the phylum *Pseudomonadota* in all three cultures; however, species of phylum *Actinobacteriota* were also observed in the E1 and E3 cultures (Figure 4.67).

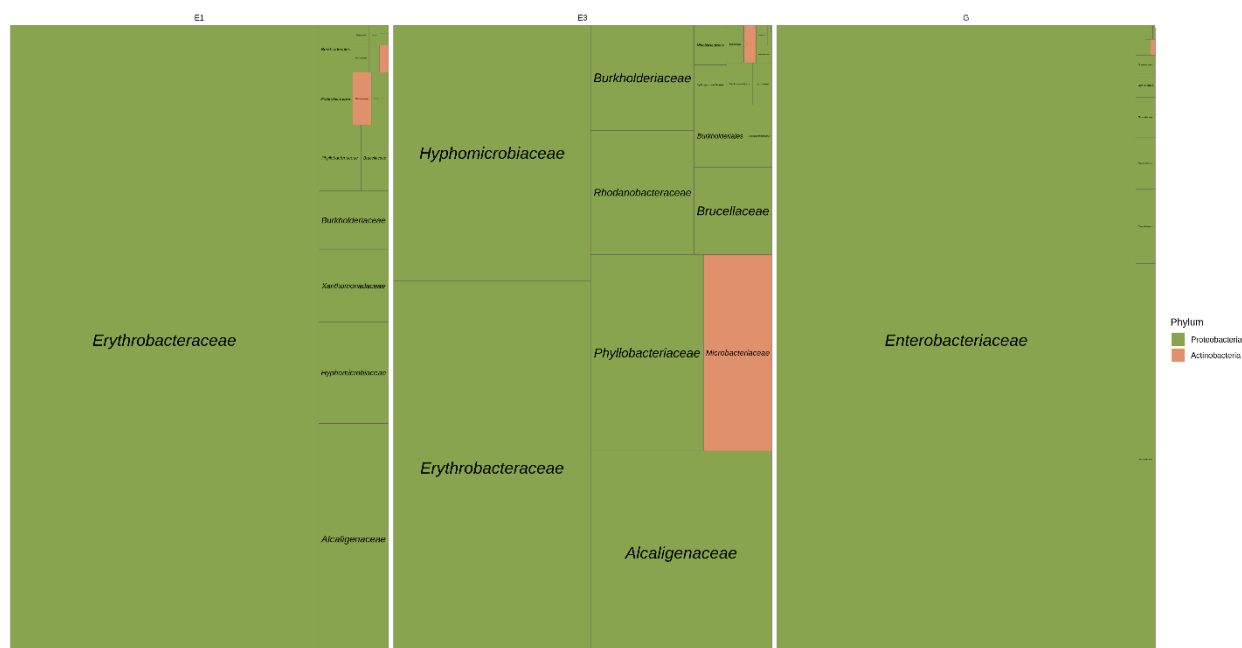


Fig. 4.67: Species relative abundance dendrogram.

Croceicoccus estronivorus was the most prevalent species across the samples grown in presence of E1 and E3. Other common species in these cultures were *Achromobacter insuavis*, *Achromobacter pulmonis* and *Hyphomicrobium denitrificans*; however, these appear to be relatively more prevalent in the E3 cultures (Figure 4.68). Interestingly, *C. estronivorus* was a prevalent species that took over other taxa in the E1 and E3 cultures from day 10 (Figure 4.69). *A. insuavis*, *A. pulmonis* and *H. denitrificans* were more prevalent in the E3 cultures than *C. estronivorus* up to day 8. Unfortunately, enough DNA required for sequencing was only obtained from the E1 cultures starting from day 10, and the distribution of other taxa between days 0 to 8 could not be determined.

The dominant taxa detected in the E1 and E3 cultures were mostly absent in the glucose (G) cultures, except for *Achromobacter insuavis* that was observed across all glucose cultures and *Hyphomicrobium denitrificans* was also observed in a single glucose culture (Figure 4.69). Three *Klebsiella* species including *Klebsiella africana*, *Klebsiella quasipneumoniae* and *Klebsiella pneumoniae*, *Citrobacter sedlakii* and *Enterobacter cloacae* were prevalent among the glucose samples.

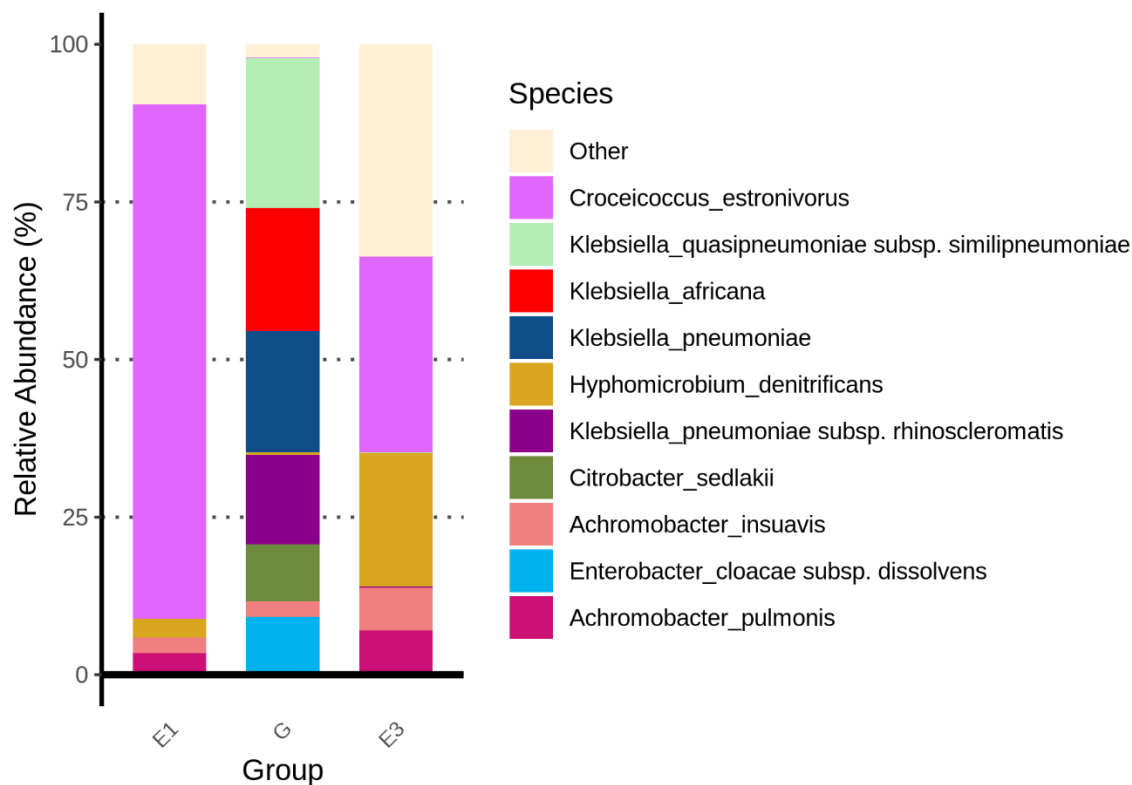


Fig. 4.68: Top 10 species identified among the cultures grown with estrone (E1), glucose (G), and estriol (E3) as carbon sources.

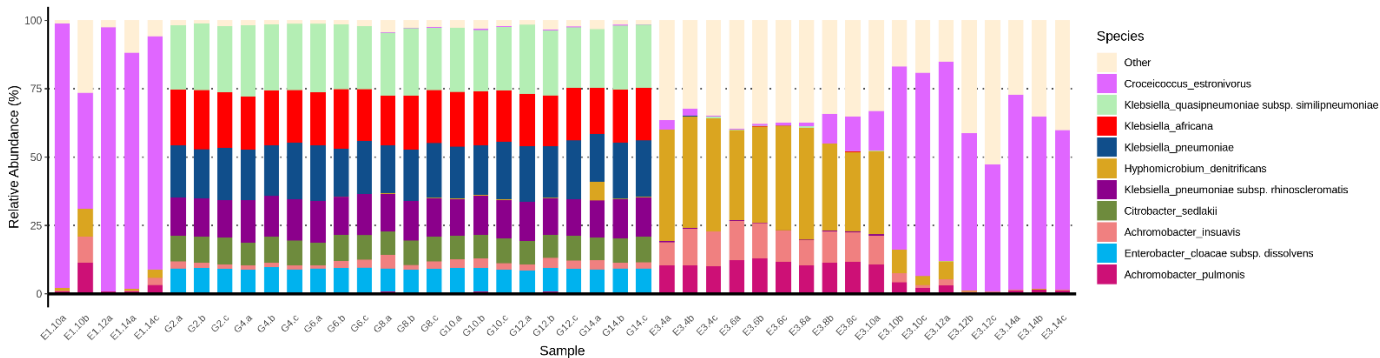


Fig. 4.69 Relative abundance of top 10 bacterial species across individual samples. E1 (estrone), G (glucose), E3 (estriol).

In total, 43 amplicon sequence variants (ASVs) were shared between the three groups. 26.3% ASVs were specific to the glucose (G) cultures and 23.4% to E3 cultures. Interestingly, only 1.8% ASVs were unique to E1) cultures (Figure 4.70A). As mentioned before, ASVs from the phylum *Pseudomonadota* (formerly *Proteobacteria*) were prevalent across all three groups but those from *Actinobacteriota* (Formerly *Actinobacteria*) were more common in E1 and E3 cultures (Figure 4.70B) At the species level, ASVs belonging to *Leucobacter massiliensis*, *Bordetella hinzi* and *Thermomonas haemolytica* were unique to E1 cultures and *Klebsiella pneumoniae* to the glucose cultures (Figure 4.70C). Some ASVs from species *Thermomonas haemolytica*, *Pseudomonas citronellolis*, *cuprividus platarum*, *Klebsiella quasipneumoniae* and *Brucella haematophila* were specific to the E3 cultures. However, DADA2 algorithm defined ASVs based on single-nucleotide difference in the full-length 16S rRNA gene, resulting in multiple ASVs within each species.

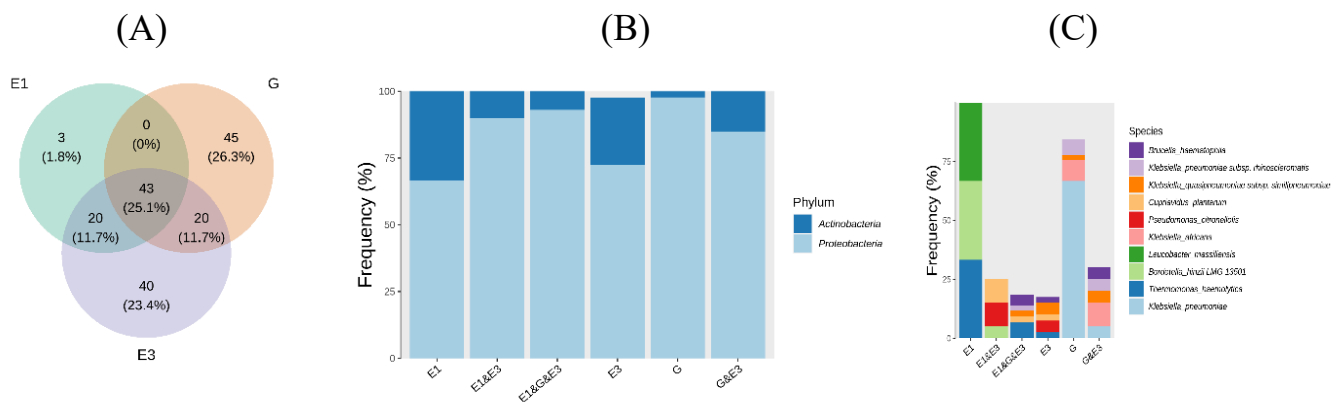


Fig. 4.70: A. Venn diagram showing the number of ASVs shared between the three groups. B. Frequency of ASVs shared at phylum and C. species level. E1 (estrone), G (glucose), E3 (estriol).

4.10.2. Microbial diversity analysis

Microbial heterogeneity within each group (culture) significantly differed from each other (Figure 4.71). The α -diversity compares richness, evenness, and microbial diversity within each sample. The E3 culture samples contained more diverse microbial taxa in

comparison to the E1 and glucose cultures, which were highly homogenous and dominated by *Klebsiella pneumoniae*.

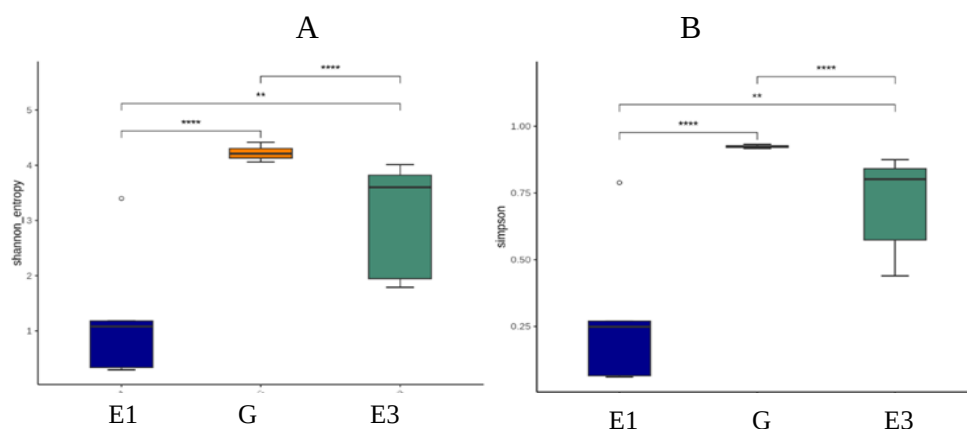


Fig. 4.71: The α -diversity between the three groups using Shannon index (A) and Simpson index method (B). E1 (estrone), G (glucose), E3 (estriol).

The β -diversity shows difference in composition of microbial community between different samples or groups. As mentioned before, microbial communities within E1 and E3 cultures were similar to each other whereas the microbial composition of glucose cultures was quite distinct (Figure 4.72).

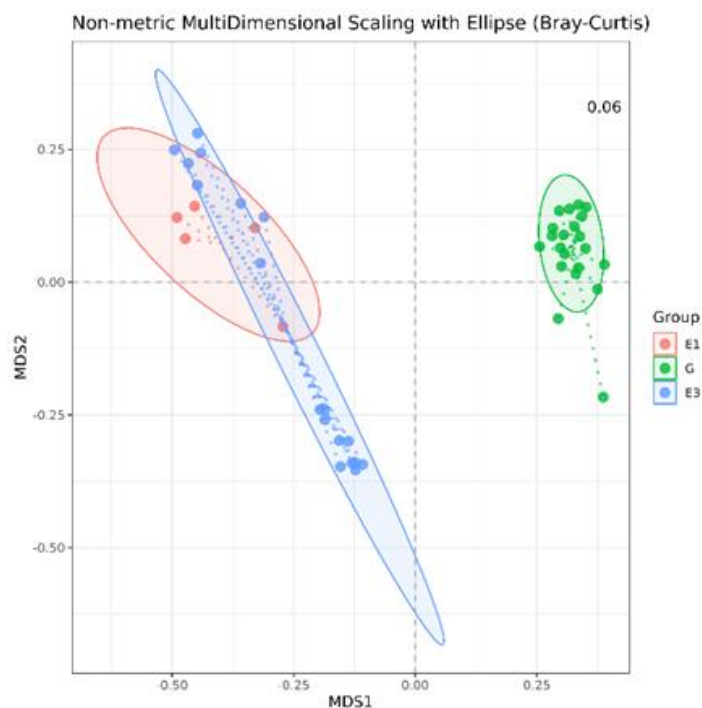


Fig. 4.72: The β -diversity showing difference in microbial community composition between the three groups.

The rarefaction curves showed that ASVs have plateaued at around 2,500 reads (Figure 4.73), suggesting that most of the microbial diversity present in the samples have been captured in this study. These results are highly robust showing the microbial communities' structures significantly vary based on the substrate used in the growth medium; however, multiple microbial taxa were common between the E1 and E3 cultures.

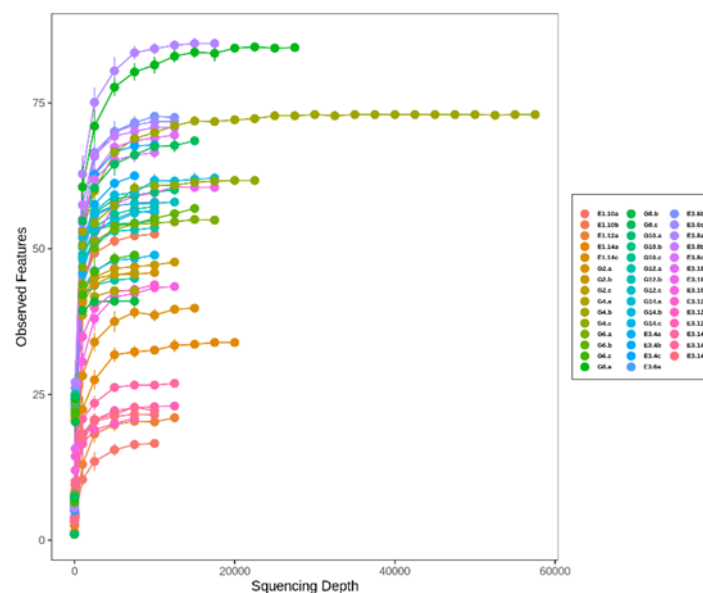


Fig. 4.73: Rarefaction curve showing ASVs against the sequencing depth.

