Evaluating Hydrophobic Bacteria as Potential Adsorbents for Estrogen Removal from Wastewater

Nur Nasimi binti Abdul Latif

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School of Engineering, Newcastle University

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Abstract

Estrogens are emerging contaminants classified as endocrine disrupting compounds (EDC). They interrupt ecological systems by altering the sex phenotype of the aquatic life, even at very low concentrations (part per trillion). Estrogens are hydrophobic compounds, which suggests that other hydrophobic materials and microorganisms could act as potential adsorbents. Consequently, several hydrophobic bacteria were evaluated as estrogen adsorbents as they are a sustainable resource, reproduce easily, and comprise high biodiversity.

The aim was to isolate an array of hydrophobic bacterial strains that could adsorb the estrogens to a level that had no health and ecological implication. The relationship between the bacterial hydrophobicity and their performance in removing estrogens was investigated. Commercial strains of *Rhodococcus erythropolis* were adapted with n-hexadecane and hexane growth substrates, and new bacterial species were isolated from the diesel-contaminated soil through a soil-enrichment process.

During the adaptation process using n-hexadecane substrate, *R. erythropolis* DSM311 showed a 6.70 % increase in cell surface hydrophobicity (CSH) compared to the parent strain. Six new strains were isolated, including the *Tsukamurella* sp. SD2-1, which showed the highest hydrophobicity at 91.33 %. Preliminary studies showed SD2-1 strain had the highest estrone removal efficiency (63.00 %) of the tested strains. A bacterial growth phase hydrophobicity study revealed that the stationary phase SD2-1 cells (collected at day 10) had higher CSH and produced higher estrone removal (95.30 %) compared to the exponential phase cells (collected at day 5). Longer incubation times (i.e. 15 and 20 days) gave no significant improvement to the CSH and estrone removal performance.

Overall, the adaptation and soil-enrichment process produced bacterial strains with higher hydrophobicity and estrone adsorption capacity than both *Escherichia coli* (control) and commercial strains (*R. erythropolis)*, removing estrone down to 1.78 – 9.31 $ng.L^{-1}$, very near to the predicted no-effect (PNEC) concentration of 3.6 $ng.L^{-1}$ for estrone reported by the European Commission.

Dedication

This thesis is dedicated to my jewel in heaven, Mohamad bin Mohamad Nadzri. This is for you, son.

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Nur Nasimi Abdul Latif

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Glossary of abbreviations

AC	Activated carbon
ANOVA	Analysis of Variance
BLAST	Basic Local Alignments Search Tool
BM	Biosurfactant-mediated uptake
BOD	Biochemical Oxygen Demand
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
CSH	Cell Surface Hydrophobicity
DCL	Direct contact of cell to large hydrocarbon drops
DCS	Direct contact of cells to submicron-sized hydrocarbon droplets
DH	Dissolved hydrocarbon
DNA	Deoxyribonucleic acid
E. Coli	*Escherichia coli*
E1	Estrone
E2	17β-Estradiol
E3	Estriol
ECD	Electrochemical detector
EDC	Endocrine Disrupting Compounds
EE2	17α-ethinylestradiol
EPS	Extracellular polymeric substance
EQS	Environmental quality standard
GAC	Granular activated carbon
HDA	Hydrocarbon surface -drops on MM1A agar
HLM	Hydrocarbon in liquid MM1
HMA	Hydrocarbon mixed in MM1A agar
HPLC	High-performance Liquid Chromatography

HRT	Hydraulic retention time
IB	Inhibited bacteria
LI	Live bacteria
MATH	Microbial attachment to hydrocarbon
MM1	Minimal media
NA	Nutrient agar
NB	Nutrient broth
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
PAC	Powdered activated carbon
PCR	Polymerase Chain Reaction
PM	Particulate matters
PNEC	Predicted no-effect concentration
PUM	Phosphate Urea Magnesium buffer
SPE	Solid Phase Extraction
TSS	Total Suspended Solids
UV	Ultraviolet
VSS	Volatile Suspended Solids
WWTP	Wastewater treatment plant

Chapter 1 Introduction

1.1 Background

Endocrine disrupting compounds (EDC) have been identified as an emerging contaminants due to their harmful effects on humans and animals. It is suspected that they are associated with the alteration of reproductive functions in males and females even at effective concentrations of parts per trillion. Estrogen is a hormonal substance which is suspected to generate the adverse effect on the endocrine system similar to an EDC. Unfortunately, inefficient wastewater treatment processes can lead to an accumulation of estrogen in effluents which potentially could pass into surface waters. This undesirable scenario has increased enthusiasm among researchers for improving techniques and exploring possibilities to enhance the efficiency of their removal and to ensure that safe water supplies reach consumers.

An appropriate method for pollutant removal is usually evaluated by assessing their key physicochemical characteristic (Hamid and Eskicioglu, 2012), which in the context of estrogens would be the water solubility index. Most estrogen compounds, namely the natural estrogens of estrone, estradiol, estriol and the synthetic estrogen ethinylestradiol, have been identified as being hydrophobic substances due to their low water solubility index (Koh *et al.*, 2008; Racz and Goel, 2010; Thomas and Potter, 2013). However, attribution of hydrophobicity creates challenges in ensuring the complete removal of estrogen via wastewater treatment. Equal or more hydrophobic materials are required to establish binding attractions with the estrogen molecules, and therefore the estrogen can be extracted from the water (Kwon *et al.*, 2006). Nevertheless, the efficient removal of estrogen is not normally achieved through conventional wastewater treatment (Bergman *et al.*, 2012) compared to a treatment plant which is additionally equipped with an advanced treatment system.

Poor estrogen removal is observed because conventional wastewater treatment was not originally designed for estrogen removal (Hamid and Eskicioglu, 2012; Cook *et al.*, 2016). Most treatment systems were designed according to wastewater treatment regulations that considered standard parameters such as concentrations of organic matter, nutrients and pathogens (Cook *et al.*, 2016) that are monitored at the level of parts per million. Conversely, estrogens are typically present in wastewater at concentrations a million-fold lower, at part per trillion. Therefore, they were not previously detected and were not considered as a pollutant of concern in the regulations.

1.2 Significance of the study

Adsorption and biodegradation are common advanced treatments that have been regularly adopted in tertiary treatment for the removal of estrogens from wastewater. An adsorption process is a promising option for estrogen removal due to its hydrophobic nature, a prominent characteristic that could be exploited in creating attraction if exposed to other hydrophobic materials. Activated carbon (AC) is a well-known commercial adsorbent effective for the physical adsorption of various polar and non-polar compounds, due to its microporous structure which provides a high surface area of binding sites. It has also been reported as a competent adsorbent for non-polar hormonal compounds, including estrogens (Rowsell *et al.*, 2009; Hartmann *et al.*, 2014; Hemidouche *et al.*, 2017). Nevertheless, alternative low-cost materials need to be discovered due to the high production costs and sustainability issues with commercial AC.

Microorganisms, and specifically bacteria are currently receiving a lot of attention in estrogen removal studies, since bacteria are a reproducible resource and can be obtained conveniently through a controlled cultivation process. However, the bacteria that may be useful for this adsorption process must have hydrophobic characteristics, so that they could interact with the hydrophobic estrogens, bind together and be extracted from water. In making use of the hydrophobic property, the aim of this study was to acquire hydrophobic bacterial species from the adaptation of commercial pure strains, and to isolate a new hydrophobic bacterial species from hydrophobic diesel-contaminated soils through an enrichment technique. Key elements involved in the adaptation and isolation process are the hydrocarbon growth substrates, which are supplied as the main carbon source for the bacteria to grow into hydrophobic cells. In addition, this research investigates the degree of estrogen removal contributed by both the adsorption and biodegradation processes of the bacteria.

The process of adapting bacteria using hydrocarbon substrates is very similar to the common approaches employed with hydrocarbon-adapting bacteria for the bioremediation process of hydrocarbon-contaminated environments (de Carvalho *et al.*, 2009). Meanwhile, the process of estrogen removal using an adsorption method is also commonly conducted in previous research. However, to the best of the present author's knowledge, no research has yet been carried out using a combination of these two processes. Furthermore, none of the hydrophobic bacterial cells obtained from hydrocarbon-adapted bacteria have previously been utilised as adsorbents in the hydrophobic estrogen removal process. The hydrophobic bacterial strains adapted in this study are expected to be compatible adsorbents in removing estrogen from the wastewater. Details of the aims and objectives of the study are explained in the next section.

1.3 Aim and Objectives

The aim of this study is to explore the potential of using hydrophobic bacteria as sustainable adsorbents for the removal of estrogenic compounds during wastewater treatment.

The research objectives were developed according to the aim of the study. The objectives are as follows:

1. To produce hydrophobic biomass of pure bacterial strains and soil-isolates by selective growth on minimal media supplemented with different hydrocarbon growth substrates.
2. To determine the cell surface hydrophobicity (CSH) characteristics during batch cultivation of the bacteria.
3. To investigate the correlation between the CSH values of the bacterial strains and estrogen adsorption performance in a treatment process, and optimize the estrogen removal performance using batch reactors.
4. To enhance the estrone removal performance using the fed-batch cultivation of bacterial biomass production, and to gain a better understanding of the effect of a longer incubation period on bacterial adaptation of cell surface hydrophobicity (CSH) and estrogen adsorption efficiency.
5. To compare the performance of the thermally pre-treated hydrophobic SD2-1 bacterial strains with commercial adsorbents, activated carbon and zeolite, for their capacity to remove E1 from a synthetic wastewater.

1.4 Hypotheses

The alternative hypotheses to be tested throughout the study are as follows:

1. Increase in the hydrocarbon uptake capability allows for the better growth of bacteria.
2. The pure strains of *R. erythropolis*, and soil-isolates, can adapt and grow in minimal media containing hydrocarbon substrates.
3. The amount of substrate used in the cultivation significantly affects the bacterial growth and biomass production.
4. The adapted and new isolated bacterial strains acquire higher cell surface hydrophobicity (CSH) compared to their parent strains after the adaptation process.
5. The cell surface hydrophobicity (CSH) of bacterial strains increases throughout the bacterial growth cycle, particularly from the exponential to the stationary phases.
6. The cell surface hydrophobicity (CSH) of the bacterial strains is correlated with their estrogen removal efficiency.
7. The degree of estrogen adsorption from wastewater is proportional to quantity of bacterial biomass used.
8. The performance of estrogen adsorption in batch reactors increases with the contact time applied.
9. The adsorption of estrogen onto bacterial biomass is affected by the stage of bacterial growth phase that the biomass was harvested.
10. The hydrophobic bacterial cell adsorbents are capable of removing estrone from wastewater below the relevant environmental quality standard values.
11. Fed-batch bacterial cultivation for periods exceeding 10 days has a significant effect on cell cell surface hydrophobicity (CSH) level, and the performance of the bacterial biomass for estrogen adsorption.
12. The removal of estrogen from wastewater using hydrophobic-adapted SD2-1 bacterial adsorbents is a viable alternative removal technology in comparison with activated carbon adsorption.
13. Temperature of thermal pre-treatment affects the E1 adsorbing performance of SD2-1 bacterial adsorbents.

1.5 Scope of the research

This research was designed to investigate and find answers to the aim and objectives planned in Section 1.3. The experimental work was divided into five stages which are: 1) a hydrophobic adaptation of bacteria; 2) the Characterisation of the isolated and adapted bacterial strains; 3) the Estrogen removal in batch reactor; 4) Fed-batch culture bacteria for estrone removal and 5) Comparison of the estrone removal efficiency of bacterial and commercial adsorbents

In Stage 1, the biological studies carried out involved the adaptation of a pure strain of the *Rhodococcus erythropolis* bacterium with different hydrocarbon substrates, and the isolation of hydrophobic bacterial species from diesel-contaminated soil (Section 3.2). Stage 2 involved the identification of isolated bacterial species, and the further molecular and physico-chemical characterisation of the adapted bacteria (Section 3.3). The bacteria isolated and adapted in Stage 1 were then used as candidate adsorbents and degrader microorganisms in the adaptation and biodegradation experiments in Stage 3. Throughout this stage, process of removal of estrogen was investigated extensively in order to determine which of the processes of biodegradation and adsorption was predominant, the optimisation of the treatment process, and the development of a quantitative analytical method for the measurement of estrogens utilising HPLC-ECD (electrochemical detection) detection instruments (Section 3.4).

The optimum conditions, as determined in Stage 3, were adopted in Stage 4, in which the bacteria were cultured in fed-batch reactors with feeding every 5 days for up to 20 days, and the bacterial biomass was then utilised to investigate the effect of this culture method on estrogen removal efficiency (Section 3.5). Finally, Stage 5 involves comparison of the efficiency of estrone removal by the bacterial strain and commercial adsorbents, desorption process and adsorption isotherm study (Section 3.6).

Chapter 2 Literature review

Wastewater can be a combination of used water from a variety of point-sources that typically include domestic, industrial and storm sewage produced by communities in residential, industrial or commercial areas. It may carry many types of pollutant considering it was produced from different sources and activities that without proper treatment can potentially have adverse effects on humans as well as the environment. Conventional wastewater treatment systems are established to create ideal conditions for the removal of pollutants and ultimately to ensure that safe final effluent can be discharged back to the environment.

The quality of treated wastewater from conventional wastewater treatment is monitored according to regulated standards for effluent, including parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), the nutrients phosphorous and nitrogen, total suspended solids (TSS), pathogen indicators, and pH. However, recent advances in chemical detection systems have revealed micropollutants in wastewater at extremely low concentrations of parts per trillion that were previously overlooked (Metcalf and Eddy, 2003). The micropollutants, including emerging contaminants, require more efficient removal techniques beyond the scope of conventional wastewater treatment (Barceló and Petrovic, 2008; Cook *et al.*, 2016). This has increased the significance of advanced treatment in the wastewater treatment process because these emerging contaminants, specifically endocrine disrupting compounds (EDC), can alter the genetic and physiological traits of the aquatic populations, even at very low concentrations, and affect their reproductive systems (Adeel et al., 2016).

2.1 Regulations

Estrogens are hormonal pollutants recognised as EDC due to their negative effect of interrupting the functions of endocrine glands, which are organs that synthesize hormones in the circulatory systems of humans and animals. They have been classified as micropollutants when present in the environment in soil, wastewater or water bodies. As mentioned in Section Chapter 1, estrogens have not been listed as a monitored parameter in wastewater treatment regulations due to their typically low concentrations. Nevertheless, given the harmful effect these compounds can cause, many environmental agencies have started to develop guidelines and scientific reports related to the adverse implications of EDC (including estrogen) and to define acceptable threshold levels.

2.1.1 Guidelines of EQS and PNEC level

In England and Wales, the monitoring of the emerging contaminants (ECs), including estrogens, has been managed by the European Commission, in which a watch lists of emerging

contaminants of concern were identified in the Water Framework Directive (European Commission, 2016). An Environmental Quality Standard (EQS) level of estrogens was derived from information on the predicted no-effect concentration (PNEC) made by the researchers and agencies. Usually, in monitoring levels of emerging contaminants, EQS is classified as both the long-term effect concentration through an Annual Average concentration (AA-EQS), and a short-term effect level, the Maximum Acceptable Concentration (MAC-EQS). The AA-EQS was derived from a monthly monitoring of the adverse effect concentration towards aquatic ecosystems including water, sediment and biotic. Presence of 17α-ethinylestradiol (EE2) in water is considered to be safe at a concentration below 0.1 $ng.L^{-1}$ by AA-EQS monitoring (European Commission, 2016) (Table 2.1). Moreover, 17β-estradiol (E2) which usually monitored together with estrone (E1) (as E2 can potentially be converted to E1) has been recommended safe when present at a level below 0.4 $ng.L^{-1}$ (Section 2.2.1). However, no EQS level has been established for E1, hence the PNEC of 3.6 $ng.L^{-1}$ was recommended to be used in place of a defined EQS (European Commission, 2016). Any concentration lower than these AA-EQS and PNEC values was considered safe and protected against the occurrences of chronic effect (Lepper, 2005). Furthermore, higher threshold level for MAC-EQS usually set because any exposure at this concentration may lead to acute toxicity effect to the aquatic life. However, the acute effects for E1, E2 or EE2 have been considered not to be significant, and therefore no MAC-EQS has been derived for these compounds (European Commission, 2011).

Table 2.1 The proposed monitoring levels of estrogens in water (European Commission, 2016).

Substance	Environmental quality standard (EQS) ($ng.L^{-1}$)		Additional info by EC
	Long-term exposure (AA-EQS)	Short-term exposure (MAC-EQS)	
Estrone (E1)	No established value	Not relevant	PNEC of 3.6 $ng.L^{-1}$ was recommended
17β-estradiol (E2)	0.4	Not relevant	-
17α-ethinylestradiol (EE2)	0.1	Not relevant	-

In addition, the World Health Organization (WHO) and United Nations Environment Program (UNEP) have also published EDC guidelines in 2012, in which E2 and E1 were classified as natural hormones that lead to the feminisation of fish populations (Bergman *et al.*, 2012), without addressing any limit of concentration for adverse effects.

2.2 Estrogens in wastewater

The removal of the main pollution load of wastewater, namely grease, suspended solids and some organic matter, can be accomplished through primary treatment, leaving the remaining suspended solids and volatile suspended solids and organic matter to be further removed in the secondary treatment of conventional wastewater (Metcalf *et al.*, 2003). Nevertheless, in many cases the effluent collected after the secondary treatment process will contain emerging contaminants (ECs) which could ultimately pass into the water body without additional tertiary treatment.

2.2.1 Detection of estrogens in conventional wastewater treatment plants

With regard to the inefficiency of estrogen removal during conventional treatment, estrogens have been detected as being present in the effluents from many wastewater treatment plants around the world. Table 2.2 lists some of the data on estrogen content of conventional wastewater treatment effluents. The Environment Agency of the United Kingdom detected the presence of estrogen compounds in the final effluents of sewage treatment plants around the UK, notably E1, E2 and EE2 (1 - 100, 1 – 22, and 1 - 3.2 $ng.L^{-1}$, respectively), and all were at higher concentrations than the EQS and PNEC (Gross-Sorokin *et al.*, 2004). In addition, an average of 20 $ng.L^{-1}$ of E1 was found in the final effluents from 25 treatment plants, well above its PNEC of 3.6 $ng.L^{-1}$. Four years later, lower amounts of estrogen were detected in effluents from activated sludge treatment plants in urban areas in the UK Midlands compared to the amounts found by Gross-Sorokin *et al.* (2004), with concentrations detected being 22.4, 1.3, and 1.5 $ng.L^{-1}$ for E1, E2 and EE2, respectively (Ifelebuegu, 2011). These are also above the recommended EQS and PNEC levels (Section 2.1.1).

E1 was also found to be present at the highest levels compared to other estrogens in the effluent of domestic treatment plant treating the wastewater produced by a community of approximately 30,000 people in Japan (Nakada *et al.*, 2006). Levels of 22.2 - 154 $ng.L^{-1}$ of E1 was found in this effluent, but no E2 and EE2 was detected, which was assumed to be due to successful removal during the treatment process. Moreover, Pessoa *et al.* (2014) measured the amounts of estrogens in wastewater treatment plants in Brazil, and found very high amounts of estrogens present in effluents of five treatment plants of mainly contained domestic sewage. E1 had the highest concentration among all estrogens (1 - 2080 $ng.L^{-1}$), compared to 1 – 387 for E2, and 1 - 176 $ng.L^{-1}$ for EE2. This extremely high level of E1 was suspected to occur due to low rainfall and high temperatures 25 – 29 °C during the sampling period that consequently reduced the dilution factor of the wastewater. These levels are considered to be very serious by the present

author, as the estrogens are present in effluents at levels of over hundred times higher than their EQS and PNECs, and consequently may carry serious risks to human health and aquatic life.

In a more recent study in South Africa, moderate amounts of 3 – 78, 4 – 107, and 1 - 8 $ng.L^{-1}$ for E1, E2 and EE2, respectively, were found in the final effluent of wastewater treatment plant treating domestic sewage from 300,000 people, including storm water (Manickum and John, 2014). The amounts present in this wastewater treatment plant however, were still above the PNEC for all of the estrogens. Similar concentrations of estrogens were found in a wastewater treatment plant in Switzerland, namely 71 - 154, 91, and 18 $ng.L^{-1}$ for E1, E2 and EE2, respectively (Margot *et al.*, 2013b).

Overall, the amount of estrogens present in effluents often exceeded the PNEC level, except for the treatment plants in Japan in which E2 and EE2 were not detected. These findings indicate that most conventional treatment systems, both conventional activated sludge (CAS) (Nakada *et al.*, 2006; Ifelebuegu, 2011; Margot *et al.*, 2013a; Manickum and John, 2014) and waste stabilization ponds (Pessoa *et al.*, 2014) were inefficient in removing estrogen to the recommended safe levels (limited to these case studies only).

In addition, based on these case studies, E1 had the highest concentration in all of the treatment plants, at average concentrations of 182.62 $ng.L^{-1}$ in influents and 88.51 $ng.L^{-1}$ in effluents (Table 2.2). For the purpose of conducting research into estrogen removal, a slightly higher level such as 200 $ng.L^{-1}$ could be a realistic initial concentration for these estrogens.

Table 2.2. Levels of estrogens concentration detected in effluent of conventional wastewater treatment plants.

WWTP location	Year of sampling conducted	Concentration of estrogen (ng.L^{-1})						Detection instrument	References [e]
		Influent			Effluent				
		E1	E2	EE2	E1	E2	EE2		
UK	March 2002	NR[b]	NR[b]	NR[b]	1.0 - 100.0 Mean: 20.0	1.0 - 22.0 Mean: NR	1.0 - 3.2 Mean: NR	SPE and GC-MS	1
Japan	January and July 2004	15.1 - 18.2 Mean: 16.6	3.9 - 23.4 Mean:13.6	ND[c] Mean: 0	22.2 - 154.0 Mean:88.1	ND[c] - 7.0 Mean: 3.5	ND[c] Mean: 0	SPE and HPLC LC/MS/MS	2
UK	2008	109.1 - 116.1 Mean: 112.5	74.4 - 82.6 Mean: 78.5	1.1 - 1.5 Mean: 1.3	14.5 - 22.4 Mean: 18.45	1.3 (± 0.6) Mean: 1.3	0.4 (± 0.5) Mean: 0.4	SPE and LCMS/MS	3
Brazil	April 2012	LOD[a] - 3050 Mean: 566	LOD[a] - 776 Mean: 143	LOD[a] - 3180 Mean: 421	LOD[a] - 2080 Mean: 242	LOD[a] - 397 Mean: 48	LOD[a] - 176 Mean: 124	SP and GC/MS	4
South Africa	March-June 2012	13 - 351 Mean: 84	20 - 199 Mean: 119	10 - 95 Mean: 30	3 - 78 Mean: 23	4 - 107 Mean: 20	1 - 8 Mean: 3	SPE and ELISA	5
Switzerland	June - October 2010	Mean: 134	Mean: 14	Mean: 5.3	Mean: 71	Mean: 1.3	Mean: <1.9	SPE and LC-MS/MS	6
Average[d]	2002 - 2012	**182.62**	**73.62**	**91.52**	**88.51**	**14.82**	**25.86**		

[a], 1 ng.L^{-1}; [b], no record; [c], not detected; [d], prorate data from references 2 to 6 only because data in reference 1 was not completed; 1, Environment Agency (2007); 2, Nakada *et al.* (2006); 3, Ifelebuegu (2011); 4, Pessoa *et al.* (2014); 5, Manickum and John (2014); 6, Margot *et al.* (2013b)

2.2.2 Fate of estrogens in conventional wastewater treatment systems

Conventional wastewater treatment systems have been identified as giving inefficient estrogen removal (Section 2.2.1). Therefore, further investigation on the performance of wastewater treatment unit processes has been conducted by several researchers to find the most efficient and inefficient estrogen removal points. Figure 2.1 schematically illustrates a model of conventional wastewater treatment units and additions of tertiary treatment, with their targeted pollutant removal. Functions of each unit operation were compared against the level of estrogens removal efficiencies (Table 2.3).

According to Bevan *et al.* (2012) and Grassi *et al.* (2013), estrogens cannot be efficiently removed from wastewater through the coagulation process employed in primary treatment because the treatment unit is designed for the purpose of removing colloidal and suspended solids only. Moreover, in a wastewater treatment plant (WWTP) in China, only 6.4 % of E1 and E2, and 39.6 % of EE2 were removed from wastewater following its primary treatment (Zhang *et al.*, 2011). The levels of estrogen removal were found to increase to 49.5, 69.2, and 100 % for E1, E2, and EE2, respectively, after the secondary treatment was completed, achieving final concentrations of 10.2 $ng.L^{-1}$ of E1, and 19.2 $ng.L^{-1}$ of E2 and undetected levels of EE2. Therefore, this conventional WWTP was considered to achieve complete removal of only for EE2, whereas E1 and E2 remained above their recommended PNEC and EQS levels.

A study of treatment unit performance in two WWTPs (WWTP A and WWTP B) in the UK also recorded very poor estrogen removal in the primary treatment unit, contributing to only 0 – 13.3 % removal (Ifelebuegu, 2011). In WWTP B, a conventional plant, the degree of estrogen removal was found to improve in a secondary treatment unit giving 79.5, 98.4, and 73.3 % removal of E1, E2, and EE2, respectively. However, the final concentrations of 22.4, 1.3, and 0.4 $ng.L^{-1}$ of E1, E2 and EE2, respectively, were still above the PNEC levels, and can be considered to still have potentially harmful effects on aquatic populations. However WWTP A with tertiary sand filters showed higher estrogen removal occurred in the secondary treatment unit followed by further reduction in the tertiary treatment. These findings proved that additional advanced treatment can increase the performance of estrogen removal, even though the final concentration in the effluents were still above the recommended levels of the EQS and PNEC. The work of Margot *et al.* (2013b) supported the findings of Ifelebuegu (2011), showing the removal of E1 and E2 could be further improved after advanced treatment using powdered activated carbon (PAC) was carried out. An additional 43 % of EE2 has been removed in advanced treatment (adsorption process) using the PAC.

Therefore, the tertiary treatment case studies conducted by Bevan *et al.* (2012), Ifelebuegu (2011) WWTP A, and Margot *et al.* (2013b) have shown tertiary treatment can give an additional degree of estrogen removal. These findings proved that primary treatment such as sedimentation and coagulation process (Figure 2.1) were designed only to fulfil its main purpose of removing solids and is not suitable for the removal of smaller molecules, notably micropollutants such as estrogens. In addition, adopting secondary treatment in conventional WWTPs can only fulfil the purpose of achieving water quality based on standard parameters in the current regulations that include TSS, VSS, BOD, COD, total N, NH_3, total P and pathogen indicators, but not the removal of micropollutants such as estrogens. An enhancement in estrogens removal has been detected whenever an advanced treatment unit operation was adopted after the secondary treatment process. This indicates the significance of supporting a conventional wastewater treatment with a tertiary treatment system, with regard to achieving more efficient estrogen removal. Even though the final concentration of the estrogens did not reach the required EQS and PNEC recommended by the European Commission, some of the concentrations were very close to the proposed safe level, therefore further improvements of water quality could potentially be achieved by using advanced treatment methods.

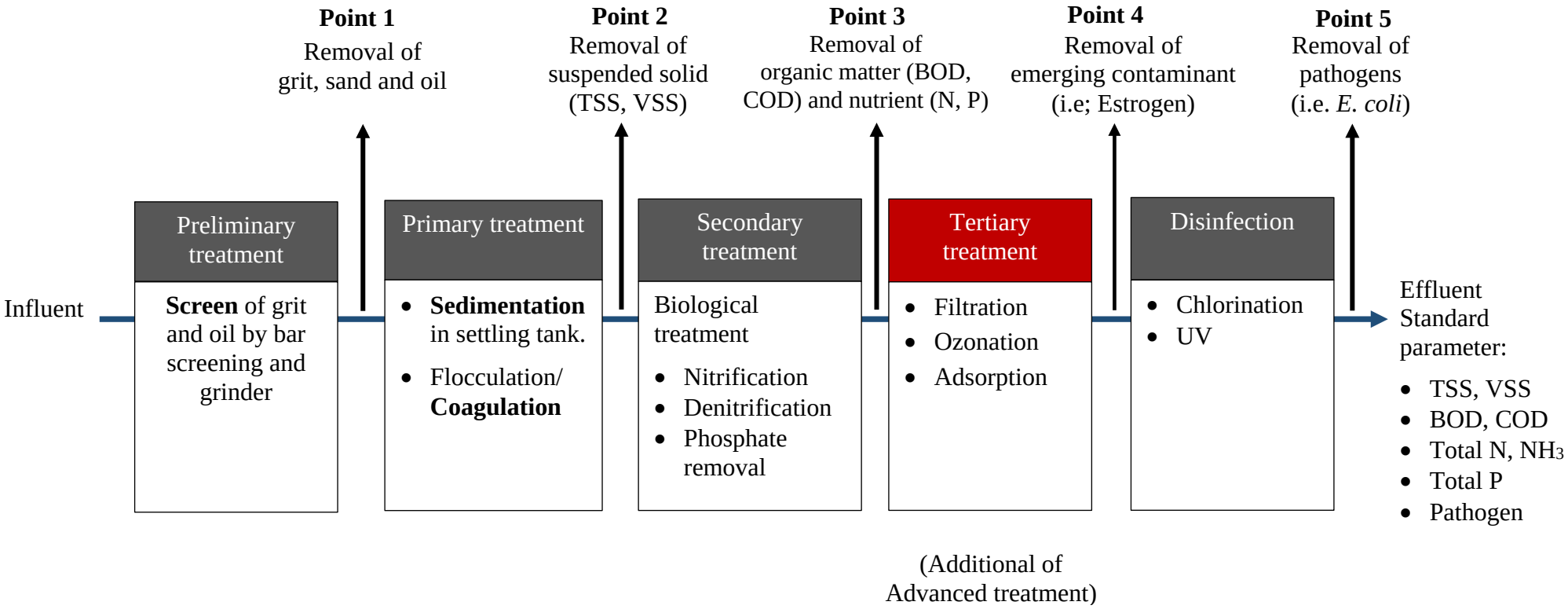


Figure 2.1. Removal of pollutants and standard parameters in conventional wastewater treatment systems and the addition of advanced treatment processes. Source: Barceló and Petrovic (2008) and Water Environment Federation (2009).