4.10.3. Differential abundance of microbial communities

LEfSe (Linear discriminant analysis effect size) was performed to identify significantly different taxa between the three cultures. This approach is extensively used in identifying biomarkers from metagenomic data. As previously observed, the taxa belonging to the phylum *Pseudomonadota* (particularly γ -*Proteobacteria*) were significantly more abundant in the glucose cultures including *K. pneumoniae*; subsp. *rhinoscleromatis* and *similipneumoniae*, *K. Africana*, *Citrobacter sedlakii* and *Enterobacter cloacae* subsp. *dissolvens* (Figure 4.74). Members of order *Sphingomonadales*, families *Erythrobacteraceae* and *Xanthomonadaceae* and the genera

Croceicoccus and *Thermomonas* were prevalent in the E1 cultures, whereas the E3 cultures harboured more diverse community including taxa belonging to orders *Hyphomicrobiales*, *Micrococcales* and *Xanthomonadales*, families *Alcaligenaceae*, *Burkholderiaceae*, *Rhodanobacteraceae*, *Phyllobacteriaceae*, *Hyphomicrobiaceae*, and *Microbacteriaceae* and genera *Achromobacter*, *Aminobacter*, *Cupriavidus*, *Dyella*, *Hyphomicrobium* and *Microbacterium*. However, taxonomic prevalence in E3 culture switched after day 10 and prevalent taxa were similar to those observed in the E1 cultures, especially *C. estronivorus* (Figures 4.74, 4.75).

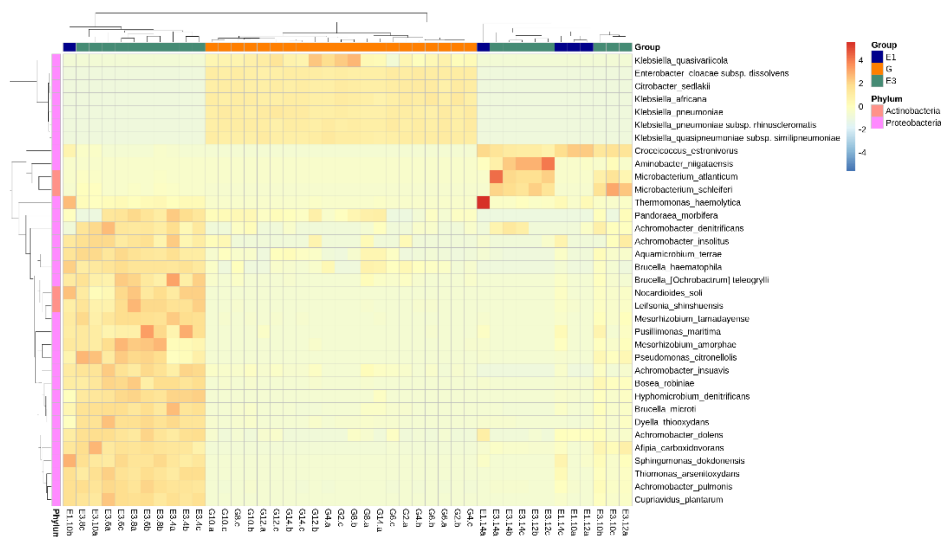


Fig. 4.74. Differentially abundant taxa between the three cultures.

4.10.4. Putative functional characteristics of the microbial communities

FAPROTAX software was used in converting taxonomic microbial community profiles into putative functional profiles. The taxa identified in all three cultures are aerobic chemoheterotrophs (Figures 4.76, 4.77). However, the dominant taxa the in the glucose cultures have more capacity to reduce nitrate and ferment sugars (Figure 4.76). These taxa also have pathogenic capacities, including potential to cause pneumonia and septicemia in humans (Figure 4.77). In contrast, bacteria dominating the E1 and E3

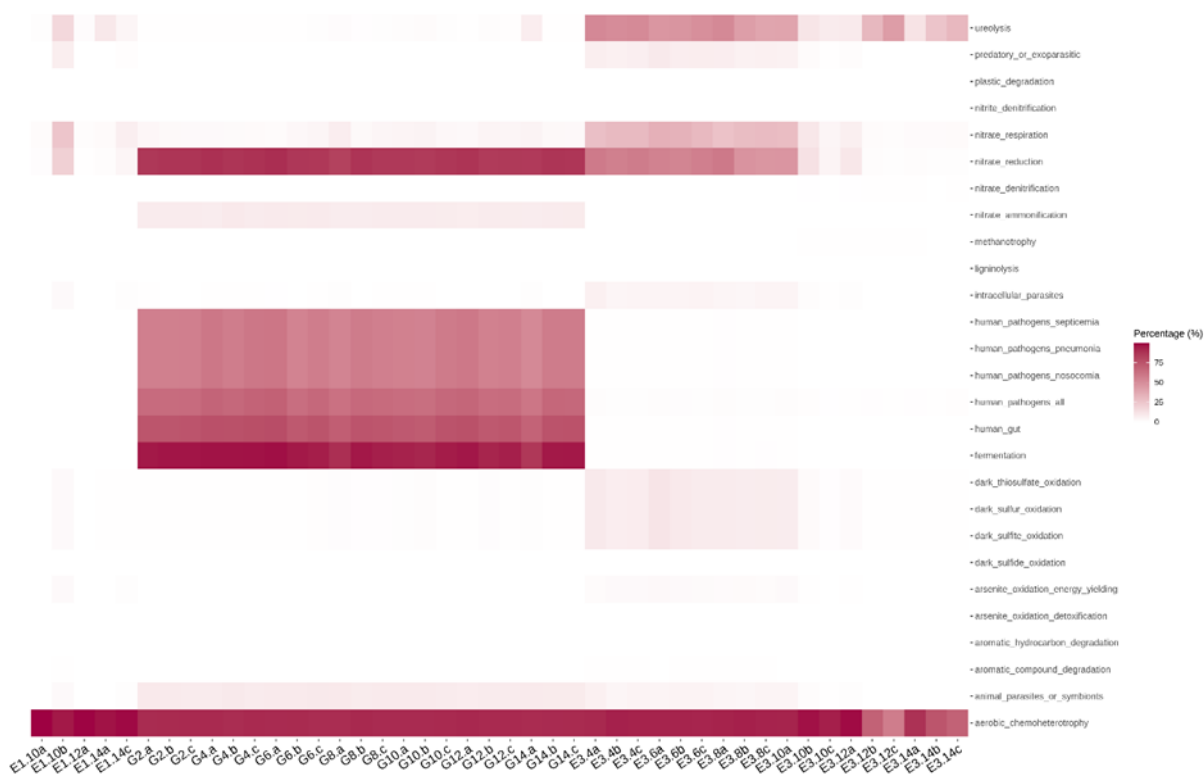


Fig. 4.77: Key metabolic variations between the microbial communities detected in the glucose, E1 and E3 cultures.

4.11. Estrogen biodegradation in wastewater microcosms

Raw sewage (inflow wastewater) and activated sludge microcosms were spiked with different estrogen concentrations dissolved in DMSO. GC-MS analysis revealed that the three estrogens E1, E2, and E3 were degraded in both microcosms at 1 mM and 0.1 mM final concentration (Figures 4.78 and 4.79). After two days of incubation, no estrogens could be detected in either microcosm.

No degradation products could be detected even after two weeks of incubation, except product-1, which was detected only in the activated sludge microcosms after 2 h of incubation and subsequently disappeared.

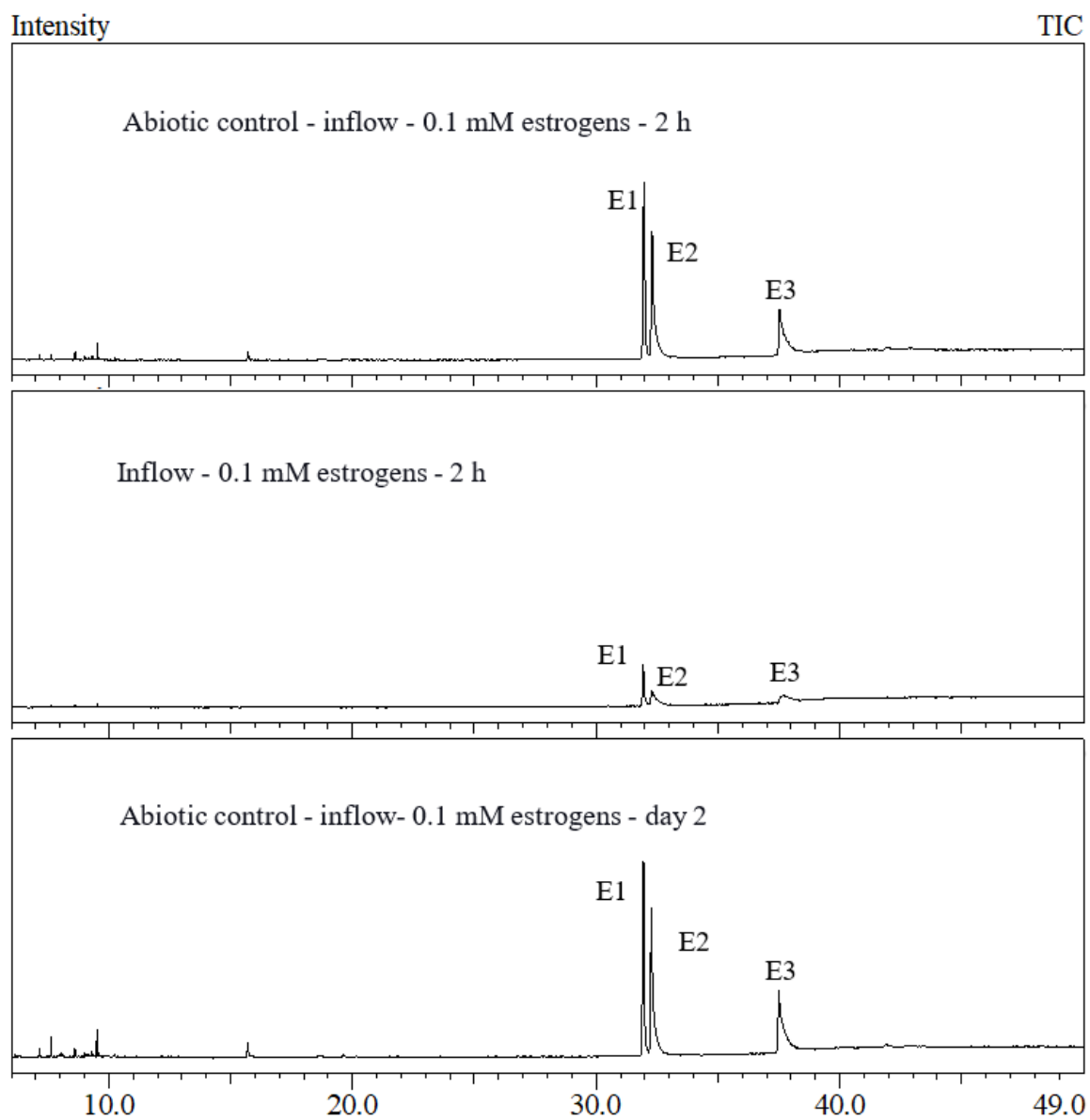


Fig. 4.78: Legend on next page

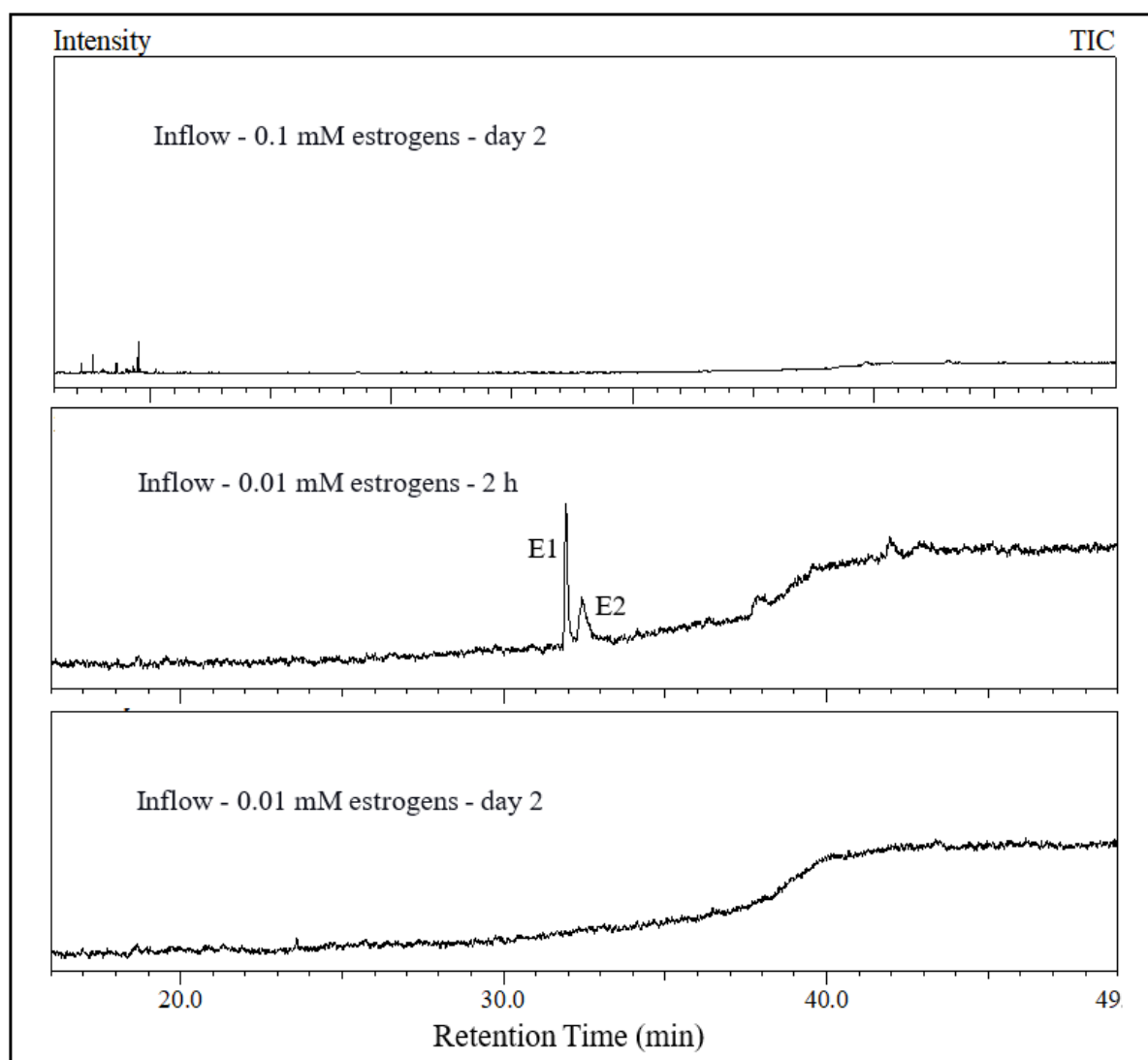


Fig. 4.78: Total ion chromatograms from GC-MS analysis of the inflow microcosms. Each microcosm contained 500 mL of freshly collected inflow wastewater in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 at two concentrations (0.1 mM or 0.01 mM each) and added from DMSO stock solutions.