Table 2.3. Average concentrations of estrogens ($ng.L^{-1}$) and percentage of estrogen removal in different treatment units of WWTPs. Point 2 to point 4 mentioned are points after unit operation for the measurements of estrogen. [1], concentration ($ng.L^{-1}$).

References	Types of estrogens	Unit process of wastewater treatment plant						
		Influents [1]Conc.	Point 2: Primary treatment		Point 3: Secondary treatment		Point 4: Tertiary treatment		Advanced treatment process
			[1]Conc.	Removal	[1]Conc.	Removal	[1]Conc.	Removal	
Bevan *et al.* (2012)	Estrone (E1)	No record		0 %		0 %		50.0 %	Chlorination
	17β-estradiol (E2)			0 %		0 %		50.0 %	
	17α-ethinylestradiol (EE2)			0 %		0 %		50.0 %	
Zhang 2011	Estrone (E1)	20.2	18.9	6.4 %	10.2	49.5 %		-	-
	17β-estradiol (E2)	62.5	58.5	6.4 %	19.2	69.2 %			
	17α-ethinylestradiol (EE2)	6.3	3.8	39.6 %	ND	ND			
Ifelebuegu (2011) WWT B	Estrone (E1)	109.1	112.3	-2.9 %	22.4	79.5 %		-	-
	17β-estradiol (E2)	82.6	72.6	12.1 %	1.3	98.4 %			
	17α-ethinylestradiol (EE2)	1.5	1.3	13.3 %	0.4	73.3 %			
Ifelebuegu (2011) WWT A	Estrone (E1)	119.3	121.0	-1.0 %	54.3	54.5 %	20.3	83.0 %	Sand filters
	17β-estradiol (E2)	44.2	42.8	3.0 %	1.1	97.5 %	<1.0	97.7 %	
	17α-ethinylestradiol (EE2)	0.9	0.9	0 %	0.5	44.4 %	0.4	55.6 %	
Margot *et al.* (2013b)	Estrone (E1)	134.0	No record		71	58.0 %		92.0 %	Adsorption by powdered activated carbon
	17β-estradiol (E2)	14.0	No record		1.9	91.0 %		>83.0 %	
	17α-ethinylestradiol (EE2)	5.3	No record		1.3	18.0 %		61.0 %	

2.2.3 Fate of estrogens

The release of estrogens into the environment has been associated with various sources; namely domestic sewage, industrial sewage, animal production, agriculture and landfill leachate. Estrogens can be either be natural hormones produced by humans and animals such as estrone (E1), 17β-estradiol (E2) and estriol (E3) or synthetic hormones 17α-ethinylestradiol (EE2). Figure 2^{-1} illustrates the entry points of estrogens into water bodies.

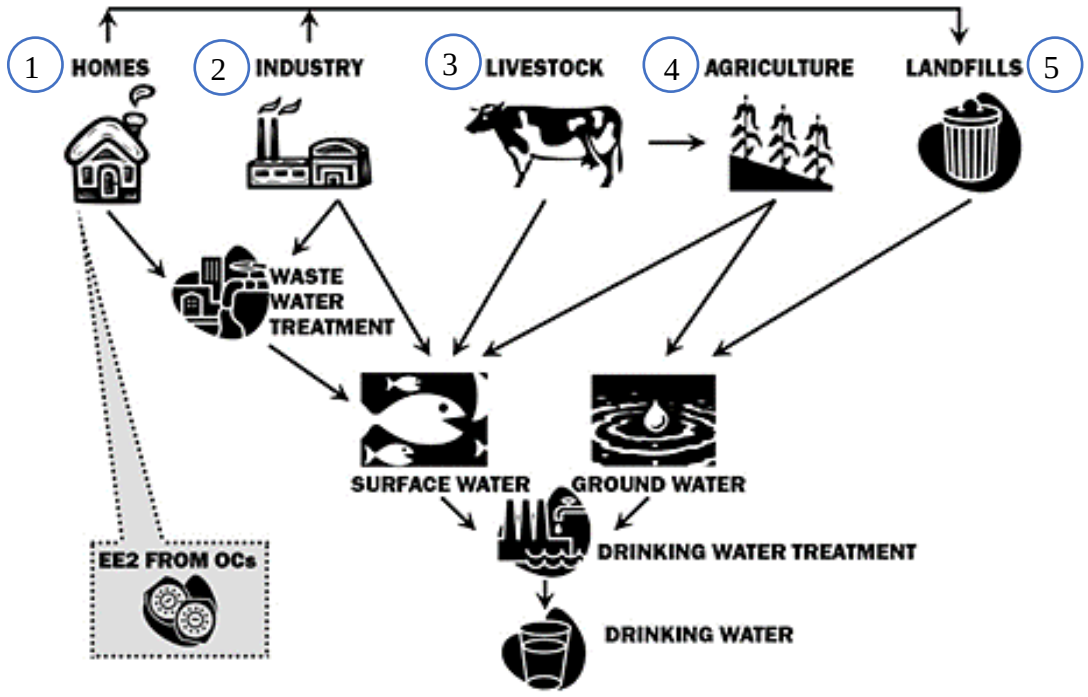


Figure 2.2 Points of entry of endocrine-disrupting compounds into the water supply (Moore *et al.*, 2011).

Natural hormones are naturally produced in the female body for the maintenance of health and tissue reproduction (Silva et al., 2012). These hormones are passed into domestic sewage through urine and faeces, as illustrated as Point 1 in Figure 2.2. Nevertheless, estrogens that are produced by either humans or animals eventually take the form of conjugated estrogens, which are physiologically inactive forms of the compound and relatively soluble in the water. The estrogens excreated through faeces and urine are either conjugated as glucuronide estrogens (GLU) or sulfate estrogens (SLU). These weakly active forms however can be associated with environmental problems because they can be readily deconjugated by the *Escherichia coli* (*E.coli*) in the faeces itself and eventually derived into free estrogens notably E1, E2 and E3 (Hamid and Eskicioglu, 2012). Further details on the mechanism of the adverse effects of hormones are discussed in Section 2.3.

In addition, EE2 is a synthetic hormone the excretion of which is normally associated with the consumption of contraceptive pills, and it is also released at Point 1. Furthermore, it can also be released into water bodies due to poor wastewater treatment by the pharmaceutical industry (Point 2) (Barceló and Petrovic, 2008). Many studies relating to the natural production of estrogens by women have been conducted as this is believed to be the main contributor to the release of estrogens in sewage. Estrogens have also been found to be produced by males, however the amounts excreted are considered very small compared to the amounts excreated by women, especially pregnant women who produce E1 at 550 - 787 $\mu g.day^{-1}$ per capita, and E2 at 277 - 393 $\mu g.day^{-1}$ (Fleming, 2015; Adeel *et al.*, 2016). Nevertheless, the accumulation of estrogens in wastewater are also believed to include contributions from the livestock industry that exceed the amounts produced by women (Moore *et al.*, 2011; Adeel *et al.*, 2016). The entry of such estrogens (Point 3) into wastewater has been detected at extremely high levels of approximately 83,000 kg/year of estrogen, which is claimed to be produced by livestock farming around the world compared to 30,000 kg/year of estrogen produced by the entire human population of 7 billion, with a further 700 kg/year of EE2 coming from oral contraceptives (Adeel *et al.*, 2016). Agriculture (Point 4) has also been recorded as producing large amounts of estrogen waste due to the practice of using livestock manure as a fertiliser in organic farming. Finally, estrogens are also released into the environment from the disposal of sewage sludge to landfill and its land application (Hamid and Eskicioglu, 2012).

There are a variety of sources of estrogen entering the environment which will pass to water bodies if not treated adequately, with livestock and domestic sewage providing the highest contribution of estrogens to the environment. The background characteristics of wastewater containing estrogens will also vary widely; vegetable oils, protein and urea are present in domestic wastewater, high levels of specific chemicals in wastewater from industry, high biological contents in livestock wastewater, high chemical and biological contents from other agricultural sources and landfill waste. Consequently, since there is no single uniform background wastewater composition, a synthetic wastewater was chosen as a model in the present study of estrogen removal (Section 6.3.1).

2.3 Adverse effects of estrogens

2.3.1 Estrogens as endocrine disrupting compounds

Estrogens are endocrine disrupting compounds that have been reported to interrupt functions of hormones in living organisms by having mimicry (agonistic) or blocking (antagonistic) effects (Birkett and Lester, 2002), the disruption of secretion and transport of hormones, or the disruption of hormone receptor functions (Acerini and Hughes, 2006; Goksøyr, 2006). The

endocrine system consists of a set of glands that produce hormones essential for body functions such as growth, reproduction, cell maintenance, metabolism, etc. Cells contain receptor binding sites as illustrated in Figure 2.3.

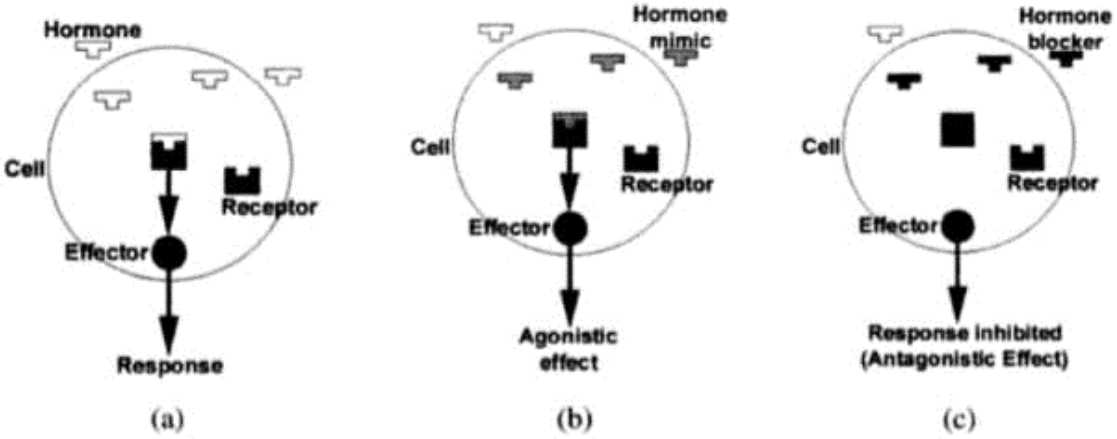


Figure 2.3 The targeted cell with its binding site: (a) natural response (b) agonistic effect (c) antagonistic effect by endocrine disrupting compounds (Birkett and Lester, 2002)

A certain low concentration of natural hormones is required to eventually become attached to the receptor in order to produce the desired effect in the body. The receptor has a high affinity for a specific hormone, and only a small quantity of the hormone can produce a response in the cell when it has bound to the receptor (Birkett and Lester, 2002). However, the presence of an EDC as a hormone mimic, may ultimately bind itself to the receptor and produce an effect that can be harmful to the body, such as binding of estrogen to receptors in male organisms. This mechanism is recognised to be an agonistic effect (Figure 2.3) that eventually produces feminisation in male fish. This effect could take place at very small concentrations of parts per trillion due to the high affinity of the cell receptor towards hormonal substances (Mortensen and Arukwe, 2007)

This agonistic effect can take place with levels of estrogens present in water bodies or drinking water and alter the sexuality of aquatic populations and cause long-term adverse effects in humans. Studies of fish populations affected by exposure to estrogens have proven the existence of the feminisation effect. Kirby *et al.* (2004) found a feminising effect which was observed to reduce male fish sperm quality and inhibit testicular growth. Reductions in sperm quality, as well as quantity in male fish correlated with the presence of estrogen compounds in the liver. The estrogen had stimulated liver tissue to produce vitellogenin, an egg yolk protein, which was later transported into the testes tissue and developing eggs which interrupted testicular

functions. This feminisation could take place whenever fish were exposed to estrogens E1, E2 and EE2 in the long-term, particularly when exceeding their specific PNECs.

2.3.2 Case studies related to the feminisation effect

Concentrations of 31.8, 5.0 and 10 ng.L^{-1} for E1, E2 and EE2, respectively, have been reported to cause vitellogenin induction in several species of fish, whereas concentrations of 318.0, 320.0 and 10.0 ng.L^{-1} can inhibit testicular growth in male fish (Gross-Sorokin *et al.*, 2004). However, these values that found to affect the sexual trait of fish are higher than the recommended EQS values of 0.4 and 0.1 ng.L^{-1} for E2 and EE2 respectively, and PNEC of 3.6 ng.L^{-1} for E1 (Section 2.1.1). This difference exists to ensure a good safety margin is achieved between the known estrogenic effect concentration and the EQS threshold concentrations.

Estrogens have also been reported to affect the populations of wildlife species other than fish, with mussels (*Elliptio complanata)* being found to experience a feminisation effects as they grew in water suspected of containing estrogens from two municipal effluents, and the feminisation bio-marker vitellogenin also being recorded in male mussels (Goksøyr, 2006; Gagné *et al.*, 2011). However, no measurements of estrogen concentration were made in this study, but the production of vitellogenin was considered by the researchers to be connected with the presence of estradiol. Feminisation was found to occur at rates 1.7 - 2.3-fold higher downstream of the discharge compared to upstream, confirming the effluent affected the mussels.

Evidence of feminisation in mussels and fish populations has strongly supported the theory that concentrations of low parts per trillion (ng.L^{-1}) of hormone mimic substances, but at levels higher than the EQS and PNEC, were replacing natural hormones and bonded to receptor cells producing an adverse response in the production of vitellogenin in the male fish or mussel cells. Naturally, Vitellogenin would only be present in females for the purpose of producing eggs. In addition, the synthetic estrogen 17α-ethinylestradiol (EE2) has been shown to be 11 - 27 times more harmful than the natural estrogen E2, which is itself is 2.3 to 3.2 times more potent than E1 for aquatic populations (Thorpe *et al.*, 2003). But, even though E1 has been reported to be the least potent estrogen with regard to feminisation, the relatively high concentrations of E1 found in typical wastewaters (Section 2.2.1) are typically double the amounts of E2 and EE2, making it equally harmful in the environment, and requiring its reduction or removed.

2.4 Characteristics of Estrogens

Effective treatment of wastewater effluents is clearly needed to prevent feminisation effects in ecosystems, and successful estrogen removal can be achieved by obtaining adequate information on its characteristics allowing for effective treatment design.

2.4.1 Hydrophobicity of estrogen compounds

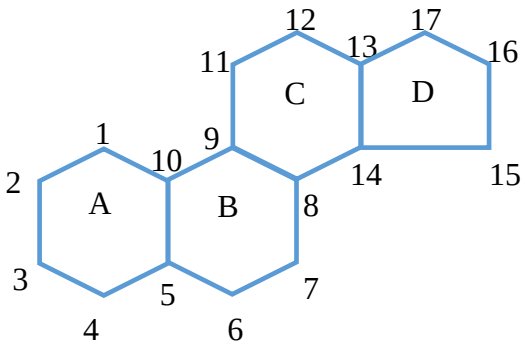
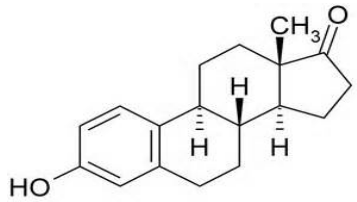
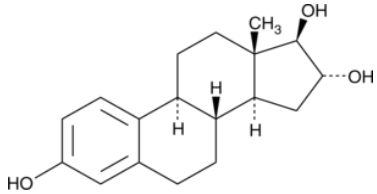
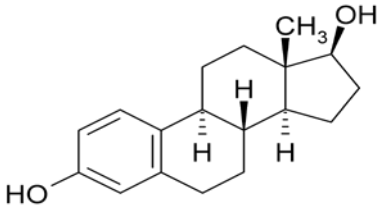
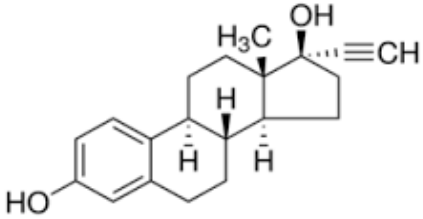


Figure 2.4 Cyclopentanoperhydrophenanthrene: the basic structure of steroidal compounds.

Estrogen is a steroidal hormone comprising the basic cyclopentanoperhydrophenanthrene molecular structure (Zarrow, 2012). The aromatic structure has four fused carbon rings as illustrated in Figure 2.4. Addition of a hydrophobic side chain functional group to cyclopentanoperhydrophenanthrene actually contributes to increase hydrophobicity in estrogen compounds (Koga *et al.*, 2011), and the degree of estrogenicity has been reported to rely on the size and degree of branching of the alkyl group, and its positioning on the phenol ring (Routledge and Sumpter, 1997; Racz and Goel, 2010). The E1, E2 and EE2 estrogens have been found to be hydrophobic due to the presence of several methyl attached to their phenolic structure, as can be observed in Figure 2.5. In addition, their water solubility indices were detected to be very low in the range of 0.8 - 3.3 $mg.L^{-1}$ at 25 °C (Table 2.4). Their hydrophobic character is a key attribute to be taken into account in developing any estrogen removal strategy, so that targeted compatible materials or techniques can be employed to extract them from wastewater or other environmental samples.

Based on the hydrophobicity index listed in Table 2.4, estrone (E1), 17β-estradiol (E2) and estriol (E3) have similar water solubility (13.0 $mg.L^{-1}$). In terms of octanol-water partition (Log K_{ow}), E2 has the highest value followed by E1 for typical natural estrogens detected in the wastewater. Therefore, both of E2 and E1 were evaluated to find which one is more suitable to be employed as estrogen model in the current study, since the proposed removal process was based on a hydrophobic mechanism.

Table 2.4 List of selected estrogen compounds and their main characteristics.

Compounds	Empirical Formula	Source/ application	Molecular Weight ($g.mol^{-1}$)	Water solubility ($mg.L^{-1}$)	Log K_{ow}	Structure
Estriol (E3)	$C_{18}H_{24}O_3$	Natural hormone	288.4	13.0[b]	2.81[a]	
Estrone (E1)	$C_{18}H_{22}O_2$	Natural hormone	270.4	13.0[a]	3.43 [a]	
17β-estradiol (E2)	$C_{18}H_{24}O_2$	Natural hormone	272.4	13.0[a]	3.94 [a]	
17α-ethinylestradiol (EE2)	$C_{20}H_{24}O_2$	Synthetic hormone / Contraceptive pills	296.4	4.8[a]	3.67 [a]	

a, Adeel *et al.* (2016); b, Silva *et al.* (2012)

2.5 Advanced wastewater treatment processes

There are many options for advanced treatment that could potentially be employed to ensure estrogen removal from wastewater. These include adsorption, biodegradation, membrane treatment, ozonation, advanced oxidation and chlorination (Hemidouche *et al.*, 2017) (Table 2.5.).

2.5.1 Adsorption

Commercial activated carbon, such as powdered activated carbon (PAC), has been reported as an excellent adsorbent material for many substances, including polar and non-polar compounds such as estrogens (Snyder *et al.*, 2007; Hartmann *et al.*, 2014). Complete removal of 100 $ng.L^{-1}$ estrone (E1) was achieved using PAC, and 97 % of E2 was removed. However the final E2 concentration was above the PNEC. The degree of estrogen removal attributed to the PAC was considered to be high, and the PNEC levels were almost achieved for all of the estrogens. Thus the PAC adsorption process can be considered to be an efficient technique for the removal of estrogens. However, there are some drawbacks to the commercial use of activated carbon (AC) such as high energy use in its production, disposal and regeneration issues, and selectivity concerns given that the adsorption of hydrophobic compounds could be limited when both polar and non-polar substances are present in the same environmental sample (Snyder *et al.*, 2007; Hartmann *et al.*, 2014).

Using material with the same hydrophobic characteristics as AC, neutral hydrophobic resin was considered as a good alternative adsorbent to PAC, with a comparable adsorption capacity for E2 of 1359 $\mu g.g^{-1}$ in distilled water. Lower capacity occurred in wastewater samples due to competition for adsorption sites by background organic substances in the wastewater (Hartmann *et al.*, 2014). In addition, granular activated carbon (GAC) was also shown to be a good adsorbent for estrogens (Ifelebuegu *et al.*, 2015). To overcome the high production cost for GAC, an adsorbent produced from black tea leaves was successfully found to have a good adsorption capacity for E2 (3.46 $mg.g^{-1}$) with 96 % E2 removal demonstrated. Use of hydrophobic adsorbents (GAC, PAC, neutral resin and black tea leaves) were considered to be excellent options for advanced treatment of estrogens through the adsorption mechanism.

Major drawback of high production cost of AC, and interference of polar compound in AC adsorption can be overcome by using alternative cheaper material with high selectivity to hydrophobic compound. Therefore removal of estrogens by adsorption is a process worthy of further investigation.

2.5.2 Membrane separation

Use of membrane separation of microfiltration or ultrafiltration was previously recommended for the removal of contaminants in treatment plants with limited space. However, this technology was found to have high selectivity for particles larger than estrogens, which limits its estrogen removal efficiency (Snyder *et al.*, 2007). Interestingly, the use of a membrane bioreactor (MBR), a combination of membrane filter with an activated sludge reactor, enhanced its performance relative to conventional activated sludge treatment, giving complete removal of EE2 and E3, and almost 97 % for the E1 (Trinh *et al.*, 2012).

2.5.3 Biodegradation

Biodegradation by bacterial co-culture (LM1 and LY1 strains) was found efficient for 98 % of E2 removal (Li *et al.*, 2018). In addition, activated sludge (AS) that usually contains mixure of diverse bacterial biomass, including estrogen-degrading bacteria that can completely remove E2 and E1 through a biodegradation process (Yoshimoto *et al.*, 2004). Moreover, biodegradation usually coexists with adsorption in AS, immediate removal of estrogen being found to be largely attributed to adsorption with longer treatment processes allowing for enhanced removal by biodegradation. Longer treatment allows the bacteria community to keep growing while degrading the estrogen as their growth substrate (Racz and Goel, 2010).

2.5.4 Ozonation

Removal of estrogens by ozonation was found to completely remove EE2, however, the estrogenic activity was still present after the treatment due to the production of a by-product following the treatment process (Larcher *et al.*, 2012). Unfortunately, the feasibility of adopting ozonation is limited by economic factors, as use of pure ozone (O_3) involves high cost operations. Despite that, the use of lower amounts of O_3 in combination with UV, can produce lower but effective degrees of estrogen removal (Pešoutová *et al.*, 2014). However, estrogen adsorption by the PAC was found to be a more efficient process for E1 removal than ozonation (Margot *et al.*, 2013a).

2.5.5 Advanced oxidation

Advanced treatment for estrogen removal by the advanced oxidation process of UV/H_2O_2 has been proven to completely remove E1, and give 90 % removal of EE2 from wastewater. However, its major shortcoming was its high energy and maintenance cost process (Hansen and Andersen, 2012).

2.5.6 Chlorination

Chlorination is claimed to remove estrogen completely from wastewater. However, the presence of by-products (recalcitrant chlorinated compounds) generated during the treatment process has been found to exert estrogenic effects, and thus it is deemed inappropriate for treating estrogen compounds (Pauwels and Verstraete, 2006).

2.5.7 Evaluation of the potential advanced treatment for estrogen removal

Based on the evaluation of each advanced treatment process listed above, it is concluded that most of the processes can remove the estrogens efficiently. However, the current study aimed to develop a treatment that did not have any associated production of harmful by-products, and one that could utilise lower cost sustainable materials and processes. Consequently, Chlorination and ozonation were excluded as they produced estrogenic by-products.

Moreover, treatment processes that classified as having high efficiency in removing estrogen but require either high cost for energy supply or maintenance were also avoided, which eliminated advanced oxidation. Furthermore, use of commercial activated carbon in an adsorption process was also deemed to have a high cost process despite being a potential advanced treatment that can target emerging hydrophobic contaminants like estrogens (Table 2.5). Biodegradation was the basis for several estrogen removal studies (Racz and Goel, 2010; Hamid and Eskicioglu, 2012; Brasil Bernardelli *et al.*, 2015) and is sustainable due to its use of natural bacteria, consequently this process was one of the processes investigated in the current research. To overcome the main drawback of high cost for the adsorption process, the identification of cheaper adsorbent materials was seen as a potential solution worthy of further investigation. Therefore advanced treatment of adsorption as well as biodegradation were selected as the estrogen removal processes in this study.

Table 2.5 Advantages and drawback of advanced treatment

Treatment process	Advantages/disadvantages of process	Estrogen removal achieved	Reference
Adsorption	Advantages: • Activated Carbon (AC) is an excellent commercial adsorbent for the polar and non-polar compounds. Disadvantages; • Thermal regeneration of AC requires a significant amount of energy and cost. Main drawback. • AC also attract hydrophilic contaminants that attach faster than hydrophobic compounds and occupied the binding site.	• Initial concentration of 100 $ng.L^{-1}$. • 5 $mg.L^{-1}$ PAC has lower removal percentage compare to 35 $mg.L^{-1}$. • E1 100 % removal (reduced to undetected level) • E2 97 % removal. 3 $ng.L^{-1}$ • EE2 96 % removal. 4 $ng.L^{-1}$	(Snyder *et al.*, 2007)
	Advantages reported: • Use of hydrophobic resin and AC. • Hydrophobic interaction as adsorption mechanism. Disadvantages reported: • Factor of competitive adsorption of other substances in real wastewater may reduce the adsorption capacity.	• Capacity of adsorption for E2: Neutral resin 1359 $\mu g.g^{-1}$; PAC 1322 $\mu g.g^{-1}$. • Complete removal of E2 by resin and 98 % removal by PAC.	Hartmann *et al.* (2014)
	Advantages reported: • Granular activated carbon (GAC) is an excellent commercial adsorbent for the removal of EDCs. • Drawback of high production cost of GAC. • Alternative cheaper material of black tea leaves (TL_H) was found to be an excellent adsorbent.	• TL_H has an almost comparable adsorption capacity (K_f) to GAC. • K_f value for the adsorption of E2 are 3.46 $mg.g^{-1}$ for TL_H and 4.01 $mg.g^{-1}$ for GAC. • GAC produced 96.98 % E2 removal and TL_H produced 95.75 %.	Ifelebuegu *et al.* (2015)

Treatment process	Advantages/disadvantages of process	Estrogen removal achieved	Reference
Membrane Separation	Advantages reported: • MBR: A combination of conventional activated sludge with Microfiltration / ultrafiltration (MF / UF). • High performance of removal for most trace organic contaminant	• Membrane bioreactor has 97-100 % removal for estrogen (E1, E2 and E3). • Final concentration of 1.5 ng.L^{-1} for E1 and complete removal for EE2 and E3.	(Trinh *et al.*, 2012)
	Disadvantages reported: • Microfiltration / ultrafiltration (MF / UF) are selective for big molecules and inefficient for estrogen removal unless membrane bioreactor (MBR) was adopted.		(Snyder *et al.*, 2007)
Biodegradation	Advantages reported: • Biodegradation by co-culture is efficient for the estradiol removal.	• 98 % of E2 removal by the co-culture	(Li *et al.*, 2018)
	Advantages reported: • Biodegradation by activated sludge is an efficient estrogen removal mechanism. • Adsorption co-exist with biodegradation in activated sludge. Other information: • Adsorption is predominant process compare to biodegradation for a high K_{ow} compound.	• Complete removal of E2 and E1	(Yoshimoto *et al.*, 2004; Racz and Goel, 2010)

Treatment process	Advantages/disadvantages of process	Estrogen removal achieved	Reference
Ozonation	• Comparison of performance between AC and ozonation.	• 92 % of E1 removal via adsorption by PAC compare to 90 % ozonation	(Margot *et al.*, 2013b)
	Advantages reported: • The O_3 treatment for estrogens of E2 and EE2 produced a less estrogenic by product. Disadvantages reported: • Feasibility of process depends on the economic factor as ozone treatment is a high cost process.	• Complete removal of EE2 with formation of by-product.	(Larcher *et al.*, 2012)
Advanced oxidation	Advantages reported: • Use of UV/H_2O_2 process • High estrogen removal. Disadvantages reported: • High energy and maintenance cost process.	• 90 % removal of EE2 • Complete removal of E1	(Hansen and Andersen, 2012)
	Advantages reported: • Effective process for estrogen removal • Removal by the O_3 is higher than the O_3/UV.	• 99 % removal of E1, E2, E3 and EE2 at 4.4 $mg.L^{-1}$ of O_3	(Pešoutová *et al.*, 2014)
Chlorination	Disadvantages: • Removed all E2, but treatment produced by-product of recalcitrant chlorinated that persists in the environment. i.e. mono- and dichlorinated E2; 4-chloro-E2 (estrogenic compound)	• Complete removal but produced other estrogenic by product	(Pauwels and Verstraete, 2006)

2.6 Potential of biodegradation and adsorption for estrogen removal

In an advanced treatment such as activated sludge, the biodegradation and sorption processes are responsible for most of the estrogen removal. Nevertheless, the degree of estrogen removal by these mechanisms is uncertain (Ren *et al.*, 2007a). Several researchers have reported that sorption typically accounts for less than 10.0 % of estrogen removal and other researchers have concluded that more than 30.0 % of estrogen removal (Table 2.6) can occur due to sorption (Racz and Goel, 2010). In addition, estrogen removal was reported to be largely attributed by the adsorption process compare to the biodegradation due to the high octanol-water coefficients of estrogen and inhibited activated sludge successfully removed 98.0 % of estrogen through the adsorption process (Ren *et al.*, 2007b). In addition, a study conducted by Brasil Bernardelli *et al.* (2015) showed that 94.0 % of EE2 was removed after 1 hour of contact time and an additional 4.0 % after 24-hour incubation. This indicates estrogen removal is largely achieved by adsorption as it is known to be a spontaneous process, compared to biodegradation.