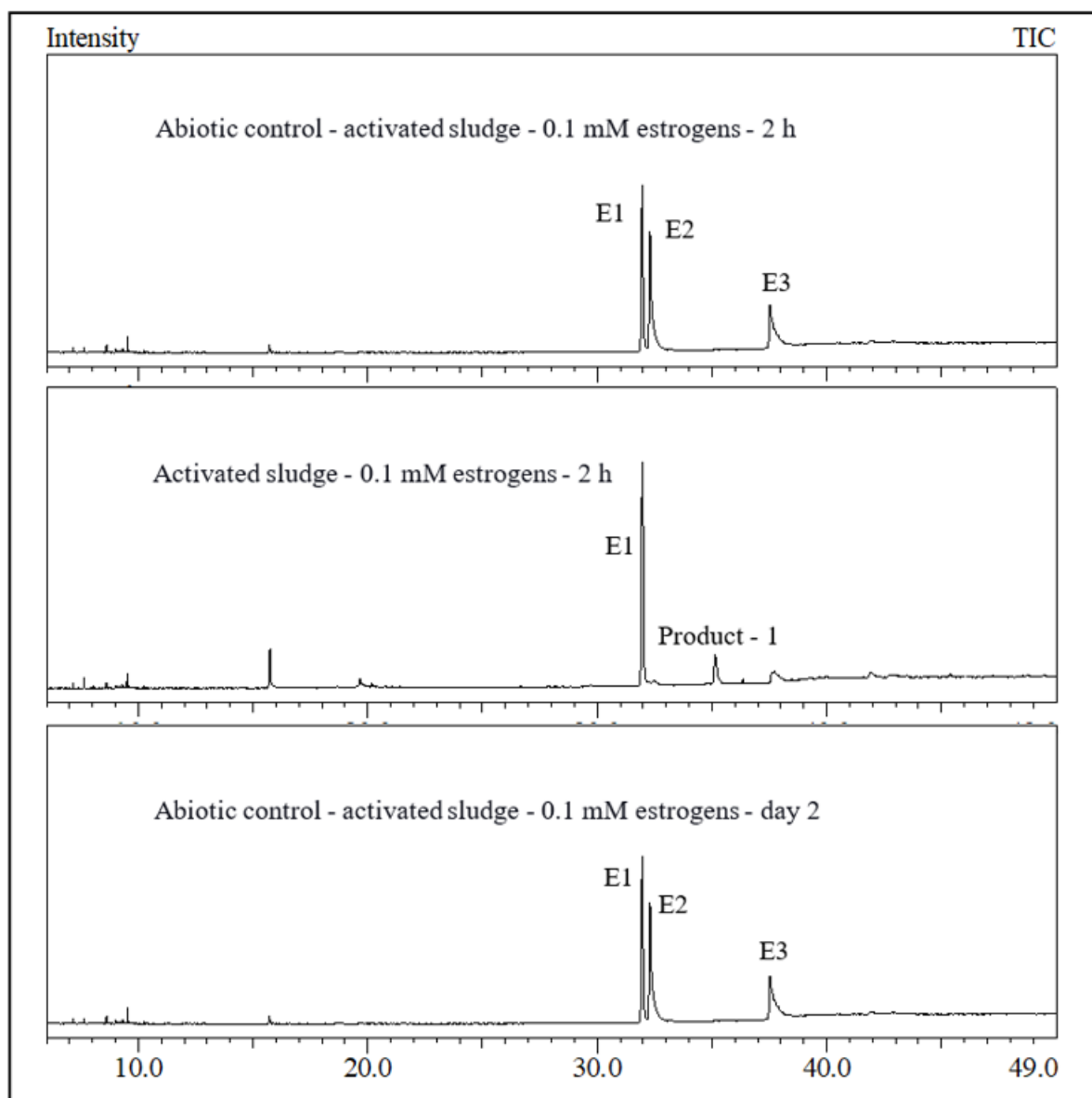


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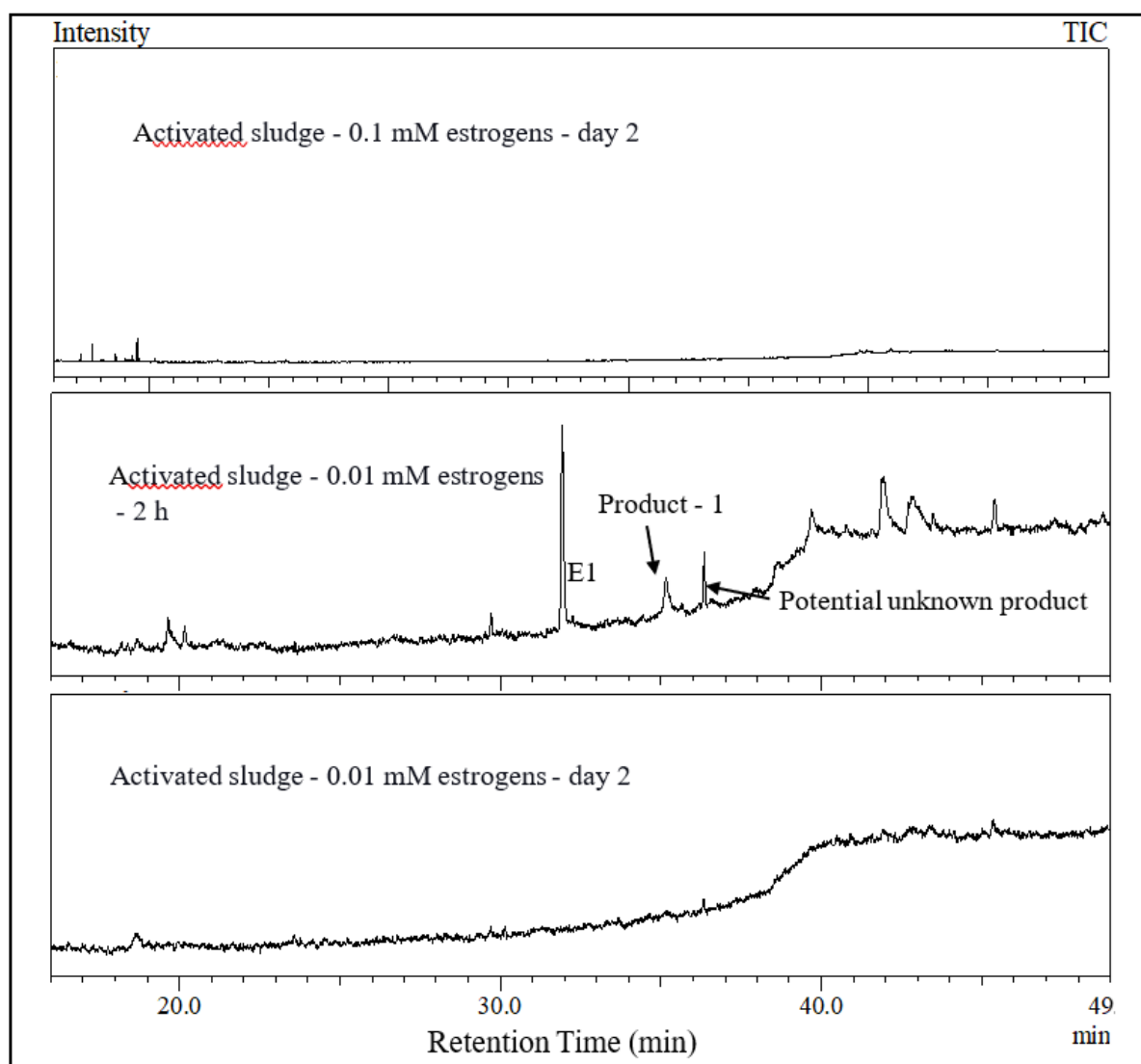


Fig. 4.79 Total ion chromatograms from GC-MS analysis of the activated sludge microcosms. Each microcosm contained 500 mL of freshly collected activated sludge in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 at two concentrations (0.1 mM or 0.01 mM each) and added from DMSO stock solutions.

To unravel whether the inflow consortium enriched in this study can degrade estrogens in wastewater samples, it was prepared as an inoculum and added to autoclaved inflow wastewater and activated sludge microcosms containing E1, E2, and E3. As shown in Figures 4.80-4.81, 4.83-4.84, the estrogens were degraded and consumed within 2 days of incubation, indicating the functionality of the inflow consortium in real wastewater samples. As observed in the previous experiment (without augmentation) no degradation products could be detected. To find out whether combining the inflow

consortium with a living estrogen-degrading indigenous community, the experiment was performed in another batch by inoculating the inflow consortium without autoclaving the wastewater samples using the estrogens in 0.1 mM concentration. Similar to the previous experiments, the estrogens were degraded and could not be detected after two days in all treatments (Figures 4.82 and 4.85). However, in the activated sludge microcosms, we noticed that after two hours, the E2 and E3 peaks decreased while that of E1 increased. Moreover, product-1 could be detected. These findings are similar to those of the activated sludge microcosms without augmentation by the inflow consortium.

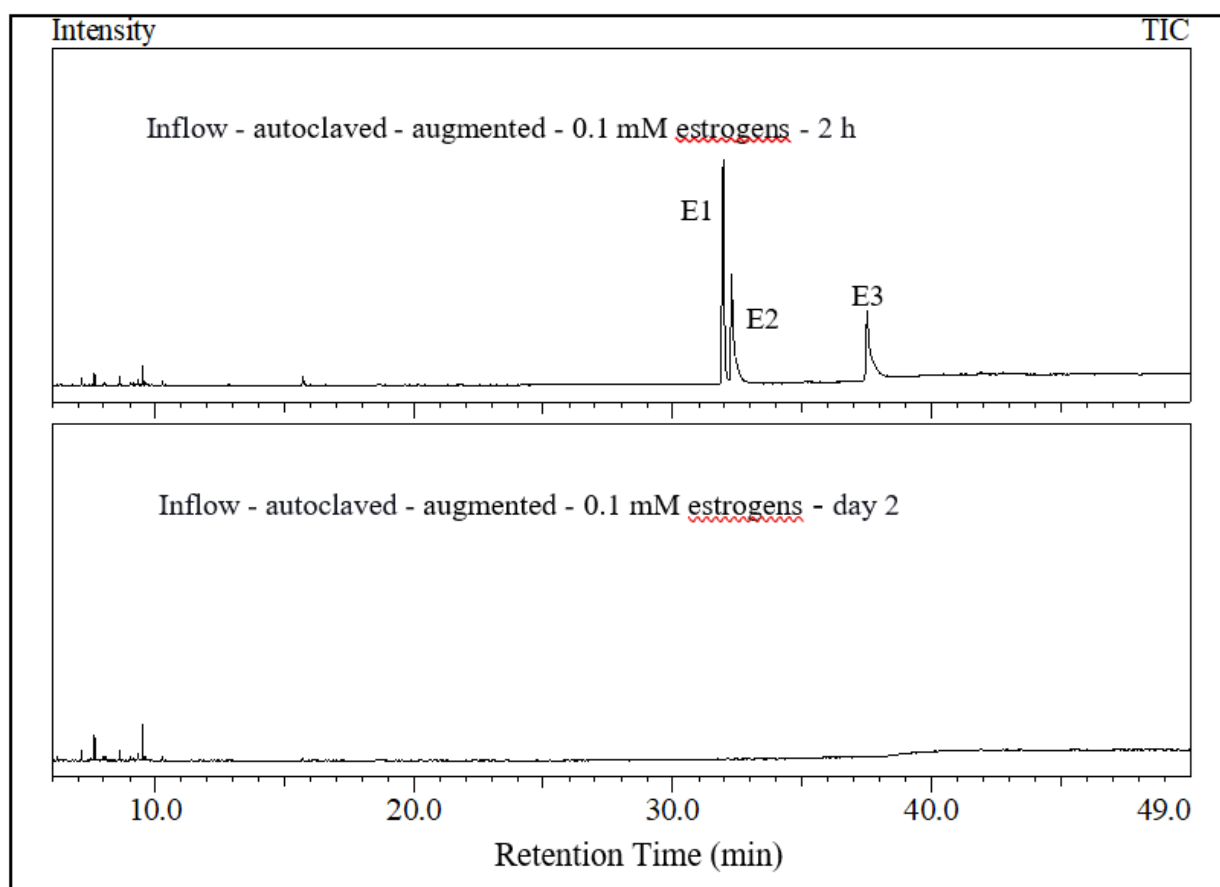


Fig. 4.80: Total ion chromatograms from GC-MS analysis of the inflow microcosms. Each microcosm contained 500 mL of freshly collected and autoclaved inflow wastewater in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.1 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

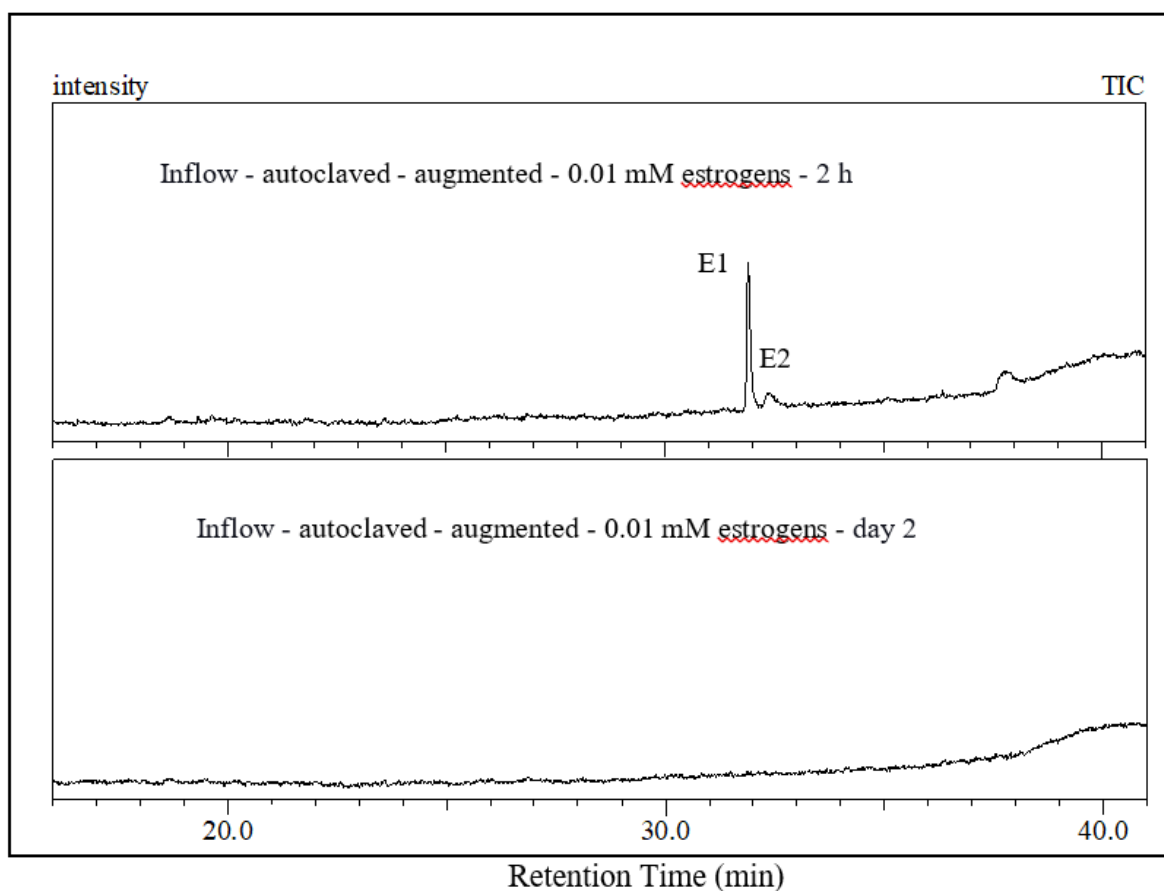


Fig. 4.81: Total ion chromatograms from GC-MS analysis of the inflow microcosms. Each microcosm contained 500 mL of freshly collected and autoclaved inflow wastewater in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.01 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

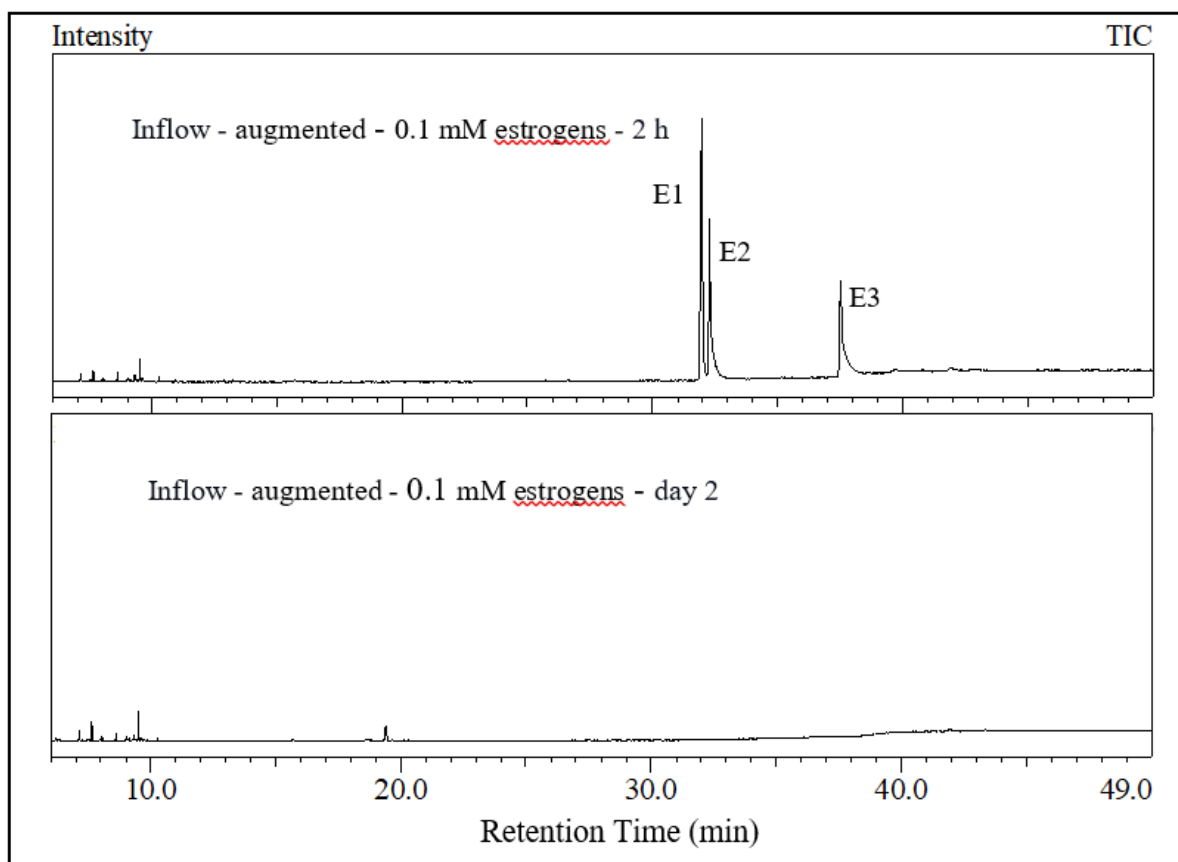


Fig. 4.82: Total ion chromatograms from GC-MS analysis of the inflow microcosms. Each microcosm contained 500 mL of freshly collected inflow wastewater in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.1 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

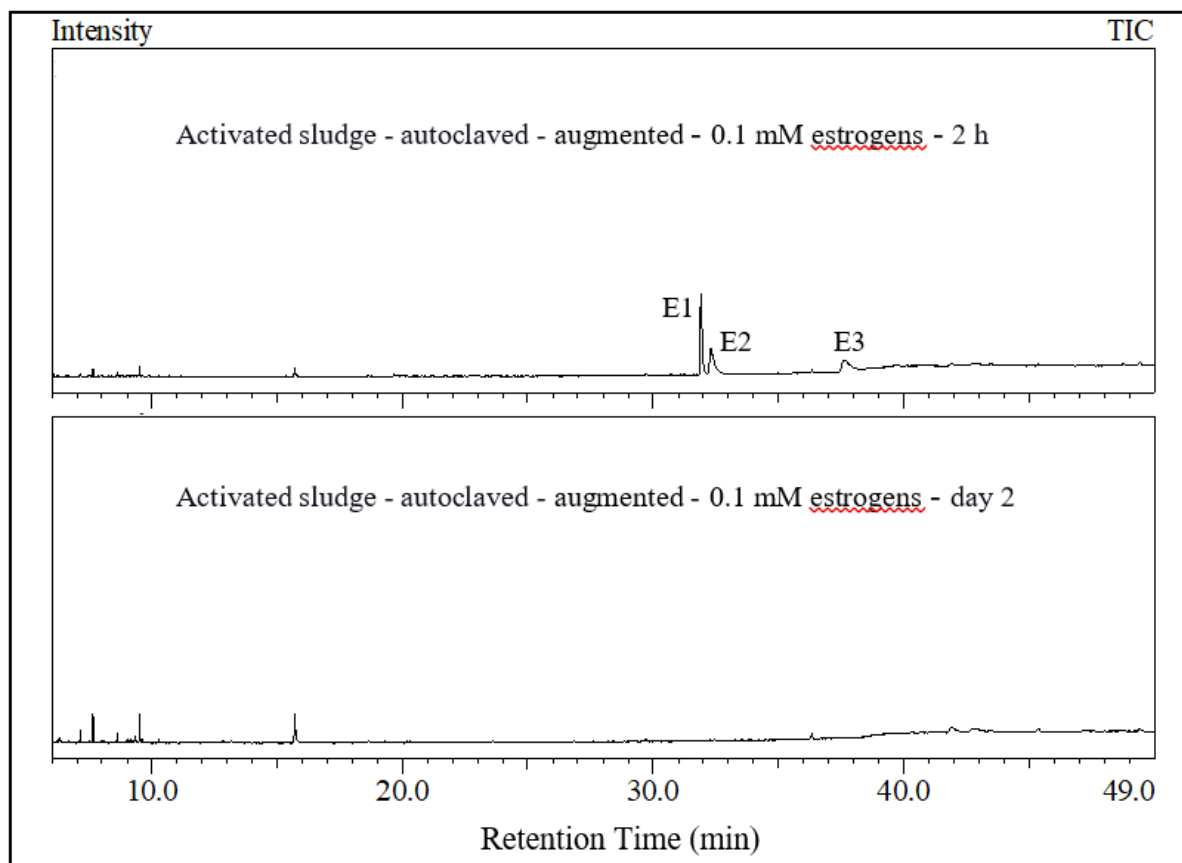


Fig. 4.83: Total ion chromatograms from GC-MS analysis of the activated sludge microcosms. Each microcosm contained 500 mL of freshly collected and autoclaved activated sludge in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.1 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

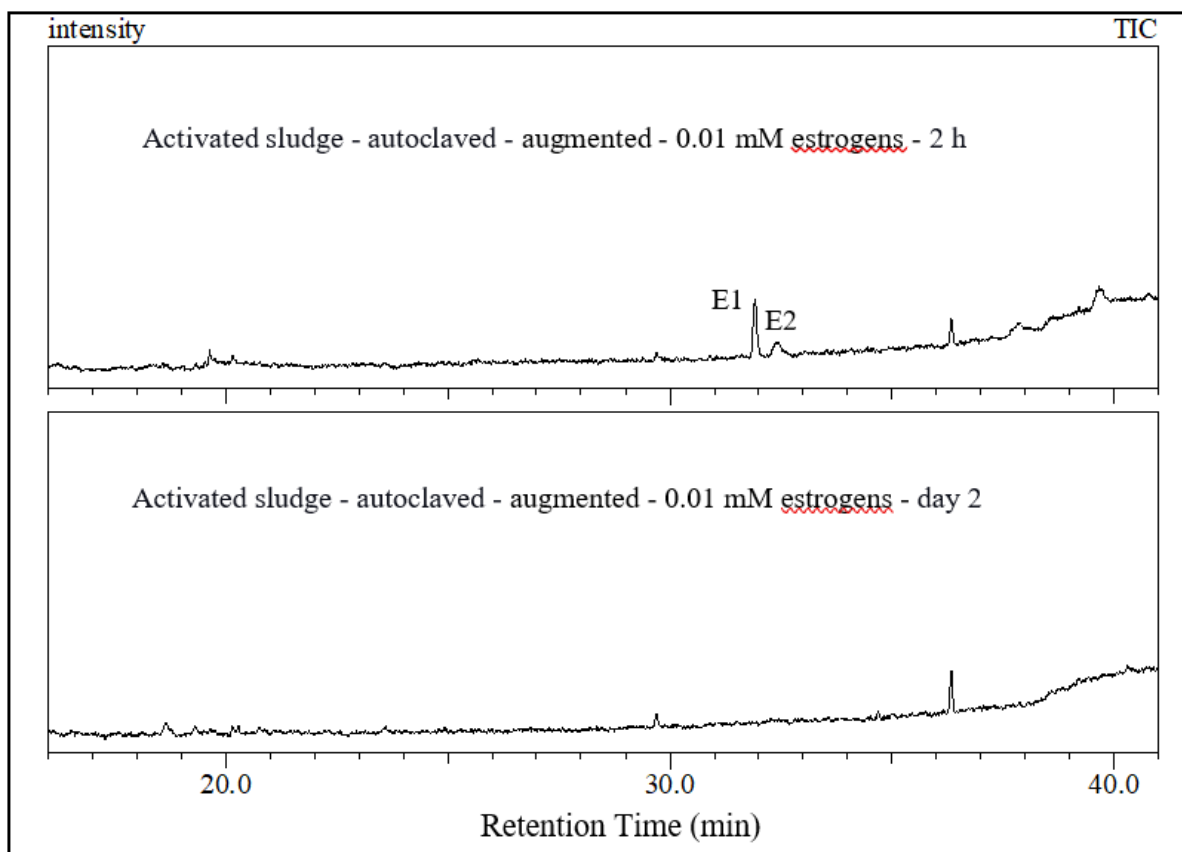


Fig. 4.84: Total ion chromatograms from GC-MS analysis of the activated sludge microcosms. Each microcosm contained 500 mL of freshly collected and autoclaved activated sludge in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.01 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

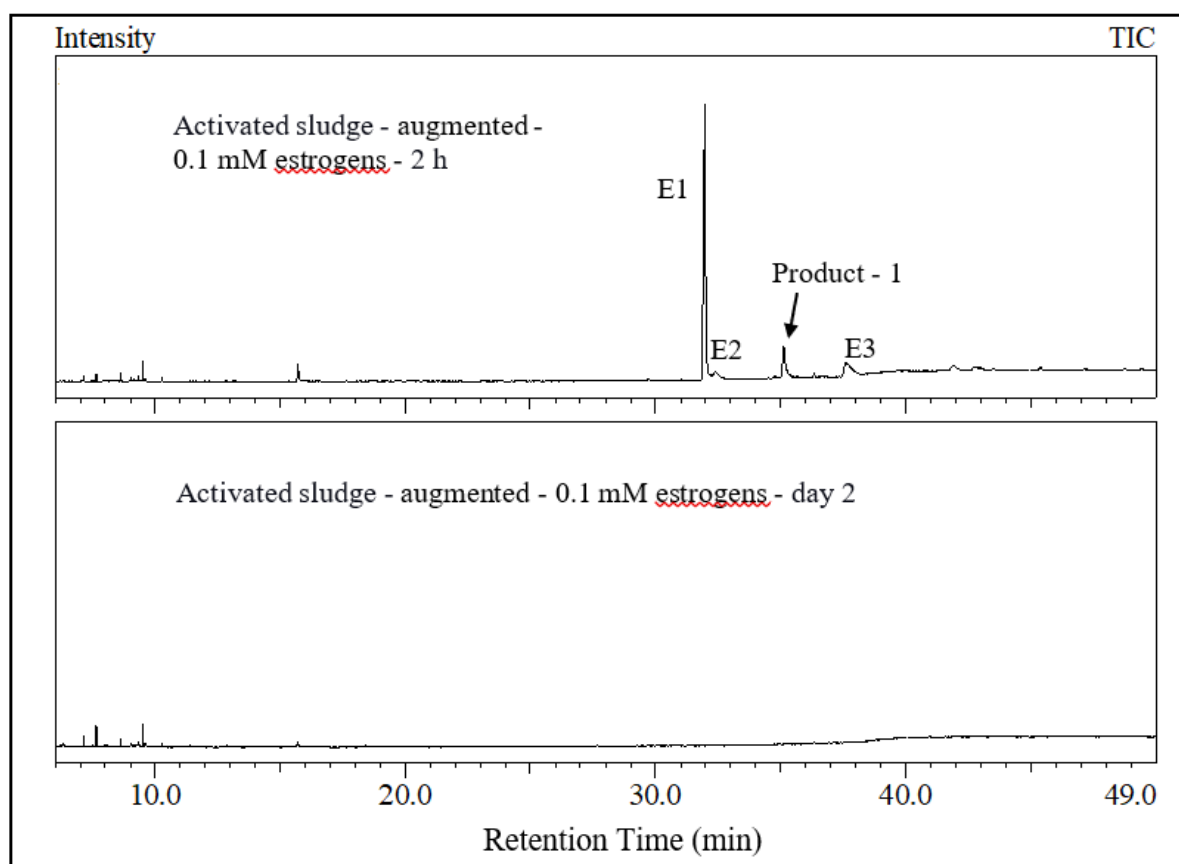


Fig. 4.85: Total ion chromatograms from GC-MS analysis of the activated sludge microcosms. Each microcosm contained 500 mL of freshly collected activated sludge in 2 L flasks and spiked with a mixture of three estrogens E1, E2, and E3 (0.1 mM each) added from DMSO stock solutions. The microcosms were inoculated with the inflow consortium.

On the other hand, EE2 did not show any detectable signs of degradation or transformation after 14 days of incubation in activated sludge or inflow wastewater microcosms (Figures 4.86, 4.87).

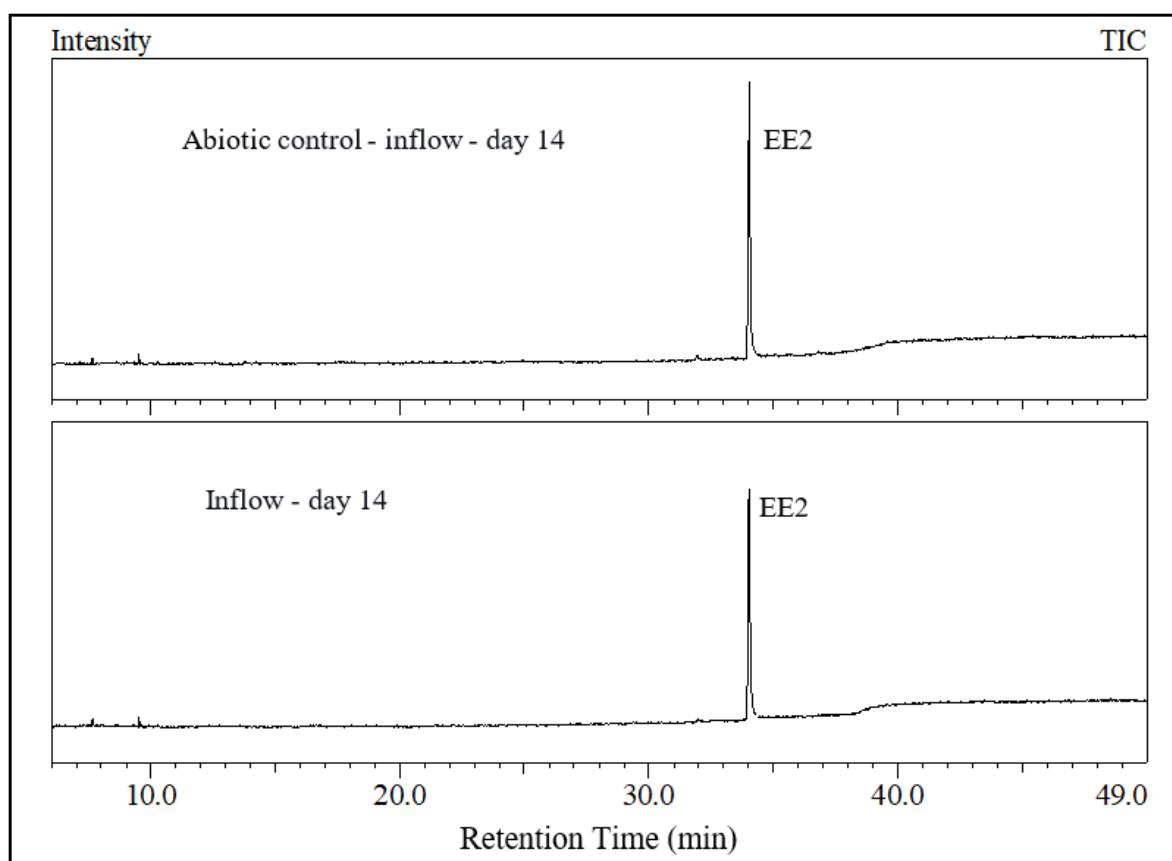


Fig. 4.86: Total ion chromatograms from GC-MS analysis of the EE2 microcosms containing 250 mL of freshly collected inflow wastewater in 1 L flasks and spiked with 0.1 mM EE2 added from a DMSO stock solution.

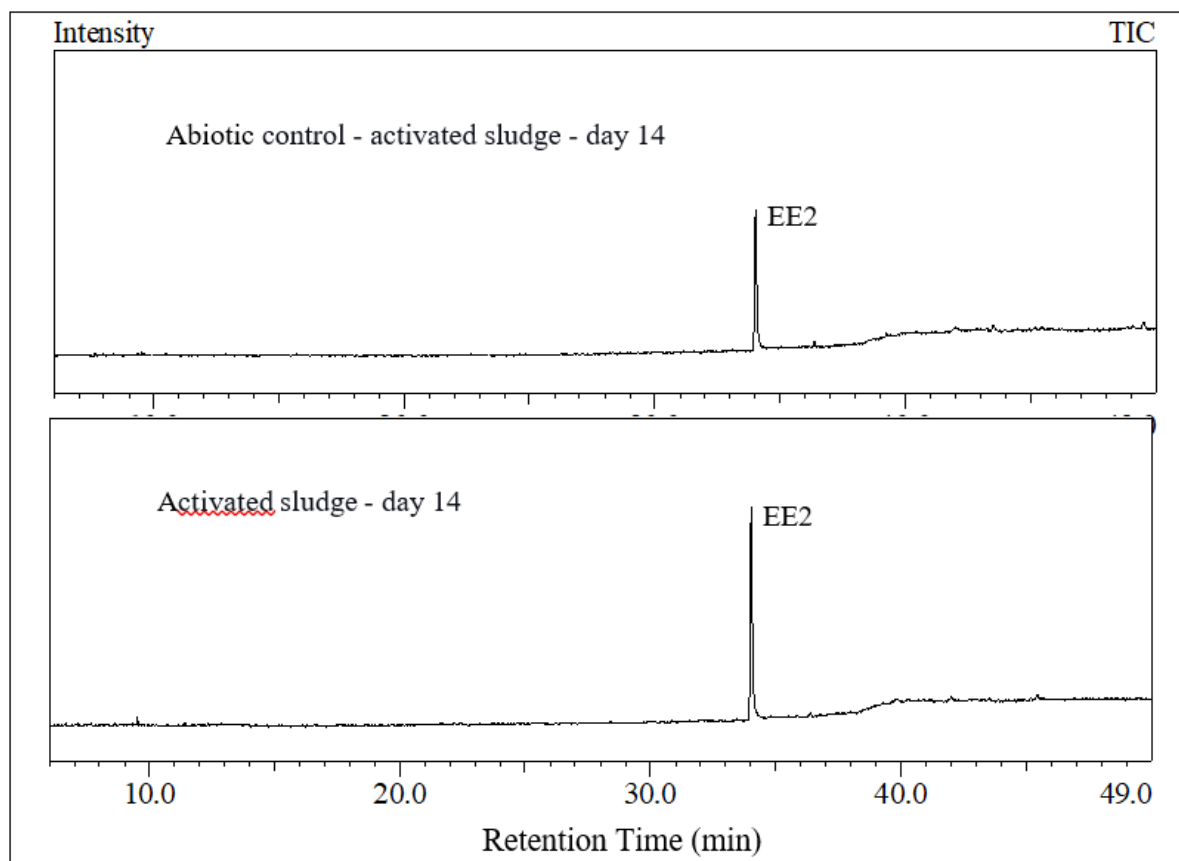


Fig. 4.87: Total ion chromatograms from GC-MS analysis of the EE2 microcosms containing 250 mL of freshly collected activated sludge in 1 L flasks and spiked with 0.1 mM EE2 added from a DMSO stock solution.