Table 2.6. Contribution of biodegradation and adsorption processes in estrogen removal.

References	Contribution to estrogen removal (%)	
	Adsorption	Biodegradation
Ren et al. (2007a)	98.0	2.0
Racz and Goel (2010)	30.0	
Brasil Bernardelli et al. (2015)	94.0	4.0

Generally, estrogen removal has been reported to be influenced by many factors including the characteristic of the sorbent (Brasil Bernardelli *et al.*, 2015). A hydrophobic sorbent undoubtedly is an excellent material to interact with the hydrophobic estrogen. It has been suggested that hydrophobic estrogens such as E1, E2 and EE2 are theoretically drawn to be adsorbed by a hydrophobic solid, suggesting adsorption as predominant mechanism in estrogen removal, compared to biodegradation (Margot *et al.*, 2013b). In addition, the percentage of estrogen removal due to adsorption and biodegradation also depends on the reactor design. In a typical activated sludge system, adsorption is a spontaneous process and it takes less than 1 hour to achieve equilibrium for estrogen removal. On the other hand, biodegradation needs a longer time to be completed due to the enrichment of slow-growing bacterial culture (Racz and Goel, 2010). Sludge age (Solids retention time, SRT) may also restrict the growth of some organisms in AS plants that are potentially capable of biodegradation. Therefore, removal of estrogen in a treatment system designed with a short hydraulic retention time (HRT) of less than 1 hour, removal is presumed to occur mainly through the adsorption process. In contrast,

an HRT longer than 1 hour allows for adsorption as well as biodegradation to occur, particularly if SRT is also long. Nevertheless, it is reported that adsorption should be achieved within 24 hours to allow for maximum removal (Ren *et al.*, 2007b).

In conclusion, adsorption and biodegradation have been proven to be efficient advanced treatment processes for estrogen removal. However, adsorption has been shown to be more effective compared to biodegradation whenever hydrophobic adsorbents are utilised. Even though adsorption has been reported to be complete within a short duration of less than 1 hour, an increase in hydraulic retention time will enhance estrogen removal, allowing higher degree of estrogen to be adsorbed.

2.7 Adsorption process for estrogen removal

Adsorption by activated carbon (AC) material is a known excellent process to increase efficiency of conventional treatment, which cheaper adsorbent materials is sought for alternative of AC. To evaluate potential of the alternative materials, understanding on mechanism of the adsorption by adsorbent of AC is to be investigated.

2.7.1 Adsorption mechanism

Adsorption is a mass transfer process associated with the accumulations of an adsorbate at the interface of two phases, such as liquid-solid phases in the removal of pollutants from wastewater. An adsorption mechanism can be classified according to its interaction mechanism as either a physisorption or chemisorption process.

Physical adsorption takes place whenever forces between the sorbent and pollutant are greater than the forces among the particle molecules, and multi-layer attachment could build-up on the surface of an adsorbent through a physisorption process (Dąbrowski, 2001). In contrast, chemisorption occurs whenever electrons from the functional group in an adsorbent interact with a pollutant particle to form a strong bond (Ruthven, 2001). Both physisorption and chemisorption could happen on the outer surface of the adsorbent by surface diffusion as well as in its pores (pore diffusion) whenever the pollutant particles are small enough. Unfortunately, the accumulation of pollutants or molecules of other substances in wastewater could potentially block the pores (Figure 2.5) and reduce the rate of adsorption in a porous material such as activated carbon.

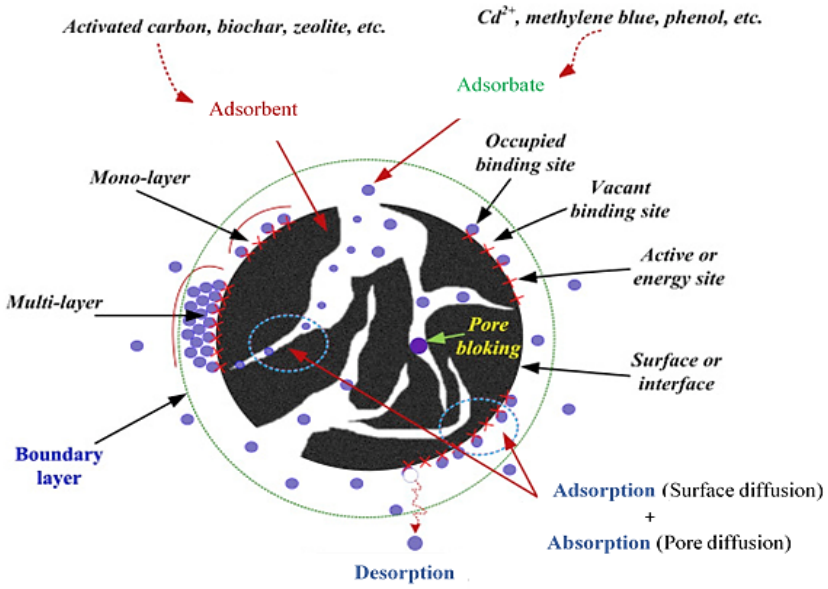


Figure 2.5 Mechanism of adsorption in an adsorbent particle (Tran *et al.*, 2017).

On the other hand, a porous adsorbent with micropores less than 2 nm in diameter contains a very high surface area for the binding sites (Ferhan Cecen, 2011). The physisorption process has also been described in terms of weak adsorbent-pollutant binding and it may be a reversible process (De Gisi *et al.*, 2016). In terms of thermodynamics, an adsorption process that spontaneously occurs without an energy supply can be classified as exothermic, whereas endothermic adsorption must be supported with an external energy supply to initiate the bonding process (De Gisi *et al.*, 2016).

Physisorption occurring via exothermic adsorption is a preferable mechanism for this study as the spontaneous process would not require additional cost in providing energy to drive the process. The common use of activated carbon (AC) could also be replaced by a lower cost hydrophobic adsorbent. In addition, an alternative material without a pore structure but with a surface area almost similar to AC would be advantageous in avoiding the adverse effects of pore blockage in the sorption process. Apart from that, AC also has an affinity for hydrophilic molecules, which could reduce the availability of binding sites for hydrophobic molecules, as stated in Section 2.5.1.

2.7.2 Adsorption isotherm

The adsorption of estrogen from wastewater onto an adsorbent such as AC involves a solid-liquid adsorption system. The amount of contaminant (q_t) which can be adsorbed on to a solid adsorbent at equilibrium can be represented by the following equation (Grassi *et al.*, 2012):

$$q_t = \frac{(C_0 - C_e)\,V}{m} \qquad \text{(Equation 2.1)}$$

where C_e and C_0 are the final and initial concentrations of the contaminant in the aqueous phase respectively. V is the solution volume and m is the adsorbent mass.

The behaviour and adsorption characteristics can be predicted by using models of adsorption by using a series of experiments. The information are given as parameters within each adsorption model, whenever the experimental data fits well into it (small regression value). Selected isotherm models that has been commonly used for removal of pollutant from wastewater are shown in Table 2.7 (Grassi *et al.*, 2012).

In addition, the sorption behaviour of the contaminant can also be predicted by its specific sorption coefficient (Gomes *et al.*, 2011). This sorption coefficient (K_d) indicates the ratio of estrogen's total equilibrium concentration in solids (C_s) to that in the aqueous matrix (C_w):

$$K_d = C_s / C_w \qquad \text{(Equation 2.2)}$$

Based on the specific sorption coefficient equation, the higher the K_d value of a pollutant like estrogen, the higher its possibility to be adsorbed into the solid phase rather than remain in the wastewater. In addition, Racz and Goel (2010) reported that sorption is only negligible if a substance possesses a value of log K_d less than 2. According to Gomes *et al.* (2011), the log K_d values of E1, E2 and EE2 were 2.10, 2.27 and 2.45, respectively, with an activated sludge adsorbent. This indicates that adsorption can be a relevant mechanism for estrogen removal and should be investigated further. However, the value of log K_d for estrogen removal depends on the adsorbent material that is employed.

Table 2.7 Isotherm models and parameters for adsorption characteristic.

Isotherm model	Formula	Parameter of adsorption
Langmuir	$\frac{q_e = q_m K_L C_e}{1 + K_L C_e}$	• Monolayer adsorption (Heraldy *et al.*, 2016) • Energy of adsorption (constant of K_L) • q_e (mg/g) is the amount of adsorbate per mass unit of adsorbent. q_m is the maximum adsorption capacity ($mg.g^{-1}$).
Freundlich	$q_e = K_f C_e^{1/n}$	• Adsorption capacity (K_f) • Adsorption intensity (1/n) • 1/n value gives the intensity of the adsorption (Chen, 2015). • Multi layer adsorption. q_e ($mg.g^{-1}$) is the amount of adsorbate per mass unit of adsorbent. C_e is the final concentration of estrogen in aqueous.
Brunauer, Emmet, and Teller (BET)	$Ln\ q_e = ln\ q_s - K_D\varepsilon^2$	• Free energy of adsorption (constant of K_D) • Adsorption capacity (q_s)

2.7.3 Adsorbent and estrogen characteristics for successful adsorption process

To ensure that successful interaction and binding could occur between the adsorbent and estrogen adsorbate, factors influencing the binding process need to be evaluated. These factors include adsorbent surface area, pH, contact time, water solubility of the type of adsorbent and type of estrogen, as well as the functional groups of the estrogen (Snyder *et al.*, 2007; Kyzas *et al.*, 2013).

1. Adsorbent surface area. An adsorbent that has a high surface area contributes a large number of binding sites for estrogen attachment
2. pH. Estrogens are neutral compounds compatible with neutral adsorbents for adsorption. Moreover, a high pH set in the treatment design has been detected to reduce the Log K_{ow} (octanol-water coefficient partition) of the estrogens and reduce their adsorption onto a hydrophobic adsorbent, so it is to be avoided (Borrirukwisitsak *et al.*, 2012).

3. Contact time. Adsorption is a spontaneous process that normally removes most of a pollutant within hours. Further removal is expected over a longer contact time such as between 24 to 48 hours (Racz and Goel, 2010).

4. Low water solubility of the adsorbent. Low water solubility with weak solute-solvent bonds increases the adsorption rate due to the higher magnitude of forces between the estrogen and the adsorbent compared to the estrogen in water. Therefore a highly hydrophobic adsorbent should bond well with hydrophobic estrogen in the removal process. The molecular structure and behaviour of a contaminant of interest, such as its hydrophobicity, is an important factor in the adsorption process (Racz and Goel, 2010; Jiang *et al.*, 2017)

5. The functional group in estrogen. The existence of functional groups (ortho, meta, para and other branched functional groups) in estrogen molecules contributes to weak internal bonds and will increase the external attachment to the molecules of the adsorbent (Grassi *et al.*, 2012; Hemidouche *et al.*, 2017).

2.8 Microorganisms as an adsorbent

2.8.1 Potential of microorganisms as an adsorbent

Potential materials which could act as adsorbents for estrogen removal should possess some of the criteria of ideal adsorbents mentioned in Section 2.7.3.

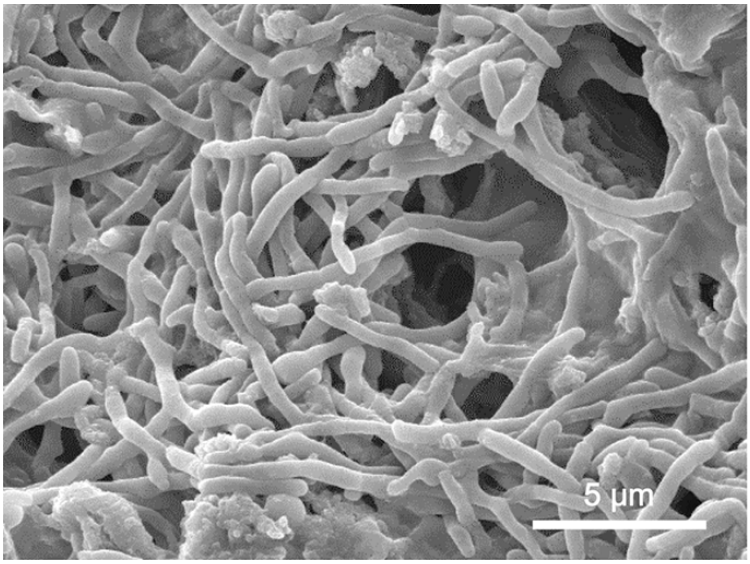


Figure 2.6 Image of gram-positive rod-shaped bacteria captured by scanning electron microscope (SEM) (Asamizu *et al.*, 2015).

High surface area and high hydrophobicity are the prominent attributions to aim for. Recently, close attention has been paid to the application of prokaryotes in wastewater treatment due to

their biodiversity and various characteristics they can offer. However, as shown in Figure 2.6, prokaryotes such as bacteria do not have a porous structure like the one that contributes to the large surface area in AC. Nevertheless, the relatively small size of bacteria (3 – 5 μm lengths) provides the high surface area required to be an efficient adsorbent. The surface areas of some bacterial species as well as granular-activated carbon (GAC) recorded in previous studies are listed in Table 2.8. According to this data, the mean surface area of bacteria is 111 $m^2.g^{-1}$, which is approximately nine times lower than the surface area of GAC (966 $m^2.g^{-1}$). Even though the surface area of bacteria may be lower than that of commercial GAC, other disadvantages of activated carbon material such as pore blocking and affinity for hydrophilic compounds (Section 2.5.1) might reduce its adsorption performance to a level comparable to that of bacterial species (Snyder *et al.*, 2007; Jiang *et al.*, 2017). In addition, hydrophilic compounds were found to break through GAC filters more rapidly than hydrophobic contaminants (Snyder *et al.*, 2007). The hydrophobic properties of some bacteria suggest they might work as an efficient adsorbent, and place them favourably in comparison with other conventional adsorbents like AC. Some bacteria that have been reported to be hydrophobic are *Rhodococcus* sp. (De Carvalho *et al.*, 2004), *Acinetobacter calcoaceticus* (Zita and Hermansson, 1997) and *Pseudomonas aeruginosa* (Bruinsma *et al.*, 2001).

Table 2.8 Surface area of potential of bacterial and commercial granular activated carbon adsorbent (GAC).

Material/ microorganism	Surface area ($m^2.g^{-1}$)	Prorate ($m^2.g^{-1}$)	References
Rhodococcus erythropolis	90	111[a]	(Revil *et al.*, 2012)
Corynebacterium DSM6688	103		(Revil *et al.*, 2012)
Bacillus subtilis	140		(Revil *et al.*, 2012)
GAC	950	966[b]	(Alhamed and Bamufleh, 2009)
GAC	798		(Jung *et al.*, 2001)
GAC	1152		(Jung *et al.*, 2001)

[a], Average surface area for bacterium; [b], Average surface area for GAC

2.8.2 Previous studies utilising bacteria for estrogen removal in wastewater treatment

Various estrogen removal studies which include the exploitation of microbial communities have been conducted by several researchers. Most of the treatments were carried out either by using pure commercial bacterial strains (Larcher and Yargeau, 2013), inocula from activated sludge (Ren *et al.*, 2007b; Zhang *et al.*, 2012; Brasil Bernardelli *et al.*, 2015; Zheng *et al.*, 2016) or the

isolation of pure bacteria from diverse sources of prokaryotes in environmental samples of soil (Kurisu *et al.*, 2010) or activated sludge (Villemur *et al.*, 2013).

Table 2.9 lists some of the research that has employed microbial communities in the estrogen removal process. An adsorption study carried out using deactivated sludge successfully obtained removal rates of 98.0, 93.6, and 98.0 % for E1, E2 and EE2 respectively (Ren *et al.*, 2007a). Nevertheless, in all cases the final concentration of estrogens was above the PNEC limits, which was believed to a result of the high initial concentrations employed. An adsorption process similar to that when adopting activated sludge was also used by Zhang *et al.* (2012) and Zheng *et al.* (2016). However lower percentages of estrogen removal were recorded at 80.0 % of EE2 and 64.7 % of E2.

A different approach has been made by Villemur *et al.* (2013) when they isolated pure strains of *Rhodococcus* sp. and other bacterial species through an enrichment culture of activated sludge. *Rhodococcus* sp. was proven to be the most competent strain in removing estrogens through a biodegradation process. The bacteria were isolated in minimal media using estrogens as their sole growth substrate. It was concluded that the bacteria had recognised estrogens as its sole carbon source during the cultivation, adapted to it and maintained high levels of the degradative enzymes subsequently, degrading the estrogens present in the test wastewater. The same principles have been employed by Kurisu *et al.* (2010) utilising E2 as the substrate in the isolation process. However, the bacteria were isolated from mixture of soil samples, instead of activated sludge. Surprisingly, similar bacterial species of *Rhodococcus* sp. were also found, and were efficient at E2 removal (85 %), compared to *Sphingomonas* sp. Another *Rhodococcus* sp. has also been detected as estrogen-degrader by Larcher and Yargeau (2013), in which the pure strain of *R. rhodochrous* reduced EE2 from 5 $mg.L^{-1}$ to levels undetectable by HPLC (limit of detection 125 $\mu g.L^{-1}$).

Table 2.9. Previous studies of estrogen removal using microbial from activated sludge, pure bacterial and isolates bacteria.

References	Pure strain/ Isolates from environmental sample	Pre-treatment/ adaptation of adsorbent material	Adsorption/ Biodegradation	Estrogen removal/ Final concentration
Ren *et al.* (2007a)	Activated sludge from sequencing batch reactor (SBR)	Deactivated in heat treatment of 80°C.	Adsorption in 24-hours (adsorption by hydrophobicity interaction)	• Initial estrogen concentration 200 µg.L^{-1} • 98 % removal for E1 and EE2 (final concentration 4.0 µg.L^{-1}) • 93.6 % removal for E2 (final concentration 12.8 µg.L^{-1})
Zhang *et al.* (2012)	Activated sludge from WWTP in China	Deactivated in heat treatment of 375 °C.	Adsorption for 24-hours	• Initial concentration 2.32 mg.L^{-1} EE2 • 80 % removal in 1 hour (final concentration 0.46 mg.L^{-1} EE2)
Zheng *et al.* (2016)	Activated aerobic granular sludge	Deactivated in autoclave 120 °C	Adsorption in 2 hours (adsorption due to hydrophobic interaction)	• Initial concentration 400 µg.L^{-1} E2 • 64.7 % removal of E2 (final concentration 141.4 µg.L^{-1} E2)
Brasil Bernardelli *et al.* (2015)	Activated sludge from WWTP in Brazil	Deactivated sludge by autoclave at 120 °C and activated sludge	Adsorption and biodegradation Batch reactor for 24-hours	• Initial 100 µg.L^{-1} • Final concentration 23 µg.L^{-1} of E1, 18 µg.L^{-1} of E2 and 2 µg.L^{-1} of EE2 in adsorption study • Adsorption provide more efficient removal.

References	Pure strain/ Isolates from environmental sample	Pre-treatment/ adaptation of adsorbent material	Adsorption/ Biodegradation	Estrogen removal/ Final concentration
Villemur *et al.* (2013)	Activated sludge contained enrichment culture *Rhodococcus* sp.	Enrichment in minimal media and estrogens substrate	Biodegradation fed-batch for 4 weeks adopting estrogen in polymer	• Initial 1000 µg.L^{-1}
Kurisu *et al.* (2010)	*Rhodococcus* sp. (*R. equi* and *R. zopfi*) and *Sphingomonas* sp. isolated from soil.	Soil-enrichment in minimal media and E2 substrate	Biodegradation	• Initial 0.8 mg/4ml of E2 (200 mg.L^{-1}) • *Rhodococcus* sp. removed 85 % E2 (final concentration 30 mg.L^{-1}) and 50 % E1 (final concentration 100 mg.L^{-1}) • *Sphingomonas* sp. removed 65 % E2 (final concentration 70 mg.L^{-1}) and 70 % E1 (final concentration 60 mg.L^{-1} E1).
Larcher and Yargeau (2013)	Pure culture of *B. subtilis, P. aeruginosa, P. putida, R. equi, R. erythropolis, R. rhodochrous, R. zopfi*	Pure culture obtained from Cedarlane Canada	Biodegradation	• Initial concentration of 5 mg.L^{-1} EE2 in 350 ml sample • *R. rhodochrous* removed EE2 to undetected level. • *R. equi* removed 61 % EE2
Fernández *et al.* (2017)	*Virgibacillus* sp., *Bacillus flexus* and *B. licheniformis* isolated from deep sea sediment of mud volcano	Enrichment in minimal media and phenanthrene (polyaromatic hydrocarbon) substrate. Then isolates were adapted in estrogen substrate	Biodegradation in batch reactor for 32 days	• Initial of 5 mg.L^{-1} E1 and E2 in media • *Virgibacillus* sp. complete removal of E2 • *Bacillus flexus* 98.8 % E2 removal • *B. licheniformis* remove 80 % of E2 • Complete removal for E1 by all species.

2.9 Adaptation of bacteria

Process of adapting bacteria utilising hydrocarbon compounds has been used in oil-spill bioremediation where ideal conditions allow bacteria to biodegrade and breakdown the targeted contaminants into environmentally safer products (Vidali, 2001). In an oil-spill bioremediation process, the bacteria are usually enriched on oil-based substrates such as alkanes or haloalkanes. Research has successfully proven that the hydrocarbon-degrading bacteria obtained using alkanes is due to their high affinity for non-polar compounds (Erable *et al.*, 2003) and consequently their hydrophobicity developed during the process.

However, to date none of these hydrocarbon-adapted bacteria have been used for estrogen removal. Nevertheless, the similarity of hydrophobic characteristics could be exploited if an adsorption process were adopted as the removal process, regardless of different substrates used. In fact, a higher log P_{ow} of hydrocarbon compound compared to estrogen could render an even more hydrophobic bacterial species than one enriched on estrogens. The cell surface hydrophobicity (CSH) of the adapted strains has been proven to increase compared to their parent strains after the adaptation process (Vasileva-Tonkova *et al.*, 2008). They could potentially be more efficient adsorbents for the estrogen removal after adaptation. Helpfully, The enhancement of bacterial hydrophobicity quantified by simple hydrophobicity tests.

2.9.1 Bacteria morphological changes

Hydrocarbon is a non-polar compound that is known to be toxic and harmful to bacteria due to its hydrophobic characteristics. However, some bacteria possess ablity in developing tolerance towards the toxicity by altering their cell surface properties (Whyte *et al.*, 1999), so they can access hydrocarbon substrates. The mechanism of alteration in bacterial cell when exposed to the toxic substrates were illustrated in Figure 2.7.

To utilize the hydrocarbon substrate, the bacterial cytoplasmic membrane lipid has to undergo an alteration process. This mechanism is essential for the bacteria to maintain its membrane fluidity, as well as structural integrity. The alteration involves transformation of unsaturated cis-fatty acid to saturated trans-fatty acid, which eventually causes the membrane to be less permeable and more hydrophobic (Figure 2.7). This bacterial protection mechanism ultimately leads to the enhancement of their cell surface hydrophobicity, that later can facilitate the hydrocarbon uptake during the adaptation process (Bredholt *et al.*, 2002).

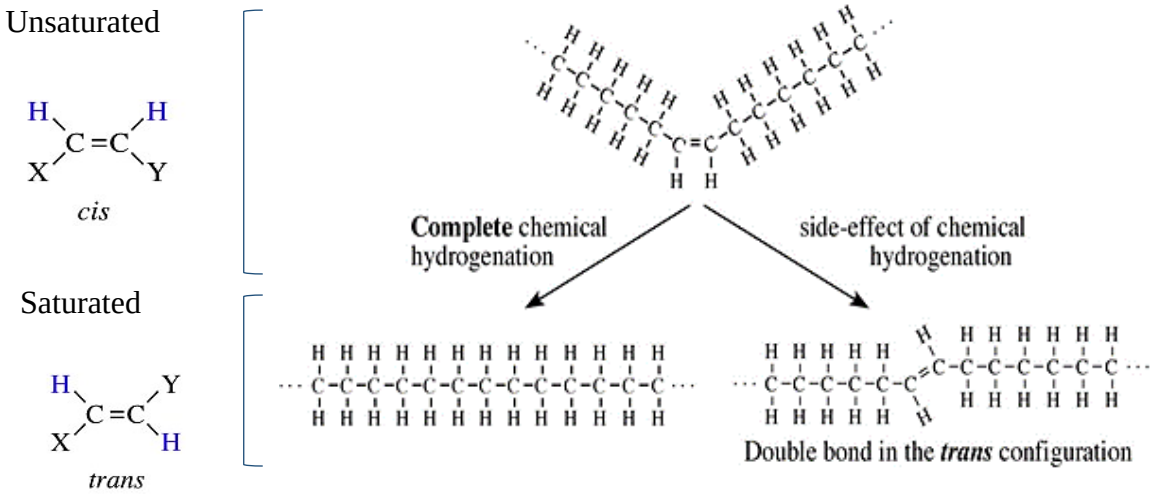


Figure 2.7 Transformation of cis-fatty acid to trans-fatty acid in bacterial cytoplasmic membrane lipid during the adaption with hydrocarbon substrate.

Enhancement of the bacterial cell surface hydrophobicity allows better cell adherence to the hydrocarbon droplets, creates direct contact (interfacial accession) between cell and hydrocarbon and allows the bacteria to access the carbon content inside the hydrocarbon. In addition, the amount of fatty acid in the bacterial cell membrane has also been reported to increase after the adaptation process is complete (Rodrigues and de Carvalho, 2015).

Due to its hydrophobic characteristic, the hydrocarbon substrate is insoluble in the liquid media and is not efficiently available for the bacteria to access. However, a hydrocarbon-degrading bacteria can increase hydrocarbon uptake through adaptation mechanisms, namely the production of EPS and biosurfactants. Besides the alteration of cell lipid composition, the adapted bacteria can also produce an extracellular polymeric substance (EPS), or mucoid secretion, to bridge gaps between the bacteria cell and hydrocarbon substrate droplet, allowing bacteria to directly access the hydrocarbon. In general, this substance can be observed as slime that ultimately forms as a biofilm on a solid surface, and holds colonies of cells together as aggregates in a liquid medium (clumping of cells) (Figure 2.8). Furthermore, bacteria can also increase their hydrocarbon uptake by emulsifying the hydrocarbon droplets with biosurfactant (Lang and Philp, 1998; Kim *et al.*, 2002). This mechanism will increase the hydrocarbon solubility for the bacteria to utilise (Tzintzun-Camacho *et al.*, 2012).

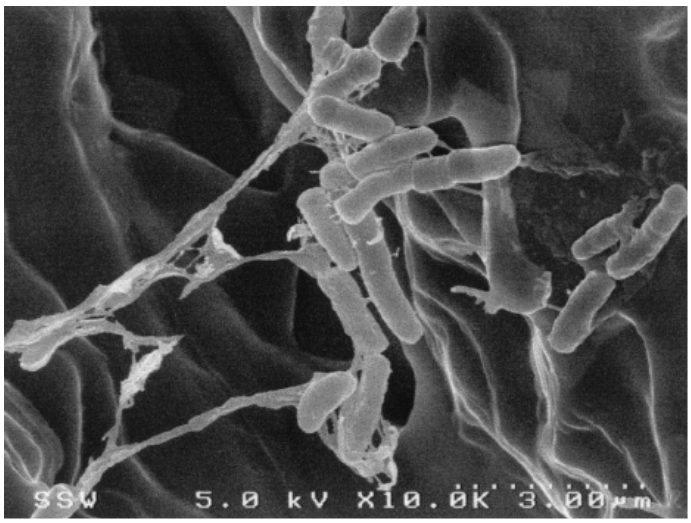


Figure 2.8 Scanning electron microscope micrograph of *Rhodococcus* sp. with the presence of EPS strands on the cell surface and between cells (Whyte *et al.*, 1999).

2.9.2 Adaptation of bacteria in previous studies

According to Table 2.10, a pure strain of *Rhodococcus erythropolis DCL14* has successfully been adapted with aliphatic alkane (n-alkanes) of C5 to C16. The Cell Surface Hydrophobicity (CSH) of the bacteria were observed to significantly increase with number of carbons in the alkane, with a CSH of 90 % recorded for *R. erythropolis* grown in hexadecane (C16) (de Carvalho *et al.*, 2009). Furthermore, C10 - C19 of n-alkanes, aromatic and polyaromatic hydrocarbon have been used for the adaptation of pure strains of *Rhodococcus ruber* and *Rhodococcus opacus* Serebrennikova *et al.* (2014). The mean CSH of these *Rhodococcus* sp. in alkanes were two-fold lower than the measurement of CSH of *Rhodococcus erythropolis* by (de Carvalho *et al.*, 2009). Nevertheless, the CSH of the *Rhodococcus ruber* was recorded to increase from 35 % in the parent strain compare to 50 % in the adapted strain, indicating that adaptation still occurred. Isolation of *Rhodococcus erythropolis* from crude oil-contaminated soil in minimal media supplied with n-hexane has been recorded to increase CSH from 16 % to 29 % in only 24 hours (Stancu, 2014), suggesting that CSH increases as the culture ages. In conclusion, the *Rhodococcus* sp. possesses tolerance to the toxicity of n-alkanes, including n-hexane and n-hexadecane. Their CSH were shown to significantly increase after an adaptation period (compared to parent strain) and adaptation increased with increasing hydrocarbon chain length (de Carvalho *et al.*, 2009) , and with incubation period (Stancu, 2014). Furthermore, isolation of other bacterial species from environmental samples in minimal media supplied with hexadecane has produced a moderate CSH species, such as *Arthrobacter* sp. at 66 % CSH (Vasileva-Tonkova *et al.*, 2008) and *A. bouvetii* achieved 72 % CSH (Tzintzun-Camacho *et al.*, 2012). Finally, the adaptation of a pure strain of *Pseudomonas frederiksbergensis* on

hexadecane substrate has shown CSH to increase from 10 % to 88 % after 10 days. This also supports the theory that CSH increases with the incubation period.