4.12. Stability of the E3-degrading inflow consortium

To unravel how stable the inflow consortium is, it was grown on two different DMSO concentrations as the sole carbon source in CDM. The inoculum used for these cultures was prepared from cultures containing E3/DMSO. As shown in Figure 4.88, the inflow consortium grew in this medium, and the growth profiles of both cultures were very similar, indicating utilization of DMSO as a carbon and energy source. Aliquots from those DMSO cultures were then transferred to fresh CDM containing E3/DMSO and growth was monitored. These cultures grew and their growth profiles were similar to the DMSO cultures from which the inoculum was taken (Figure 4.89). To unravel whether E3 was utilized by the inflow consortium, culture samples were retrieved and analyzed by GC-MS, which revealed that E3 was not degraded in these cultures even

after 12 days incubation, indicating that the inflow consortium lost the E3 degradation phenotype (Figures 4.90-4.92).

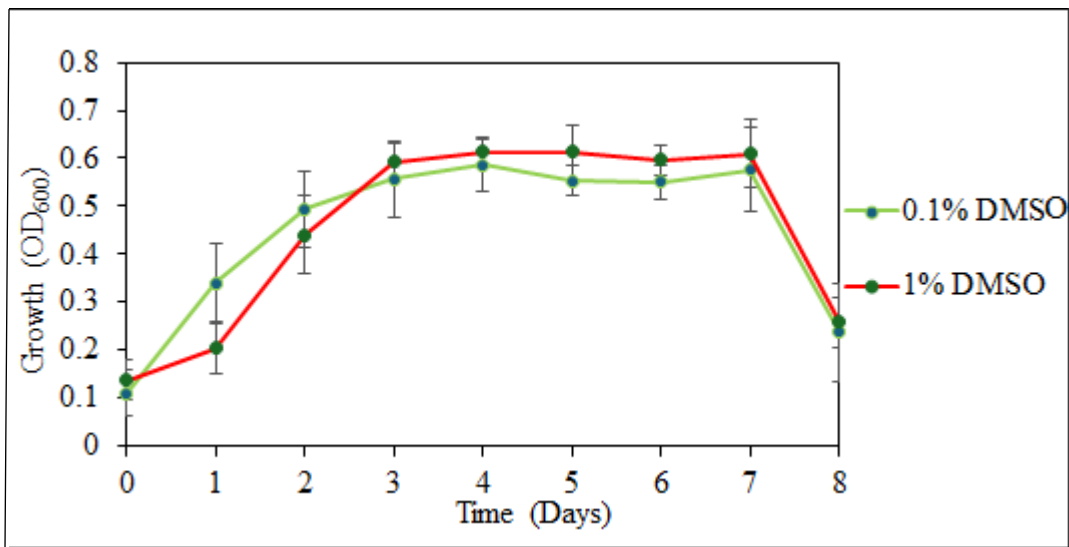


Fig. 4.88: Growth curves of the IF-consortium on 0.1% and 1.0 % autoclaved DMSO (v/v) as the sole carbon source. Data presented as mean of biological triplicates and error bars represent standard error. The cultures were in 100 mL CDM, and the inoculum was prepared from precultures grown in the presence of 0.1 mM E3.

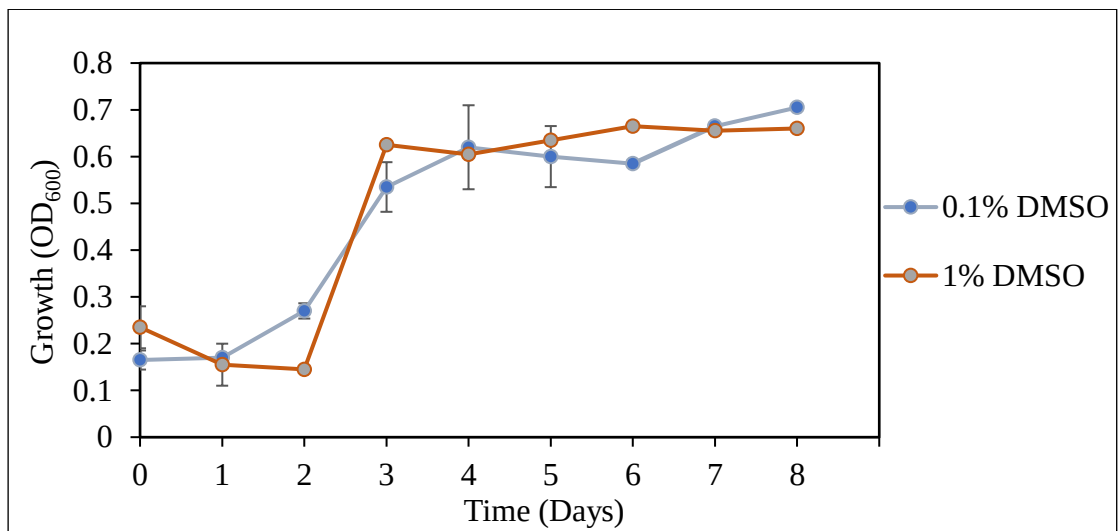


Fig. 4.89: Growth curves of the IF-consortium in CDM cultures containing 0.1 mM E3 and inoculated from precultures grown on either 0.1% or 1.0 % autoclaved DMSO (v/v) as the sole carbon source. E3 was added from a DMSO stock solution. Data presented as mean of biological triplicates and error bars represent standard error.

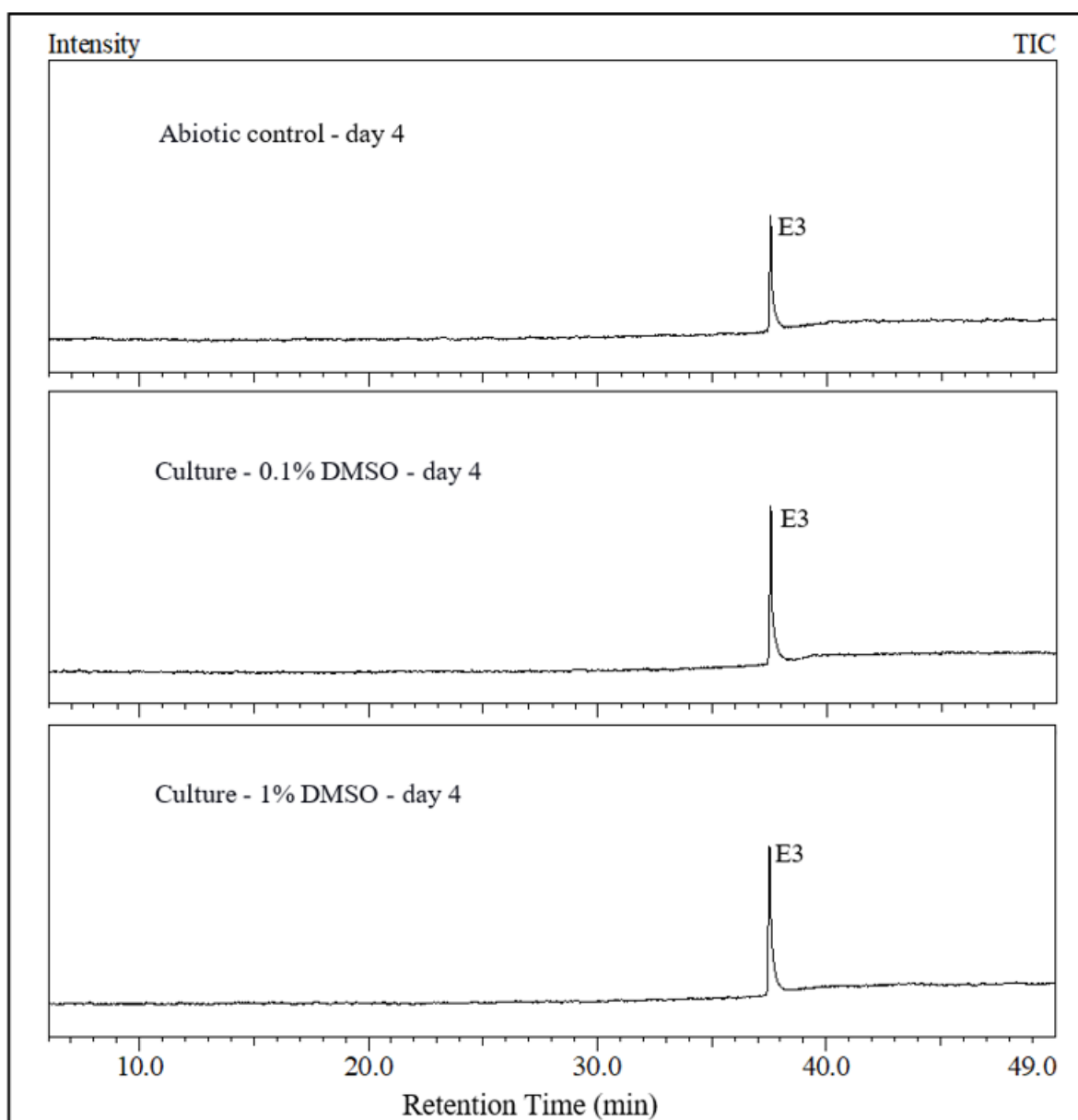


Fig. 4.90: Total ion chromatogram from GC-MS analysis of E3 (0.1 mM) cultures of the inflow consortium. The inoculum of these cultures was from precultures grown on either 0.1 or 1.0 % DMSO as the sole carbon source. Cultures samples were retrieved after 4 days of incubation, extracted with ethylacetate and analyzed by GC-MS.

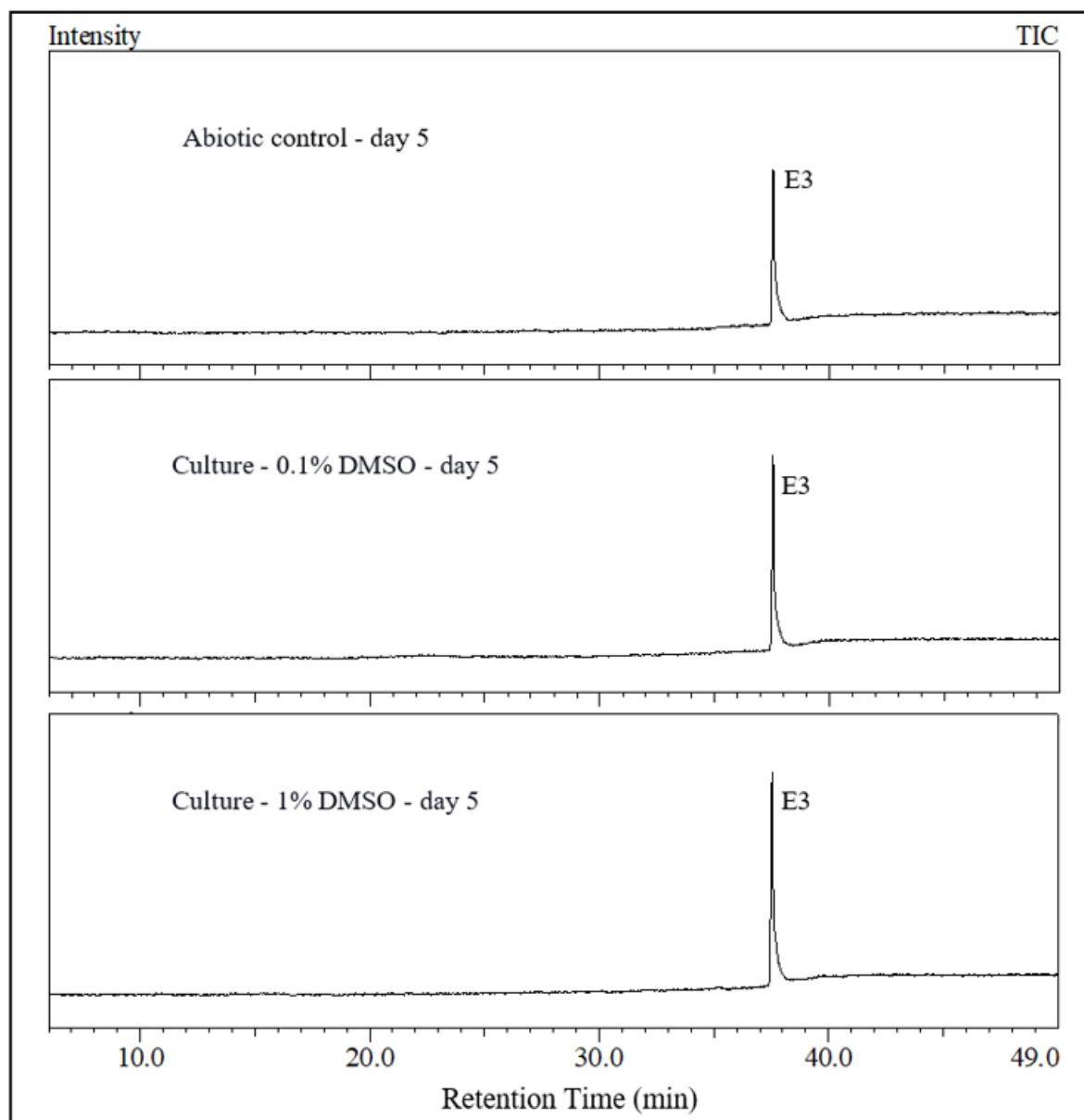


Fig. 4.91: Total ion chromatogram from GC-MS analysis of E3 (0.1 mM) cultures of the inflow consortium. The inoculum of these cultures was from precultures grown on either 0.1 or 1.0 % DMSO as the sole carbon source. Cultures samples were retrieved after 5 days of incubation, extracted with ethylacetate and analyzed by GC-MS.

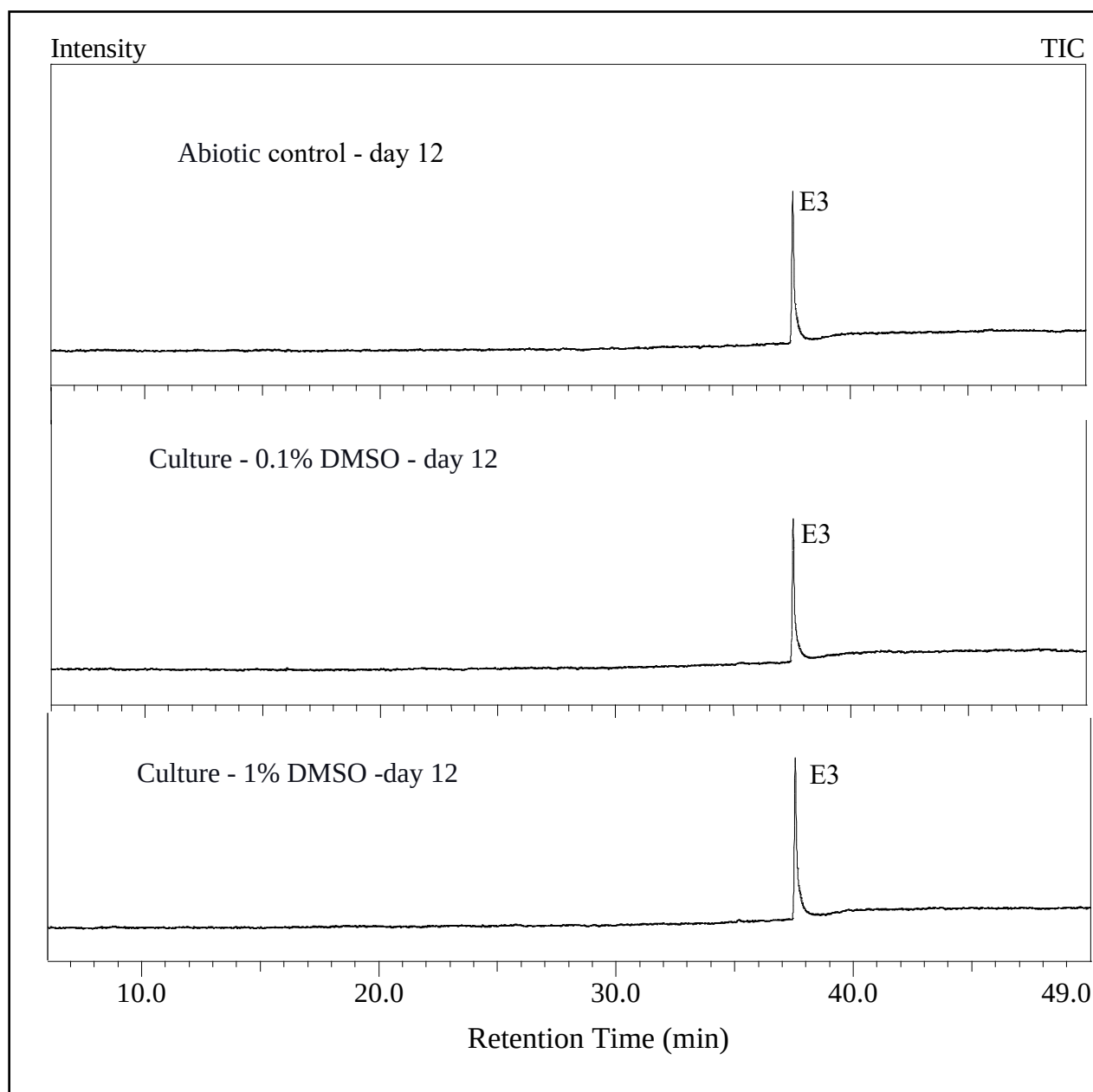


Fig. 4.92: Total ion chromatogram from GC-MS analysis of E3 (0.1 mM) cultures of the inflow consortium. The inoculum of these cultures was from precultures grown on either 0.1 or 1.0 % DMSO as the sole carbon source. Cultures samples were retrieved after 12 days of incubation, extracted with ethyl acetate and analyzed by GC-MS.

5- Discussion

In this study, the selection of EE2 and E3 as model estrogens for biodegradation studies was based on the fact that they are rarely reported in the literature, compared to their counterparts E1 and E2. This under representation could be due to the very high structural similarity among the different estrogens, which leads to the perception that E3 and EE2 biodegradation may proceed similarly to E1 and E2. Furthermore, the relatively high recalcitrance of EE2, due to the ethynyl group, rendered the isolation of EE2-degrading microbes a challenge. However, the large diversity of the reported biotransformation reactions and complete biodegradation pathways justifies more in-depth studies to unravel specific biodegradation mechanisms and key degrading microbes for E3 and EE2. As domestic wastewater treatment plants (WWTPs) are key source of estrogen pollution in the environment, we selected a major WWTP in Bahrain to study EE2 and E3 degradation in wastewater samples collected therefrom. In addition to activated sludge samples, which were frequently studied, raw sewage (inflow wastewater) samples were also collected from WWTP and used as a source of estrogen-degrading bacteria. The study started by trying to enrich EE2-degrading bacteria.

5.1. Attempts to enrich and isolate EE2-degrading bacteria

The various attempts in this study to enrich and isolate EE2-degrading cultures were not successful. This could be attributed to the recalcitrance of EE2 compared to the natural estrogens (Olivera & Luengo, 2019; Zhang et al., 2016). Moreover, the applied growth conditions were probably not conducive to growth of EE2 degraders. For instance, the incubation period was too short, the EE2 concentration was relatively high, an essential growth factor was lacking in the culture medium, etc. In addition, most of the available studies reported microbial cultures that can utilize/transform EE2 in the presence of co-substrates, thus following a cometabolic mode of degradation (O'Grady et al., 2009; Pauwels et al., 2008).

In the current study, the objective was to isolate bacterial cultures that can utilize EE2 as a sole carbon and energy source for growth. Therefore, no other carbon sources were added to the enrichment cultures or bacterial cultures of the tested pure isolates. Although DMSO was present, as the EE2 solvent, to all cultures, it did not play a role as a facilitator of EE2 utilization.

Co-substrates may strengthen central metabolism, promote cell growth, and supply reducing power, and can even induce the expression of some enzymes that play a role in EE2 metabolism (Fischer & Majewsky, 2014; Gröning et al., 2007; Tran et al., 2013). The number of reported microorganisms that can transform or cometabolize EE2 is very limited including *Sphingomonas* spp., *Pseudomonas* spp., *Nitrosomonas europaea*, the cyanobacterium *Microcystis novacekii*, the microalgal strain *Selenastrum capricornutum*, and the fungi *Phoma* sp. and *Pleurotus ostreatus* (Flint, 2019; Pratush et al., 2020a).

Pseudomonas spp. and *Acinetobacter* sp. metabolized EE2 only in the presence of E2 (Pauwels et al., 2008). *Pseudomonas citronellolis* SJTE-3 cometabolized EE2 in the presence of ethanol or glucose (Peng et al., 2023). Moreover, *R. erythropolis* and *R. equi* removed EE2 from minimal medium when adipic acid and glucose were added as co-substrates (O'Grady et al., 2009). Only a few bacteria were reported to degrade EE2 as a sole carbon and energy source such as *Sphingobacterium* sp. JCR5 (Haiyan et al., 2007) and *Rhodococcus equi* (Yoshimoto et al., 2004).

Recently, Palma & Costa (2024) isolated several EE2-degrading bacteria from an acid mine drainage. These bacteria were affiliated with the genera *Aeromonas*, *Rhizobium*, and *Paraburkholderia*. They degraded EE2 in minimal medium and produced several degradation metabolites which were mainly ring cleavage products. The authors mentioned that EE2 was the sole carbon source in the cultures. However, EE2 was added from a methanol stock solution, and it was not clear whether it was evaporated before inoculation. The work on EE2 was terminated at this stage as there is a long list of parameters and factors to be optimized or investigated.

5.2. E3-degrading consortia were enriched from raw sewage and sludge samples

On the contrary to EE2, E3-degrading bacterial cultures could be more readily obtained from raw sewage (inflow wastewater), activated sludge, and thickened sludge samples. This suggests that E3 is less resistant to microbial degradation (Coello-Garcia et al., 2019) and probably E3-degrading microbes are more common in wastewater treatment plants. At this stage it was assumed that the enriched microbial cultures are consortia consisting of many bacteria, and differences in the growth profiles could be due to different bacterial composition of the consortia obtained from the three sources of inocula. Although the similarity between the E3 GC-MS analysis profiles may argue against this postulation, there are some differences that are in favor of it. For instance, in the cultures of the activated sludge consortium, E3 appeared to be consumed faster than the inflow and thickened sludge cultures. Moreover, only in the thickened sludge cultures, one product, designated product-1, could be detected concomitant with E3 consumption. Hence these apparent differences could be due to different bacterial consortia and/or different E3 metabolic pathways. However, to validate these postulations, community structure and degradation pathways data are needed, which is currently lacking.

The literature has a very limited number of studies reporting specifically on E3 biodegradation. E3 was mostly reported in substrate spectrum experiments in research articles addressing biodegradation of other estrogens (Hsiao et al., 2021; Wang et al., 2019c; Ye et al., 2019). It was also reported in some studies as a transformation product of E2 (Hao et al., 2022; Liu et al., 2020), and EE2 (Li et al., 2013a, 2013b; Ma & Yates, 2018). Most of the reported E3-degrading bacteria are axenic cultures isolated from activated sludge, compost, or soil (Flint, 2019). This is in contrast to this study where E3-degrading consortia were obtained from raw domestic sewage in addition to activated sludge and thickened sludge.

The ability of all three enriched consortia to retain E3 degradation capability after repeated subculturing of the initial enrichments indicates utilization of E3 as a carbon and energy source. However, all cultures of the consortia contained DMSO as the solvent of E3 and glycerol carry over from the inocula obtained from frozen stocks. This raised the question whether DMSO and/or glycerol served as a carbon source for growth of the consortia and whether they were indispensable for E3 co- metabolism. Exclusion of glycerol by modifying the inoculum preparation procedure did not affect the E3 degradation profile as revealed by GC-MS analysis. However, it affected the growth profiles, indicating that glycerol could be utilized by the enriched consortia as a carbon source. This was confirmed by growth of the consortia in CDM with glycerol as the sole carbon and energy source.

Accordingly, it can be proposed that in the cultures containing E3/DMSO, the consortia degrade and utilize E3 as a carbon source. The exhaustion of E3 added to the cultures and absence of degradation products as revealed by GC-MS analysis further confirms utilization and mineralization of E3, which in line with growth-associated metabolism of E3. At this stage, however, it could not be excluded that the consortia can also utilize DMSO as a carbon or a sulfur source. Hence, each consortium might consist of E3-degrading and DMSO-degrading members or subpopulations. Alternatively, the same consortium members may be using both E3 and DMSO simultaneously. Some of the pure isolates reported in the literature can utilize E3 as a sole carbon and energy source (Hsiao et al., 2021; Ke et al., 2007; Li et al., 2012; Liu et al., 2021).

Further evidence for mineralization of E3 by the enriched consortia could be envisaged from the GC-MS analysis showing formation of product-1 in the cultures of the thickened sludge consortium. This product did not accumulate in the culture and could not be detected after 7 days of incubation indicating that it was further metabolized. According to mass spectral search, it appears that product-1 is most probably 16 α -OH-E1. It can be produced from E3 by oxidation or dehydrogenation of the C17 hydroxyl group, a reaction that is catalyzed by 17 β -hydroxysteroid dehydrogenase (Ye et al., 2019).

It is well-known that estrogen-degrading/transforming bacteria have several hydroxysteroid dehydrogenases (Pratish, et al., 2020a). These findings are in line with those reported by Ke et al. (2007) where an *Acinetobacter* sp. degraded E3 as a sole carbon source and transiently produced 16 α -OH-E1. This compound was reported before as a metabolite of E2 degradation by sewage bacteria (Lee & Liu, 2002) and of E1 degradation by intestinal bacteria (Järvenpää et al., 1980). Moreover, 16 α -OH-E1 can be reduced to E3 under anaerobic conditions by *Staphylococcus aureus* (Järvenpää et al., 1980).

At this stage, it was postulated that 16 α -OH-E1 is a key intermediate in the E3 degradation pathway, at least by the thickened sludge consortium. The subsequent investigations aimed at answering the question: Is it possible to isolate pure E3-degrading bacteria from the enriched consortia?

5.3. Isolation and identification of E3-transforming bacteria

Despite the isolation of many bacterial strains from the three enriched consortia, none of them could grow on E3 or the solvent DMSO as the sole carbon and energy source. However, five isolates transformed E3 to product-1, which accumulated in the cultures whether E3 was added with or without the solvent. These findings provide several clues.

1. Complete E3 degradation or mineralization requires several members of the consortia.
2. The five isolates may play a role in E3 biodegradation by their respective consortia.
3. 16 α -OH-E1 is a key intermediate in E3 biodegradation by the enriched consortia.

It is, however, worth noting that isolation of bacteria from the three consortia was done on LB-agar plates, without addition of any estrogens. This could explain why E3-utilizing/mineralizing bacteria could not be isolated, as the medium was probably not sufficiently selective. The majority of E3-degrading bacteria reported to date were pure

cultures and were isolated mainly from activated sludge, farmland soil, compost, and animal waste (Flint et al., 2019; Li et al., 2021; Tian et al., 2020). On the contrary, to the best of our knowledge, no E3- degrading consortia have been reported to date.

In addition to product-1, two additional products were detected in cultures of the isolate AS-31, which was isolated from the activated sludge consortium. Product-2 was detected only when this isolate was included with 1 mM E3/DMSO, and it has a higher molecular mass than E3. The similarity index to the closest matches from the mass spectral library was very low and suggests that product-2 could be obtained from E3 via several structural modifications including alkylation and hydroxylation reactions. Hence, product-2 may be a biotransformation product of E3. Production of estrogen biotransformation products with higher molecular mass than the estrogen substrate has been reported (Bilal et al., 2021; Pratush, et al., 2020a).

Another minor product, product-3, was also detected in the AS-31 cultures only when supplied with E3 as solid crystals (without DMSO). Similar to product-2, the mass spectral similarity index of the closest matches was low. However, product-3 appeared to have 4 mass units less than E3 and could be another oxidation product. Currently, the available information is not sufficient to elucidate more precisely the chemical structure of these to products, and therefore, their potential role in E3 biodegradation/biotransformation awaits further investigation.

The 16S rRNA gene sequences and phylogenetic analysis revealed that the 5 E3-transforming isolates belong to the genera *Gordonia*, *Microbacterium*, and *Hydrogenophaga*. Some studies reported E2 degradation by *Microbacterium* sp. (Hao et al., 2022, 2023; Xiong et al., 2023). Moreover, *Gordonia* sp. degraded the four estrogens as a sole carbon source (Liu et al., 2020), cholesterol (Drzyzga et al., 2011), and bile acids (Chamizo-Ampudia et al., 2023).

Hydrogenophaga spp. were also implicated in the degradation of E1 (Du et al., 2017), bisphenols (Huang et al., 2019) sulfamethoxazole (Tian et al., 2024), and phthalic acid (Wang et al., 2022). Moreover, *Hydrogenophaga* spp. were reported among the

dominant members of a bacterial community in a membrane bioreactor treating synthetic hospital wastewater (Tiwari et al., 2021).

5.4. The inflow consortium degrades natural estrogens

One of the three enriched consortia, namely, the inflow consortium, was selected for further in-depth investigations. Growth profiles and GC-MS analysis confirmed the ability of the inflow consortium to grow on and utilize the natural estrogens E1 and E2 either individually or in a mixture with E3. This finding shows a broad substrate spectrum of the inflow consortium. In addition, it excludes co-metabolic degradation of these natural estrogens. On the contrary, the inflow consortium could not grow on the synthetic estrogen EE2 or utilize it either individually or in a mixture with the three natural estrogens. This indicates that growth observed in EE2 cultures was on DMSO. In contrast, in cultures containing a mixture of the four estrogens only E2 and E3 were utilized and both E1 and EE2 accumulated in the cultures. Formation of product-1 in this culture was concomitant with E3 degradation, further confirming that product-1 is an intermediate of E3 biodegradation. Furthermore, utilization of E2 may explain why E1 peak increased with time, which is in line with the reported metabolic transformation of E2 via E1 (Chen et al., 2018).

Accumulation of E1 and product-1 for two months could be due to competitive substrate inhibition of E1 and product-1 metabolism by EE2. Alternatively, an essential nutrient was potentially exhausted from the culture medium. In either case it can be postulated that E2 and E3 were only transformed, and no further metabolism occurred. This explains why the culture entered the stationary phase after 48 h.

5.5. E3 is degraded via the E1 degradation 4,5-*seco* pathway

Analysis of the E3 and E1 degradation metabolites by UPLC-HRMS provided two key evidence. The first confirmed that E1 and E3 were mineralized as carbon sources in the inflow consortium cultures, despite the presence of DMSO. The second clearly proves that E3 and E1 are metabolized via the 4,5-*seco* pathway. Although E1 metabolism via

the 4,5-*seco* pathway was reported by some bacteria (Chen et al., 2018; Wu et al., 2019) this was not the case for E3 until very recently (Liu et al., 2024).

Detection of E1 as a key metabolite in the E3-grown cultures of the inflow consortium suggests that transformation of E3 to E1 is one of the initial reactions in the E3 catabolic pathway. We can envisage several possible scenarios on how E3 can be transformed to E1. Based on our findings, E3 is first oxidized by a putative hydroxysteroid dehydrogenase at either C17 or C16 to produce 16 α -OH-E1 or 16-keto-E2, respectively (product-1) (Ke et al., 2007; Pratush et al., 2020a; Ye et al., 2019).

As we did not detect any E2 derivatives, we postulate that product-1 is 16 α -OH-E1. Further metabolism of product-1 via D-ring cleavage (Pratush et al., 2020a) can be excluded as we did not detect D- ring cleavage products. In contrast, detection of E1 and 4-OH-E1 strongly suggests that product-1, 16 α -OH-E1 is transformed to E1 via removal of the C16 hydroxyl group by a putative dehydroxylase/dehydratase. Then, E1 is further metabolized via the 4,5-*seco* pathway. While this work was in progress, a very recent study by Liu et al. (2024) reported the degradation of E3 by *Novosphingobium* sp. ES2-1 and proposed two alternative pathways for E3 degradation based on metabolite analysis.

The first proposed pathway proceeds via E3 transformation to E1, which is further degraded via the 4,5-*seco* pathway, as we proposed in this study. However, the authors did not clarify how E3 was transformed to E1. They proposed oxidation and dehydration reactions, albeit no biochemical evidence was presented. The other pathway proposed by Liu et al. (2024) also proceeds by transforming E3 to E1, then to 4-OH-E1, which undergoes B-ring cleavage, resembling the 9,10-*seco* pathway. However, nothing was reported on the genes and enzymes involved in this later pathway. Moreover, the mechanism of the B-ring cleavage was not discussed. The various detected metabolites were not quantified, which is essential to reflect on the relative significance or dominance of the two proposed pathways.

Although the involvement of a dehydroxylation reaction in aerobic or anaerobic microbial estrogen metabolism has not been reported, biological dehydroxylation is known to occur in bile acids metabolism by gut-associated bacteria and in particular via the Hylemon-Björkhem 7 α -dehydroxylase pathway of bile acid degradation (Ridlon et al., 2023; Wise & Cummings, 2023). Moreover, biological dehydroxylation is a key step in bacterial anaerobic degradation of 4-hydroxybenzoyl-CoA and other aromatic compounds which are metabolized via 4-hydroxybenzoyl-CoA such as phenol, 4-hydroxybenzoate, *p*-cresol, and 4-hydroxyphenylacetate (Brackmann & Fuchs, 1993), in a Birch-reduction-like mechanism.

It is worth noting that the E3 concentration (1 mM and 0.1 mM) used in the different enrichment and biodegradation experiments in this study was several orders of magnitude higher than the actual E3 concentration detected in the activated sludge (Chen et al., 2017; Chiang et al., 2020). Although this was done on purpose to enable detection of the degradation intermediates, it might have an impact on the degradation rate, mechanisms and the involved microbial communities (AlDhafiri et al., 2022; Chen et al., 2017; Xu et al., 2009). Moreover, the laboratory conditions that were applied are different from the actual process conditions applied for the activated sludge treatment in WWTPs, which might alter the structure and function of the indigenous microbial communities. Therefore, despite the strong UPLC-HRMS-based evidence suggesting the aerobic degradation of E3 by the inflow consortium via the 4,5-*seco* pathway, the presence of alternative E3 degradation pathways in raw sewage or activated sludge can't be fully excluded.

5.6. Structural and functional analysis of the E3-degrading inflow consortium

After elucidation of the E3 biodegradation pathway by the inflow consortium, it was important to identify the key E3 degraders among the consortium community members and reveal how the community structure might evolve with time. In addition to the E3

cultures, glucose cultures were included to differentiate between estrogen degraders and non-estrogen degraders. Moreover, E1 cultures were also included in an attempt to reveal community members specifically associated with E3 degradation (if any). Culture samples from day 2 and day 4 up to day 14 could be analyzed for the glucose (G) and E3 cultures. However, from the E1 cultures, DNA of sufficient quantity could be isolated only from day 10 to day 14. This could be due to low biomass yield during the first 7-8 days of incubation.