Based on the findings that n-hexadecane is a competent hydrocarbon substrate, relative to other alkane such as n-hexane, and that *Rhodococcus erythropolis* shows the highest CSH (>90) this research investigated the adaptation of pure *Rhodococcus erythropolis* utilising n-hexadecane and n-hexane as growth substrate, and how factors influencing CSH of bacteria, such as incubation time, alkane chain length and comparison with parent strain were related to their estrogen removal properties.

Table 2.10. Adaptation of bacteria to increase hydrophobicity by cultivation in minimal media (MM1) adopting hydrocarbon substrate

Bacterium	Pure strain/ isolated from environmental sample.	**Hydrocarbon substrate**	CSH[1] (the highest)	Adaptation and hydrophobicity process	References
Rhodococcus erythropolis DCL14	Pure strain provided by Wageningen Agricultural University	C5 to C16 n-alkane	*Rhodococcus erythropolis* >90 %	MATH	de Carvalho *et al.* (2009)
Rhodococcus ruber and *Rhodococcus opacus*	Pure strain provided by the RSC[2]	C10-C19 of n-alkane, aromatic and polyaromatic hydrocarbons	*R. ruber* IEGM 615 • 35 % (parent strain) • 50 % (isolated strain) *R. opacus* IEGM 249 • 47 % (parent strain) • 15 % (isolated strain)	MATH	Serebrennikova *et al.* (2014)
Rhodococcus erythropolis	isolated from a crude oil-contaminated soil	Cyclohexane, n-hexane, n-decane and aromatics	*R. erythropolis* in n-hexane • 16 % after 1 hour • 29 % after 24 hours	Low hydrophobicity BATH	Stancu (2014)
Arthrobacter sp. HW-8 and 14 other species.	Previously isolated from lubricant-polluted WWTP in Sofi.	Hexadecane	*Arthrobacter* sp. HW-8 (66.5 %)	Minimal media supplied with 1.5 % hexadecane (15 days)	Vasileva-Tonkova *et al.* (2008)
Xanthomonas sp., *Acinetobacter bouvetii*, *Shewanella* sp. and *Defluvibacter lusatiensis*	Isolated from an oil-contaminated site Mexico	Hexadecane	*A. bouvetii* 72 %	Presence of biosurfactant by the *A. bouvetti*	Tzintzun-Camacho *et al.* (2012)
Pseudomonas frederiksbergensis	Previously isolated	Hexadecane	• 10 % after 1 day • 88 % after 10 days	MATH	Abdel-Megeed *et al.* (2014)

1, Regional Specialized Collection of Alkanotrophic Microorganisms

2.10 Cell Surface Hydrophobicity (CSH)

Cell surface hydrophobicity (CSH) is an important parameter that can be measured to investigate the physicochemical profile of the adapted bacterial strains. The hydrophobicity index can be determined by an array of techniques that have been established by previous researchers. The common techniques available are microbial adhesion to hydrocarbon (MATH) (Rosenberg *et al.*, 1980; Rosenberg, 2006), contact angle measurement (Bruinsma *et al.*, 2001), salting-out aggregation (Del Re *et al.*, 2000), and hydrophobic interaction chromatography (Oliveira *et al.*, 2001). Preferences of adopting specific techniques seem to be very closely related to the samples attribution. Nevertheless, MATH and contact angle are the most popular method in bacterial hydrophobicity study due to its simple and accurate method (Oliveira *et al.*, 2001; Rosenberg, 2006).

Figure 2.9 Microbial adhesion to hydrocarbon assay. Turbid suspension of bacteria with n-hexadecane (left), bacterial cell partitioned between aqueous and oil phase (right) (Rosenberg, 2006).

MATH is a measurement of the percentage of bacterial cell adhere in the hydrocarbon compared to the aqueous phase Figure 2.9. According to its protocol, the attachment of cells in aqueous phase represented in optical density is made with a spectrophotometer after a thorough mix of culture and separation of phase completed. The CSH can be calculated using the Equation 2.3.

$$\text{CSH (\%)} = [1 - (A_0 - A_1)/A_0] \times 100\ \%. \quad \text{(Equation 2.3)}$$

Where A_0 is the initial optical density (OD) of the bacterial culture and A_1 is the OD for the aqueous phase after separation completed. A very hydrophobic bacteria will have a higher hydrophobicity percentage.

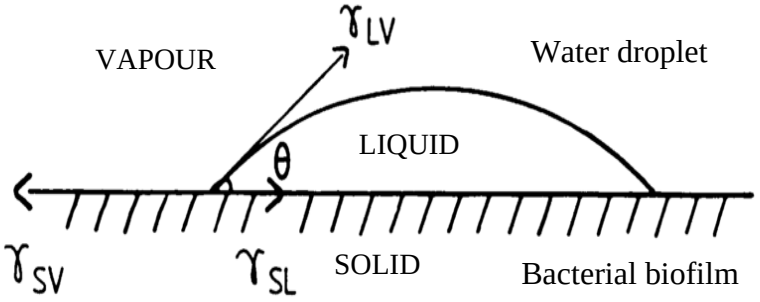


Figure 2.10 Measurement of contact angle for different interfacial tensions (Van Loosdrecht *et al.*, 1987).

Alternatively, the contact angle is a measurement of wettability of a solid surface by a liquid, which in this context means wettability of a bacteria biofilm by an axisymmetric drop of water. The contact angle can be determined by Young equation

$$\gamma_{LV} \cos \theta = \gamma_{sv} - \gamma_{SL} \quad \text{(Equation 2.4)}$$

which require information such as the surface tension between solid (S), liquid (L) and vapour (V), water drop volume and water droplet diameter as illustrated in Figure 2.10. These data can be determined in experiments utilising stereomicroscope that can capture the image of the droplet and digitise the image to measure the data (Drumm *et al.*, 1989).

Several researchers claim the MATH technique has some drawbacks due to its two-phase bacterial partitioning technique. The attachment of bacteria into the different phases was suggested to associate with attachment due to the hydrophobic effect and electrostatic forces, that interfere the actual hydrophobicity measurement (Doyle, 2000; Oliveira *et al.*, 2001). However, Rosenberg (2006) had cleared the argument by stating that the electrostatic effect will only affect the attachment process if a low ionic strength of buffer were adopted instead of the actual PUM buffer proposed in the original MATH technique (Rosenberg *et al.*, 1980). The low ionic strength buffer allows for less hydrophobic interactions and the electrostatic interaction increases (Rosenberg, 2006). Therefore, MATH technique is still reliable as a bacterial hydrophobicity assay in addition to the contact angle measurement.

Chapter 3 Experimental and analytical methods

3.1 Introduction

Five stages of experimental work were planned to explore the potential of using hydrophobic bacteria as sustainable adsorbents in estrogen removal. An overview of the workflows is summarised in Figure 3.1 and brief descriptions of each stage are given in the subsequent sections. Full details of the methodology is given in the individual sections and chapters as stated in Table 3.1- Table 3.4.

3.2 Stage 1: Hydrophobic adaptation of bacteria

This study was undertaken in order to adapt commercial strains of bacteria and soil-isolates into hydrophobic bacterial cells. The commercial strains were *Rhodococcus erythropolis* NCIMB13064 and DSM311, and the soil-isolates were obtained through a process of soil-enrichment of diesel-contaminated soils. The hydrophobic-adaptation process was conducted by culturing the strains in minimal media supplemented with hydrocarbon growth substrates. Hydrocarbons were chosen as growth substrates (main carbon source) due to their high octanol-water coefficient, which was considered a good indicator of hydrophobic compounds.

A preliminary study was initially conducted to determine the most appropriate culture method that gave optimum hydrocarbon uptake during the adaptation process (Section 4.4.3). Aliphatic hydrocarbons of n-hexadecane and n-hexane were assessed to identify the most suitable growth substrate for the bacteria to grow on, concurrently with other nutrients in the minimal media (Section 4.4.4). Furthermore, the optimum concentration of hydrocarbon (substrate) giving maximum biomass yield was also investigated (Section 4.4.5). Monitoring of the bacterial growth phase (Section 4.4.6) and morphological characteristic were also carried out (Section 4.4.7). Details of the methodology used to investigate the bacterial adaptation process in Stage 1 are described in Table 3.1.

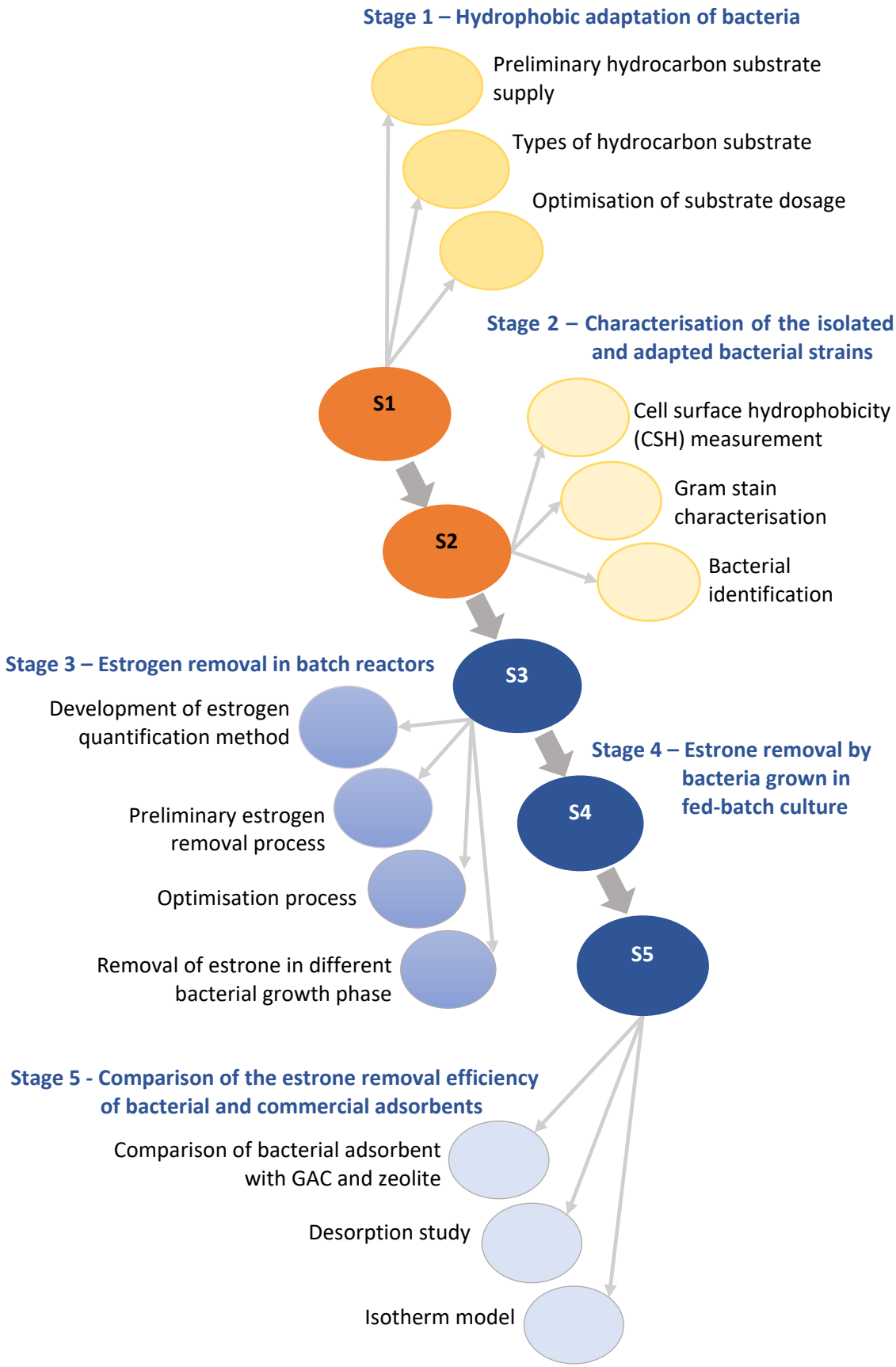


Figure 3.1 Summary of the workflow for evaluating hydrophobic bacterial adsorbents for estrogens removal. S1-S5 represent Stage 1 to Stage 5 of the experimental study.

Table 3.1 Methodology for hydrophobic adaptation of bacteria (Stage 1).

Experimental study	Details of methodology
Preparation of minimal media	Section 4.3
General method of the bacterial adaptation process	Section 4.4
Preliminary study of cultivation on n-hexadecane	Section 4.4.3
Types of growth substrate study	Section 4.4.4
Optimization of growth substrate concentration	Section 4.4.5
Growth monitoring	Section 4.4.6
Microscopic observation	Section 4.4.7
Isolation of bacteria from soil- soil enrichment study	Section 4.6

3.3 Stage 2: Characterization of the isolated and adapted bacterial strains

Characteristics of the hydrophobic-adapted bacterial strains *R. erythropolis* DSM311, NCIMB13064 and soil-isolates PD2-1, PD2-1, PD2-3, SD1-1 and SD2-1 obtained from Stage 1, were investigated further in Stage 2.

The hydrophobicity of the bacteria can be represented by their cell surface hydrophobicity (CSH). Therefore the measurement of the bacterial CSH for culture incubation period of 3, 5 and 10 days were carried out using microbial attachment to hydrocarbon (MATH) method (Section 5.3.1). The most hydrophobic strains were identified and the effect of the bacterial growth phase on the CSH of the bacteria was also assessed. Gram stain characterisation was also carried out to differentiate the bacteria based on their cell wall constituents (Section 5.3.2), Gram-positive bacteria having a thicker cell wall, in contrast to Gram-negative. Finally, a process of identification of the bacterial genera or species was conducted by DNA extraction, PCR amplification, and Sanger sequencing (out-sourced) (Section 5.3.3). DNA sequence data and DNA chromatograms of the bacteria were then analysed (Section 5.3.4). Experimental tasks that were developed for Stage 2 are listed in Table 3.2 and details of the methodology is discussed in Chapter 5.

Table 3.2. Methodology for the characterisation of bacterial strains (Stage 2).