The adopted experimental approach was effective, and the findings revealed key aspects of the community structure dynamics and the key functional members. In general, the remarkable differences between the glucose cultures and the estrogen-containing cultures indicate uniqueness of the estrogen-degrading community whose members were strongly enriched in the presence of estrogens. The glucose- metabolizing community was fairly stable during the time frame of the experiment, whereas strong compositional shift occurred in the estrogen cultures after day 8, highlighting the possibility that different sub-populations or different community members are involved in estrogen degradation at the different time intervals.

This could be due to change of the estrogen and metabolite concentrations with time. Another plausible explanation is that complete degradation or mineralization of the estrogen substrate may require concerted action of various community members. This postulation is coherent with the finding that E3 was not mineralized by the isolated pure cultures. Indeed, the remarkable shift in the community composition which happened after 8 days was coincident with the detection of E1 and the 4-OH-E1 in the E3 cultures. It is thus tempting to propose that the initial stages of E3 metabolism, which prevailed at high E3 concentration, are mediated by the community members which dominated the culture up to day 8. Then further metabolism requires a community that evolves as the initial intermediates start to accumulate and/or as the E3 concentration decreases.

Considering that the inoculum used for this experiment was prepared in E3-containing culture, it can be postulated that original composition of the inflow consortium

is similar to the composition of the consortium revealed at day 4 in the E3 cultures, because data for day 0 and day 2 are lacking. Hence, the inflow consortium originally consisted mainly of members of the genera *Hyphomicrobium*, *Achromobacter*, *Microbacterium*, and *Cupriavidus* as the most dominant members. This community structure appears to be the key E3-degrading community up to day 8, where the community became strongly dominated by *Croceicoccus estronivorus*, an α -proteobacterial member of the *Erythrobacteraceae* (Kim et al., 2021) that appears to be the key E3 degrader during the late stages of the culture. Interestingly, similar trend was observed in the E1 culture, indicating that *Croceicoccus estronivorus* is a key estrogen degrader in the inflow consortium.

Detection of *Microbacterium schleiferi* as one of the dominant members in the E3 culture is consistent with the isolation of *Microbacterium schleiferi* DSM 20489 from the inflow consortium grown on E3 (IF-20) and suggests a key role in E3 metabolism. Among the dominant bacteria identified in the estrogen-grown cultures, members of the genera *Achromobacter*, *Microbacterium*, and *Aminobacter* were reported in estrogen biodegradation studies (Flint, 2019). Also, *Hyphomicrobium* spp. were detected among the dominant members of estrogen-degrading consortia enriched from activated sludge (Villemur et al., 2013). On the contrary, *Croceicoccus estronivorus* (Formerly, *Altererythrobacter estronivorus*) has been very rarely reported in estrogen biodegradation studies (Qin et al., 2016). However, *Croceicoccus* spp. were studied for the biodegradation of bisphenol-A (an endocrine disruptor) (Li et al., 2021) and hydrocarbons (Huang et al., 2015).

To our knowledge, biodegradation of E3 by microbial consortia has not been reported. In contrast, E1 biodegradation via the 4,5-seco pathway by a bacterial consortium in activated sludge microcosms was reported (AlDhafiri et al., 2022). This study was conducted with activated sludge samples collected from Tubli WWTP in Bahrain, which is the same WWTP studied in the current investigation. Although the key E1 degraders in the consortium were Alphaproteobacterial taxa, in line with the current

study, the dominant bacteria were affiliated mainly with *Novosphingobium*, *Sphingoaurantiacus* and *Sphingobium* spp., unlike the current study. The authors also reported temporal shifts in the community composition.

Estrogen (E1, E2, and E3) degradation by activated sludge and mixed cultures enriched therefrom was also reported. Although the structure of the mixed culture was determined based on culture dependent isolation, the temporal and substrate-dependent dynamics were not reported (Müller et al., 2010; Weber et al., 2005). The members of these mixed cultures were affiliated with *Alcaligenes*, *Pusillimonas*, *Denitrobacter*, *Bretundimonas*, *Achromobacter* and *Ralstonia* spp.

Bernardelli et al. (2019) analyzed the structure and dynamics of a bacterial community degrading estrogens in a sequencing batch reactor with activated sludge and synthetic wastewater. The original community was dominated by *Altererythrobacter* and *Sphingopyxis*. After 30 days, the community structure shifted towards *Pseudomonas* and *Nitrospira*, while the relative abundance of *Altererythrobacter*, *Novosphingobium*, and *Sphingopyxis* significantly decreased. The authors assumed that *Pseudomonas* spp. were key estrogen degraders, and their abundance decreased with time due to exhaustion of E1 and E2 compared to EE2, thus highlighting the role of the substrate concentrations in shaping the microbial community structure. The authors also noted that some members of the community which existed in low abundance in the inoculum such as *Nubsella*, *Sphingobacterium*, *Emticicia*, and *Sphingomonas* spp. increased significantly with time and were proposed to play a role in EE2 degradation, which persisted for longer time in the reactor.

The effect of substrate concentration on the composition of E1-degrading bacterial community in activated sludge was studied by Thayanukul et al. (2010). Microautoradiography-fluorescence in situ hybridization revealed that at high concentration (200 µg/L) the E1-degrading community was dominated by members of the *Beta*- or *Gammaproteobacteria*. However, at low concentration (540 ng/L), i.e. as the E1 concentration decreased, the community shifted towards *Alphaproteobacterial* taxa,

and the relative proportion of the *Beta*- and Gammaproteobacterial taxa decreased. In mudflat sediment microcosms amended with bisphenol A, the relative proportion of *Croceicoccus* substantially increased with time, suggesting a role in bisphenol A biodegradation (Tong et al., 2021).

5.7. The inflow consortium degrades estrogens in raw sewage and activated sludge

To further explore the potential of the inflow consortium to eliminate estrogens in wastewater, it was used to bioaugment raw sewage and activated sludge microcosms spiked with a mixture of estrogens (E1, E2, E3) at two different concentrations (0.1 and 0.01 mM). Initial experiments revealed that the estrogen mixtures were degraded in raw sewage (inflow wastewater) and activated sludge microcosms without bioaugmentation, indicating the presence of estrogen-degrading bacterial communities, which is consistent with enrichment of estrogen-degrading bacterial consortia from these sources.

After showing the estrogen degradation activity of indigenous microbial communities, new microcosms were prepared, autoclaved, and inoculated with the inflow consortium. Similar to the previous experiment, elimination of the estrogen mixtures at two different concentrations confirmed the functionality of the inflow consortium not only in raw domestic sewage from which the consortium was initially obtained, but also in activated sludge. These findings show that estrogen biodegradation in raw sewage and activated sludge during wastewater treatment does not necessarily require supplementation with any nutrients such as those constituting the CDM. It was even interesting to see that the mixture of the natural estrogens was apparently removed faster in the spiked microcosms than in CDM inoculated with the inflow consortium. Combining the inflow consortium with indigenous microbial communities in the raw sewage and activated sludge did not affect the biodegradation profile remarkably as revealed by GC-MS analysis. On the contrary, to the natural estrogens, persistence of

EE2 in the microcosms excludes the presence of EE2-degrading bacteria and explains why it was not possible to enrich or isolate EE2-degrading bacteria from these sources.

While biodegradation of estrogens in activated sludge microcosms and by mixed cultures enriched from activated sludge has been studied (Bernardelli et al., 2019; Lee & Lin, 2001; Müller et al., 2010; Thayanukul et al., 2010; Weber et al., 2005), similar studies for raw domestic sewage (inflow wastewater), and particularly for E3 biodegradation are largely lacking. However, in most of those studies, the enriched consortia or isolated bacteria were not tested to reveal their functionality when re-inoculated into activated sludge samples.

5.8. The inflow consortium is not functionally resilient

One of the crucial issues challenging the application of mixed cultures or microbial consortia in bioremediation is the functional resilience/resistance, i.e. stability of the consortium. Nonetheless, this key criterion is not properly studied for estrogen-degrading bacterial consortia reported in the literature. In the current study, stability of the inflow consortium was tested by growing it in CDM with only DMSO as the sole carbon source, then aliquots from the DMSO cultures were re-inoculated into E3/DMSO cultures. This approach was based on the hypothesis that growing the inflow consortium under non-selective conditions, i.e. in the absence of estrogens, with a non-steroid carbon source will disrupt the community structure by eliminating the key-estrogen degraders.

Obviously, persistence of E3 up to day 12 indicated that the inflow consortium lost its E3 biodegradation phenotype. Loss-of-function suggests that the inflow consortium is not sufficiently resilient. It appears that growth on DMSO alone leads to extinction or elimination of the key E3 degraders from the consortium, which becomes dominated by DMSO utilizers. This explains why the inflow consortium could not degrade E3 when transferred from the DMSO to the E3/DMSO cultures. It further indicates that the growth observed in the E3/DMSO cultures was due to DMSO utilization. DMSO biodegradation by bacteria has been reported (Hwang et al., 2007). Furthermore, it can be postulated that

the key E3-degrading members are not able to metabolize DMSO as a sole carbon source, which explains why they need the presence of E3 to be retained in the consortium, i.e. E3 provides selective pressure on the consortium to maintain the E3 biodegradation phenotype even in the presence of other carbon sources.

Conclusions and future research directions

This study provided a plenty of solid evidence showing the presence of estrogen-degrading microbial communities, not only in activated sludge but also in raw sewage (inflow wastewater). These microbial communities can utilize natural estrogens as a carbon source and eliminate them in laboratory culture media as well as in real wastewater and activated sludge microcosms. On the contrary, the synthetic estrogen EE2 was recalcitrant to microbial degradation under the applied conditions, explaining why attempts to enrich and isolate EE2-degrading cultures failed.

The enriched microbial consortia consist of many bacterial taxa, some of which are directly or indirectly involved in estrogen degradation. Other members of the community are not associated with estrogen degradation, albeit retained in the community as DMSO utilizers and/or to gain energy/growth factors from the estrogen degraders. Complete degradation or mineralization of E3 by the enriched bacterial consortia requires concerted activity of different community members. This is backed by the finding that isolated bacteria obtained from the consortia could only transform E3 to another estrogen, most probably 16 α -OH-E1, which is not further metabolized by the individual strains.

The inflow consortium can utilize the three natural estrogens either individually or as a mixture, without apparent substrate preferences. However, when the synthetic estrogens EE2 is added to the mixture, the consortium preferentially utilizes E2 and E3, while E1 accumulates, probably due to competitive metabolic inhibition by EE2. E3 metabolism by the inflow consortium proceeds via the 4,5-*seco* pathway of aerobic estrogen degradation. The pathway starts by transforming E3 to E1 via the transient formation of 16 α -OH-E1. These steps require oxidation of the C17 hydroxyl group,

which can be catalyzed by a putative 17 β -hydroxysteroid dehydrogenase, followed by removal of the C16 hydroxyl group, which probably proceeds via reductive dehydroxylation.

The inflow consortium undergoes temporal compositional shifts during E3 biodegradation, reflecting contribution of different bacterial taxa to the E3 utilization depending on substrate and products concentrations. The key E3 degraders belong to the genera *Croceicoccus*, *Achromobacter*, *Microbacterium*, and *Hyphomicrobium*. In addition, *Hydrogenophaga* and *Gordonia* spp. contribute to E3 biotransformation to 16 α -OH-E1 in the bacterial consortia enriched from activated sludge and thickened sludge. Although the estrogen degradation efficiency of the inflow consortium was conclusively revealed, it lacks functional robustness, which is one of the main drawbacks of microbial consortia when implemented for environmental applications. To retain the E3 biodegradation phenotype, the inflow consortium must be always handled in the presence of E3.

The adopted experimental design and methods or techniques enabled addressing the set research questions and objectives conclusively. However, some underlying limitations need to be rectified in future studies. For instance, the presence of the estrogen solvent DMSO challenged concluding that estrogens were utilized as the sole carbon source. In addition, similar to many solvents, it may exert some toxic effect on the isolated microbial communities. The adopted estrogen concentrations in the different experiments were several orders of magnitude higher than the reported environmental concentrations in domestic sewage. This could select microbial communities which are not the actual estrogen degraders in natural and engineered polluted environments. It can also lead to molecular and biochemical conclusions which may not be necessarily applicable to natural ecosystems.

One more limitation of this study is the isolation of pure bacteria from the E3-degrading consortia on the rich medium LB-agar plates. This approach does not guarantee isolation of estrogens-mineralizing bacteria due to the absence of selective

pressure in the medium, which explains why none of the isolates could grow on E3 as the sole carbon and energy source.

Future studies should focus on the development of functionally robust microbial consortia for efficient application in bioremediation of estrogen-polluted environments. However, this is an ultimate goal, which still needs prior sufficient understanding of the estrogen degradation mechanisms. For E3, biochemical and molecular studies are still needed to unravel the enzymes and genes involved in the transformation of E3 to E1. These efforts can be supported by comparative genomics and proteomics/transcriptomics investigations. Moreover, the stability of estrogen-degrading consortia is a key research area, which should be investigated thoroughly under various physicochemical conditions. Identification of the key estrogen degraders in microbial consortia requires carefully planned metagenomics coupled to metaproteomic investigations. In addition, further attempts to enrich and isolate EE2-degrading bacterial cultures may require optimization of the culturing conditions and exploration of more polluted environmental samples. The contribution of abiotic estrogen degradation (photodegradation and chemical degradation) to their bioavailability and biodegradability is worth investigating. Eventually, more studies are needed to analyze the pathways and microbial communities involved in E3 degradation in diverse geographic locations.

Recommendations

So far, most studies have focused on the estrogenic pollution of aquatic environments. In contrast, the occurrence, and effects of estrogens in solid matrices such as sediments, sludge and soil are rarely investigated. That could be justified by the deficiency of analytical methods and the sampling complexity. Future studies need to consider the bioaccumulation of estrogenic compounds quantitatively in Bahrain's aquatic organisms and assess their ecological risk and impact on human health.

Future studies should focus on wastewater treatment plants to eliminate estrogens more efficiently. Additionally, research on the occurrence and toxicity risk of degradation or biotransformation metabolites and by-products of estrogens should be

considered in future studies to yield more safe recycled water for plant irrigation projects. To achieve goal 14 of Sustainable Development Goals (SDGs) 2030, goal 2 (zero hunger), and goal 6 (sustainable management of water), along with sustainable use of marine resources, require marine water to be free of pollutant.

This study calls for setting SDG for environmental chemical pollution to help policymakers establish regulations to manage the use of chemicals at sustainable levels on regional and global scales. Effective measures are highly recommended for the government and management to monitor and control the discharge of estrogenic compounds as environmental pollutants and decrease their hazard to the ecological environments in the coming decades. Development and implementation of efficient and green technologies such as bioremediation can enable sustainable and economic elimination of hazardous pollutants like steroid estrogens from wastewater, which is a prerequisite for safe reuse and recycling.

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Appendix

Method

1. © Sample collection and preparation

From the DNA samples to the final data, each step, including sample test, PCR, SMRTbell library preparation, and sequencing is applied gradually to environmental microbial diversity and differences of microbial community. The workflow is as following:

1. Extraction of genome DNA

Total genomic DNA from samples was extracted using the column-based method (e.g. QIAamp PowerFecal DNA Kit, Qiagen). DNA concentration was determined by Qubit 4.0 Fluorometer (Thermo Scientific) and adjusted to 1 ng/ul for the following process.

2. PCR Amplification and Purification

The full-length 16S genes (V1-V9 regions) was amplified by barcoded 16S gene-specific primers. According to the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing Procedure (PacBio), each primer is designed to contain a 5' buffer sequence (GCATC) with a 5' phosphate modification, a 16-base barcode and the degenerate 16S gene-specific forward or reverse primer sequences. (Forward: 5'Phos/GCATC- 16-base barcode - AGRGTTYGATYMTGGCTCAG -3', Reverse: 5'Phos/GCATC- 16-base barcode - RGYTACCTTGTTACGACTT -3'). Degenerate base identities are: R = A, G; Y = C, T; M

= A, C. In brief, 2 ng of gDNA was used for the PCR reaction carried out with KAPA HiFi HotStart ReadyMix (Roche) under the PCR condition: 95°C for 3 minutes; 20~30 cycles (sample dependence) of: 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 60 seconds; 72°C for 5 minutes and hold at 4°C. The PCR products were monitored on 1% agarose gel. Samples with bright main strip around 1,500bp were chosen and purified by using the AMPure PB Beads for the following library preparation.

3. SMRTbell library construction and sequencing

The SMRTbell library was prepared according to the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing Procedure (PacBio). In brief, equal Molar of each barcoded PCR product was pooled and 500-1,000 ng of pooled amplicon sample was used for DNA damage repairing following by End-repair/A-tailing and ligation steps to introduce the universal hairpin adapters onto double-stranded DNA fragments. After purification with AMPure PB beads to remove the adapter dimer, the SMRTbell library was incubated with Sequel II primer 3.1 and sequel II Binding Kit 3.1 for the primer annealing and polymerase binding. At last, sequencing was performed in the circular consensus sequence (CCS) mode on a PacBio Sequel IIe instrument to generate the HiFi reads with Predicted Accuracy (Phred Scale) = 30.

2. © Data analysis

The circular consensus sequence (CCS) reads were determined with a minimum predicted accuracy of 0.9 and the minimum number of passes set to 3 in the official workflow of PacBio through the SMRT Link software. Only CCS reads with a quality score greater than Q30, referred to as Q30 HiFi reads, will proceed to the next stage. After demultiplexing, the HiFi reads were further processed with DADA2 (version 1.20) to obtain amplicons with single- nucleotide resolution [1, 2]. DADA2 workflow include quality filtering, dereplication, learning the dataset-specific error model, ASV inference and chimera removal. The trimming and filtering were performed with a maximum of two expected errors per read (maxEE = 2). DADA2 algorithm resolves exact amplicon sequence variants (ASVs) with single-nucleotide resolution from the full-length 16S rRNA gene with a near-zero error rate. For each representative sequence, the feature-classifier [3] and classify-consensus-vsearch [4] algorithm in QIIME2(v2022.11) [5] was employed to annotate taxonomy classification based on the information retrieved from the

NCBI 16S ribosomal RNA database. To analyze the sequence similarities among different ASVs, multiple sequence alignment was conducted by using the QIIME2 alignment MAFFT [6] against the NCBI 16S ribosomal RNA database [7-9]. A phylogenetic tree was constructed with a set of sequences representative of the ASVs using the QIIME2 phylogeny fasttree [10, 11].

Alpha diversity analysis was applied to analyze species diversity within a sample. Several alpha-diversity indices were used to measure alpha diversity for each sample, including observed-species, Menhinick's richness, Margalef's richness, Shannon, Simpson, Pielou's evenness, PD whole tree, and Good's coverage index [12]. All indices were calculated with QIIME. R package ggplot2 was used to generate the plots.

Beta diversity analysis was applied to analyze the difference in species complexity between samples. Dissimilarity matrices (Bray-Curtis, weighted and unweighted Unifrac) [13, 14] were generated using microeco package in R. We used Anosim [15], MRPP,

perMANOVA [16] and PERMDISP analysis to determine whether the community structures significantly differ among and within groups. To visualize patterns in multidimensional data, dimensionality reduction methods, such as PCA [17], PCoA [18], CPCoA [19, 20], NMDS [21], tSNE [22] and PLS-DA [23] were used. In addition, we used UPGMA to construct a tree that reflects the similarities between samples presented in UniFrac distance matrix. Anosim, MRPP, perMANOVA and PERMDISP analysis were performed using vegan package in R. PCA, PCoA, CPCoA and NMDS were conducted using R package microeco and vegan. PLS-DA was performed using the R package mixOmics. t-SNE was performed using the R package Rtsne. UPGMA was performed using the R package microeco. R package ggplot2 and plotly were used to generate the plots.

For statistical analysis, significance of all species among groups at various taxonomic level was detected using differential abundance analysis with a zero-inflated Gussian (ZIG) log-normal model as implemented in the “fitFeatureModel” function of the R Bioconductor metagenomeSeq package [24]. Moreover, welch’s t-test was performed using the stat package in R. Statistically significant biomarkers were identified by the use of the LefSe analysis [25]. In brief, LefSe is an approach based on an algorithm that performs the non-parametric Kruskal-Wallis test and Wilcoxon rank-sum test to identify bacterial taxa whose relative abundance is significant difference between the control and interest. LefSe applies LDA to those bacterial taxa identified as significantly difference and further assesses the effect size of each differentially abundant taxon. In this study, taxa with LDA score (log 10)

> 2 was considered significant.

For functional prediction analysis, 16S rRNA gene sequencing data were analyzed with several software, such as PICRUSt2 (v 2.5.1) [19], Tax4Fun2 [20] and FAPROTAX (v1.2.6) [21]. These tools predicted functional abundances with different reference databases. PICRUSt2 uses the MetaCyc database for pathway prediction. The Tax4Fun2 approach implemented in R package microeco, and select Ref99NR database for prediction. FAPROTAX is a manually constructed database based on the literature on cultured representatives. To find the important functional profiles across groups, R package ggplot2 and microeco were used to generate the plots and metastat statistics. In environmental factor analysis, to calculate the factors affecting community structure with some constrained ordination methods, such as RDA, CCA and db-RDA. method. The BioENV available for find the best subset of environmental variables with vegan package in R. Mantel test is a statistical test of the correlation between environmental factor and microbial community. To deliver more information, plot correlation heatmap based on

Mantel test results with vegan and linkET package in R. For advanced analysis, microPITA version (v 1.1), with the following sample selection criteria: for groups containing 10-14 samples, 50% of samples are selected; for groups containing 15-20 samples, 40% of samples are selected; for groups containing 21-30 samples, 30% of samples are selected; for groups containing 31-60 samples, 20% of samples are selected; and for groups containing 61 or more samples, 10% of samples are selected. Except for the features method, which selects the same number of samples within each group, all other methods use a proportional filtering based on the overall samples. SourceTracker version (v1.0) is also utilized in this analysis.

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Annex 2

Sequences of the 16S rRNA genes amplified from the E3-transforming isolates

Strain TH-11

Closest member(s): *Gordonia cholesterolivorans* and *Gordonia sihwensis*

Identification percentage: 98.53% for *G. cholesterolivorans* and 98.39% for *G. sihwensis*

Size of sequence: 1437 bp

Sequence:

```
>CATGCGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGCCAGCTTGCTGG
GTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGGACTCT
GGGATAAGCCTGGGAACTGGGTCTAATACCGGATAGGACCACTGATTGCATG
GTTGGTGGTGAGAAATGCTTTTGC GGTTTCAGGAGTGGCCCCGCGGCCTATCAG
CTTTGTTGGTGGGGTAATGGCCTAACAAGGCGACGACGGGTAGCCGACCTGAA
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTCGCTAGGGACGAAGCGT
AAGTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCG
TAGGCGGTTTGTGCGGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCA
GGCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCT
GGGTAGTAACTGACGCTGAGGAGCGAAAGCGTTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACCGTTGGGTACTAGGTGTGGGGCTCATTT
CACGAGTTCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACCG
CCGCAAGGCTAAAACCTCAAAGGAATTGACCGGGGGCCCGCACAAGCCGCCGA
GCATGTGGAATAATTTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATAC
AACAGAAAGCCGTAGAGATACCGGCCCCCCTTTGTGGTTGGTGTACAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTGTCGTGAGAATGTTGGGTAAAGTCCCGCAACG
AGCGCAACCCTTGTCTGTATTGCCAGCGGGTTATGCCGGGGACTTGCAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGTCCAGGGCTTCACAACATGCTAAAATGGCGCGTACAGAGGGCTGCGA
GACCGTGAGGTGGAGCGAATCCCTTAAAGCGCGTCTCAGTTCCGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGC
```

TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAG
TCGGTAACACCCGAAGCCGGTGGCCTAACCTTGTGGAGGGAGCTGTCGAAGG
TGGATCGGAAC

Strain TH-16

Closest member(s): *Gordonia cholesterolivorans* and *Gordonia sihwensis*

Identification percentage: 97.43% for *G. cholesterolivorans* and 97.29% for *G. sihwensis*

Size of sequence: 1446 bp

Sequence:

>CAGATGGCGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGCCAGCTTGC
TGGGTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGGAC
TCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACCACTGATTGC
ATGGTTGGTGGTGGAATAACTTTGCGGTTGCAGAATGGGCCCGCGACCTAT
CAGCTTGGGGGGTGGGGTAATGGCCTAACAAAGGCGACGACGGGTAGCCACCC
TGAGAGGGTGATCGGCCACACTTGGGACTGAAACACGGCCAAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACG
CCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCGCTAGGGACGAA
GCGTAAGTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAG
CCGCGTAATACGTAGGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGA
GCTCGTAGGCGGTTTGTGCGGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGC
GTGCAGGCGATACGGGCAGACTTGAGTACTACAGGGGGAGACTGGAATTCCTG
GTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG
GTCTCTGGGTAGTAAGTACGCTGAGGAGCGAAAGCGTTGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGGTTGGGTACTAGGTGTTGGG
GCTCATTTACAGAGTTCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGG
AGTACCGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGGGCCCGCACAAAGC
GGCCGAGCATGTGGAATAATTTCGATGCAACGCGAAGAACCTTACCTGGGTTTG
ACATACCACCAGAAAGCCGTAGAGATACCGGCCCCCCTTTGTGGTTGGTGTAC
AGGTGGTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTTGGGTAAATCCC
GCAACAAGCGCAACCCTTGTCTGTATTGCCAGAGGGTTATGCCGGGGACTTG
CAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAGGAAGTCAAGTCAT
CATGCCCCCTCTATGTCCAGGGCTTCACACATGCTACAATGGCGCGTACAGAGG
GCTGCGAAGACCATGAGGTGGAGCGAAACCCTTAAAGCGCGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATC
AGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGT
CATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCTTGTGGAGGGAGCT
GTCGAAGGTGGATCGGAGTC

Strain TH-30

Closest member(s): *Gordonia cholesterolivorans* and *Gordonia sihwensis*

Identification percentage: 98.33% for *G. cholesterolivorans* and 98.19% for *G. sihwensis*

Size of sequence: 1445 bp

Sequence:

```
CAATGGCGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGCCAGCTTGCTG
GGTACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGGACTC
TGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACCACTGATTGCAT
GGTTGGTGGTGGAAAGCTTTTGCGGTTCAGGATGGGCCCCGCGGCCTATCAGCT
TGTTGTGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGT
GAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCGCTAGGGACGAAGCGTAA
GTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGTGCAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTA
GGCGGTTTGTGCGCTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCAG
GCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGC
GGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTG
GGTAGTAAGTACGCTGAGGAGCGAAAGCGTTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACCGGTTGGGTACTAGGTGTGGGGCTCATTT
CACGAGTTCGGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACCG
CCGCAAGGCTAAAACCTCAAAGGAAATTGACCGGGGGCCCGCACAAAGCCGCCG
AGCATGTGGAATAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATA
CCACCAGAAAGCCGTAGAGATACCGGCCCCCTTTGTGGTTGGTGTACAGGTG
GTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAAAGTCCCGCAAC
GAGCGCAACCCTTGTCCTGTATTGCCAGCGGGTTTATGCCGGGGACTTGCAGG
AGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
CCCCTTATGTCCAGGGCTTCACACCATGCTAAAATGGCGCGGTACAGAGGGGG
CTTGCGAGACACGTGAGGTGTGAGCAGAAACCCTTAAAGCGCGTCTCAGTTCC
GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA
TCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC
GTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCTTGTGGAGGGAG
CTGTCTGAAGGTGGATCGAAC
```

Strain IF-20

Closest member(s): *Microbacterium schleiferi* DSMZ 20489^T (Y17237)

Identification percentage: 99.16%

Size of sequence: 1431 bp

Sequence:

```
>GATTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAGCAGAGCTTGCT
CTGTGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAATCTGCCCCTGACT
CTGGGATAAGCGCTGGAAACGGCGTCTAATACCGGATACGAGCTGCGAAGGC
ATCTTCAGCAGCTGGAAAGAATTTTCGGTCAGGGATGAGCTCGCGGCCTATCAG
CTTGTTGGTGAGGTAAACGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGA
GGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGC
GTGAGGGACGACGGCCTTCGGGTGTAAACCTCTTTTAGCAGGAAAGAAGCGA
AAGTGACGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCG
TAGGCGGTCTGTGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGC
AGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTA
GCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTC
TGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAG
ATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCC
ACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGC
CGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGC
ATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATAC
GAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTAAACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCCGCAACGAGCG
CAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGATGAAGTCAAATCATCATGCCCCTTA
TGTCTTGGGCTTCACGCATGCTACAATGGCGCGGGACAAAAGGGCTGCAATAC
CGCGAGGTGGAGCGAATCCCAAAAAAACGGCCCCACTTCGCATTGAGGTCTGC
AACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGC
GGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCG
GTAACACCTGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAAGGTGG
ATCGAAG
```

Strain AS-31

Closest member(s): *Hydrogenophaga intermedia* DSMZ 5680^T (AF019037)

Identification percentage: 98.48%

Size of sequence: 1448 bp

```
>CGATGGCGGCATGCTTTACACATGCAAGTCGAACGGTAACAGGCCGC
AAGGTGCTGACGAGTGGCGAACGGGTGAGTAATGCATCGGAACGTGCC
CAGTCGTGGGGGATAACGCAGCGAAAGCTGCGCTAATACCGCATACGA
TCTGTGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCGATTGGAGCGG
CCGATGTCAGATTAGGTAGTTGGTGGGGGTAAAGGCTCACCAAGCCCG
ACGATCTGTAGCTTGGTCTCGAGAGGACGACCAGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGAC
AATGGGGGCAACCCTGATCCAGCAATGCCGCGTGCAGGAAGAAGGCCT
TCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGGCTCTGGTTAATAC
CTGGGGCACATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGTGCGCAGGCGGTGCTGTAAGACAGGCGTGAAATCCC
CGGGCTTAACCTGGGAATGGCGCTTGTGACTGCAGTGCTGGAGTGTGGC
AGAGGGGGATGGAATTCCGCGTGTAAGCAGTGAAATGCGTAGATATGCG
GAGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTC
ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATAACCCTGGTAGTCC
ACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTTTTCTGACTCAGTAA
CGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGT
TGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGT
GGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTAC
GGAAGTTGCCAGAGATGGCTTCGTGCTCGAAAGAGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTAAAGTC
CCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTA
ATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT
CCTCATGGCCCTTATAGGTGGGGGCTCACACAAGTCATACAAATGGCCG
GGTACAAAGGGCAGCCACACCCGCGAAGGGGAGCCACATCCCATAAAG
CCGGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGA
ATCGCTAGTAATCGTGGATCAGCATGTCACGGTGAATACGTTCCCGGGT
CTTGACACACCGCCCGTCACACCATGGGAGCGGGTCTCGCCAGAAGTA
GGTAGCCTAACCGCAAGGAGGGCGCTTACCACGGCGGGTTCGATGG
```

الملخص

التحلل الميكروبي هو المسار الرئيسي لإزالة الإستروجينات من محطات معالجة مياه الصرف الصحي والبيئات الملوثة لتجنب خطورتها البيئية بسبب خصائصها المسببة لاضطراب الغدد الصماء والمسرطنة. ومع ذلك، فإن المعلومات المتعلقة بالتحلل البيولوجي للإستريول (E3) والإستروجين الصناعي إيثينيل إستراديول (EE2) والكائنات الحية الدقيقة المشاركة في هذه العمليات وأهميتها البيئية محدودة للغاية. في هذه الدراسة تم جمع عينات من مياه الصرف الخام (مياه الصرف المتدفقة)، والحمأة النشطة، والحمأة السميكة من محطة معالجة مياه الصرف الصحي في البحرين لعزل وتوصيف المزارع الميكروبية المسببة لتحلل EE2 و E3.

تم الحصول على ثلاث مجتمعات ميكروبية قادرة على تحليل E3 من خلال زراعة معززة باستخدام E3 كمصدر للكربون في وسط نمو محدد بواسطة هذه المجتمعات. على النقيض، لم تتمكن مزارع البكتيريا النقية المعزولة من هذه المجتمعات من النمو باستخدام E3 كمصدر وحيد للكربون، لكنها حولت E3 إلى 16 α -OH-E1 كمنتج رئيسي. كشفت التحاليل التصنيفية أن البكتيريا القادرة على تحويل E3 تنتهي إلى أجناس *Hydrogenophaga* و *Macrobacterium* و *Gordoni*. تمكنت مجموعة المجتمع الميكروبي المستخلص من مياه الصرف الخام من استخدام E1 و E2 بالإضافة إلى E3 سواء بشكل فردي أو كمزيج في وسط نمو محدود، وفي نظم بيئية مصغرة تحتوي على مياه الصرف الخام والحمأة النشطة، إلا أن EE2 لم يتم تحليله. بينما تم تحليل E3 بواسطة مجتمع التدفق الداخلي عبر مسار 4.5-سكو، حيث يتم تحويل E3 في البداية إلى E1 خلال تجربة تحليل E3 لمدة 14 يوماً، خضع هيكل المجتمع الميكروبي لتغيرات زمنية ملحوظة حتى اليوم السادس من التجربة، حيث سيطرت أجناس *Achromobacter* و *Hyphomicrobium* و *Microbacterium* و *Cupriavidus* و *Aminobacter* على المجتمع. ثم تحول المجتمع بشكل كبير نحو سيطرة *Croceicoccus estronivorus* بدءاً من اليوم الثامن وحتى اليوم الرابع عشر. أدى نمو المجتمع الميكروبي المتدفق (من مياه الصرف الخام) والمحتوي على ثنائي ميثيل السلفوكسيد في غياب الإستروجينات إلى القضاء على أعضاء المجتمع القادرين على تحليل E3، مما أفقد المجتمع القدرة على تحليل E3.

نستنتج من ذلك أن المجتمعات الميكروبية القادرة على تحليل الإستروجينات الطبيعية لا توجد فقط في الحمأة النشطة، ولكن أيضاً في مياه الصرف الخام. يتطلب التمعدن الكامل للإستريول E3 تضافر عمل عدة أعضاء من مجتمع التدفق الداخلي في أوقات مختلفة حسب تركيزات المواد المتفاعلة والمنتجات. ومع ذلك، فإن المرونة

الوظيفية للمجتمع تُعد عاملاً حاسماً يجب معالجته بعناية. يعتبر الزمن وتركيزات المواد المتفاعلة والمنتجات عوامل مهمة تحدد تكوين المجتمع الميكروبي.

الكلمات المفتاحية: الإستروجينات، الإستريول، التحلل الحيوي، الحمأة النشطة، مسار 4،5-سكو، المجتمعات الميكروبية.

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كلية العلوم



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أطروحة مقدمة كجزء من متطلبات الحصول على درجة الدكتوراه في برنامج البيئة والتنمية المستدامة

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