Experimental study	Details of methodology
CSH measurement	Section 5.3.1
Gram stain characterisation	Section 5.3.2
DNA extraction	Section 5.3.3
PCR amplification, DNA quantification and sequencing	Section 5.3.4

3.4 Stage 3 - Estrogen removal in batch reactor

Estrogens removal (E2 and E1) was investigated using hydrophobic-adapted bacterial strains and compared to the *E.coli*. Development of the estrogen quantification method was firstly made to define the solid phase extraction (SPE) and HPLC-ECD procedures for estrogen analysis (Section 6.4). The first estrogen removal investigation, namely the preliminary study of estrogen removal (Section 6.5) was conducted using all of the adapted bacteria in order to find any relationship between degree of estrogen removal and the CSH of the bacterial strains, and to select the best bacteria for further work. A high concentration of estrogens (1 $mg.L^{-1}$) in synthetic wastewater (Section 6.3.1) was employed to evaluate the best bacterial adsorbent using thermal pre-treated bacteria (Section 6.3.2). In additional batch experiments, live bacteria were employed in the removal process to assess the mechanism of estrogen removal from the two processes of biodegradation and adsorption.

Lower concentrations (200 $ng.L^{-1}$) of estrogen was then employed in the optimisation of estrogen removal studies, i.e: effect of contact time; and adsorbent dosage (Sections 6.7.1 and 6.7.2). Effect of exponential and stationary growth phases of the bacterial cells on estrogen removal and CSH were also investigated. The experimental work is listed in Table 3.3 and details of the methodology for the Stage 3 study is given in Chapter 6.

3.5 Stage 4 – Estrone removal by bacteria grown in fed-batch culture

A potential improvement in degree of estrone (E1) removal was further evaluated by using a longer growth adaptation period with bacterial strains SD2-1 (isolate) and *R. erythropolis* DSM311. Fed-batch cultivation method was employed to achieve higher hydrocarbon uptake and growth of biomass over 15 and 20 days, and to avoid the inhibition of bacterial growth

caused by excess free phase hydrocarbons in the media. Full details of the methodology is reported in Chapter 7.

Table 3.3 Methodology for the estrogen removal in batch reactor (Stage 3).

Experimental study	Details of methodology
Quantification of bacterial biomass	Section 6.2.1
Preparation of estrogen stock and synthetic wastewater	Section 6.3.1
Pre-treatment of the bacterial adsorbent biomass	Section 6.3.2
Development of the estrogen quantification method	Section 6.4
Preliminary estrogen removal study	Section 6.5
Optimization – contact time	Section 6.7.1
Optimization – adsorbent dosage	Section 6.7.2
Removal of estrone using different bacterial growth phase	Section 6.7.3

3.6 Stage 5 - Comparison of the estrone removal efficiency of bacterial and commercial adsorbents

The performance of strain SD2-1 adsorbent for estrone (E1) removal was compared against commercial adsorbents. Bacterial biomass adsorbent from strain SD2-1, and granular activated carbon (GAC) and zeolite were added into reactors to follow E1 removal (Sections 8.2.1 and 8.2.2). In addition, the SD2-1 bacterial adsorbent was thermally pre-treated by two different methods (85 °C and 100 °C) to assess its effect on E1 removal.

To determine the E1 recovery, further experiments were carried out on the desorption of E1 from the SD2-1 bacterial adsorbent after adsorption (Section 8.2.3). Finally, an isotherm study was also conducted for the SD2-1 adsorbent to investigate its adsorption capacity and the affinity of the adsorption process using parameters obtained from isotherm models (Section 8.2.4). Full details of the methodology is given in Chapter 8 and is summarized in Table 3.4.

Table 3.4 Methodology for the comparison of performance of bacterial SD2-1 bacterial adsorbent with commercial adsorbents, desorption and isotherm studies (Stage 5).

Experimental study	Details of methodology
Preparation of bacterial adsorbent	Section 8.2.1
Comparison of adsorption properties of SD2-1 bacterial adsorbents with activated carbon and zeolite	Section 8.2.2
Desorption process	Section 8.2.3
Isotherm model	Section 8.2.4

Chapter 4 Hydrophobic adaptation of commercial strains of bacteria and hydrophobic bacteria isolated from soil

4.1 Introduction

The commercial strain of *Rhodococcus erythropolis* has previously been used in research because it has the capacity to grow on hydrocarbon substrates without suffering any toxic effects (Alvarez, 2010). However, when *R. erythropolis* undergoes growth on relatively toxic hydrocarbon substrates, it must undergo adaptation before it can grow effectively. This adaptation brings about alterations in cellular morphology and an increase in cell surface hydrophobicity. Adapted strains possess high cell surface hydrophobicity (CSH), retain their ability to grow on hydrocarbon substrates, and retain their morphology in subsequent generations (Vasileva-Tonkova *et al.,* 2008). Moreover, other microbes inhabiting hydrocarbon-contaminated soils may also retain the same hydrocarbon tolerance. Therefore, commercial strains *R. erythropolis* NCIMB3064 and DSM311, as well as isolates from soil, were used in this adaptation study. Factors affecting the adaptation were also investigated.

4.2 Aim

To generate highly hydrophobic bacterial biomass with high CSH by the adaptation of *R. erythropolis* and other bacterial isolates using extended culturing on hydrocarbons.

4.2.1 Objectives

The objectives of this study were:

1. To identify the most efficient cultivation technique that allows for optimum hydrocarbon uptake.
2. To obtain hydrophobic biomass using pure bacterial strains and soil-isolates by selective growth on minimal media supplemented with different hydrocarbon growth substrates.
3. To investigate the effect of varying substrate dosage during the bacterial growth phase, in order to find the optimum dosage for high bacterial biomass productivity.

4.2.2 Hypotheses

The following hypotheses were proposed in this study:

1. Increase in the hydrocarbon uptake capability allows for the better growth of bacteria.
2. The pure strains of *R. erythropolis,* and soil-isolates, can adapt and grow in minimal media containing hydrocarbon substrates.

3. The amount of substrate used in the cultivation significantly affects the bacterial growth and biomass production.

4.2.3 Hydrocarbon substrates

Hydrocarbons are organic compounds made of hydrogen and carbon elements, and are classified as being either saturated or unsaturated (Timmis *et al.*, 2010). The saturated hydrocarbons have no polar functional groups which makes them inert, and physically attracted towards non-polar or hydrophobic materials (Timmis *et al.*, 2010). Therefore it was considered that these would make ideal growth substrates in the bacterial hydrophobic-adaptation process.

Unfortunately, most hydrocarbons are toxic to microorganisms as they can accumulate in the bacterial cell membrane, and eventually disrupt the cell membrane (Heipieper and Martínez, 2010). Only bacteria with ability to adapt to hydrocarbon can overcome this toxicity effect, and grow on these hydrocarbons (Section 4.1). Straight chain hydrocarbons (aliphatic) have been identified to have less toxicity than aromatic hydrocarbons. High molecular weight hydrocarbons, such as n-hexadecane $CH_3(CH_2)_{12}CH_3$, have a high carbon content, and therefore represent good carbon sources for bacterial growth.

Indeed, n-hexadecane has previously been employed successfully as a growth substrate in the adaptation of *Rhodococcus erythropolis* and *Acinetobacter bouvetii* (Vasileva-Tonkova *et al.*, 2008; Tzintzun-Camacho *et al.*, 2012). The CSH of these strains was found to increase after the adaptation process. *R. erythropolis* has also been reported to be a competent hydrocarbon-degrading species, and can even degrade halogenated hydrocarbons, such as 1-chlorobutane, due to its strong dehalogenase activity (Armfield *et al.*, 1995; Erable *et al.*, 2003). Furthermore, *R. erythropolis* also can degrade n-hexane (Peng *et al.*, 2007), n-hexadecane and other n-alkane compounds (de Carvalho *et al.*, 2009), using them as sole carbon sources.

4.2.4 Mechanism of hydrocarbon uptake in the adaptation process

Cultivation of bacteria in hydrocarbons requires that they first access the hydrocarbon molecules, followed by transportation of the hydrocarbon through bacterial cell membrane to the cell cytoplasm, where degradation of the hydrocarbon by the action of bacterial intracellular enzymes (Hua and Wang, 2014).

Thus, in order to access the hydrocarbons substrate, the bacteria must have adapted their cell membranes to be tolerant to it, which consequently leads to an increase in their CSH (Section 2.9.1). Hydrocarbons are not only potentially toxic, but also have very low water solubility, which limits the concentration of dissolved molecules for the bacteria to access. Therefore, to

investigate the most efficient cultivation method, the utilisation of hydrocarbon, with its probable uptake pathways were assessed.

4.3 Preparation of growth media

Liquid Modified minimal media (MM1) was prepared according to the main components suggested by Erable *et al.* (2003), but using the trace elements reported by Gimkiewicz and Harnisch (2013), in deionized water, as specified in Table 4.1. The trace element solution was added to the minimal media at a rate of 10 ml per litre.

Table 4.1 Components in (i) Modified minimal media (MM1) and (ii) Trace elements solution.

Modified minimal medium (MM1)	**Amount ($g.L^{-1}$)**
NH_4Cl	0.85
KH_2PO_4	0.56
$Na_2HPO_4.2H_2O$	0.86
K_2SO_4	0.17
$MgSO_4.7H_2O$	0.37
Yeast Extract	0.02
Bacteriological agar (washed and used for solid media only)	12.00

Trace elements solution	**Amount ($g.L^{-1}$)**
$MgSO_4.7H_2O$	3.00
$MnSO_4.H_2O$	0.50
NaCl	1.00
$FeSO_4.7H_2O$	0.10
$CoCl_2.2H_2O$	0.10
$CaCl_2.2H_2O$	0.10
$ZnCl_2$	0.13
$CuSO_4.H_2O$	0.01
$AlK(SO_4)_2$	0.01
H_3BO_3	0.01

Solid Modified minimal media agar (MM1A) was prepared from MM1 and washed agar (12 $g.L^{-1}$) and used in agar plates. The latter was prepared beforehand by soaking agar powder (bacteriological agar Lab M Limited) for 3 days in deionized water to reduce its nutrient content to the lowest concentration. The solution was sterilized by autoclaving at 121 ºC for 15 minutes immediately after preparation.

4.4 Hydrophobic adaptation method

4.4.1 Hydrophobic adaptation of pure bacterial strains (R. erythropolis)

This section is about pure bacteria, an equivalent study of soil bacteria is reported in Section 4.6. Adaptation of pure bacterial strains obtained as freeze-dried ampoules was carried out according to the experimental flow in Figure 4.1.

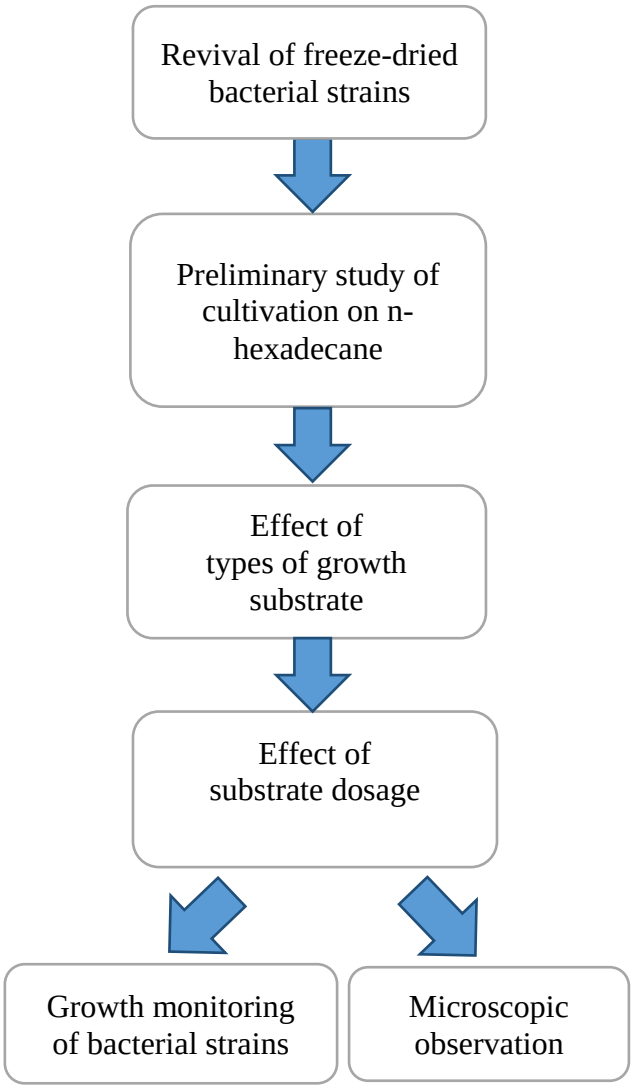


Figure 4.1 Workflow for the pure bacterial strain (*R. erythropolis*) adaptation process

4.4.2 Revival of freeze-dried bacterial strains

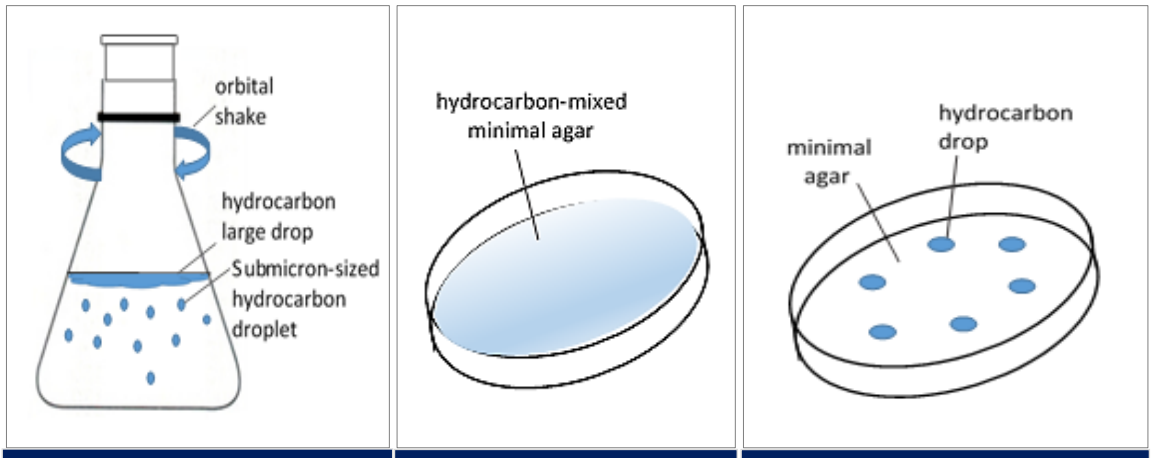
The commercial strain of *R. erythropolis* NCIMB 13064 was purchased from NCIMB Ltd, and another five *R. erythropolis* strains, namely NCIMB10422, NCIMB11148, NCIMB9905, NCIMB9409 and DSM311 were provided by Bath University. All of the strains were cultured initially in nutrient agar (NA) (Nutrient agar LAB 8, Lab M Limited) and incubated at 26°C for 3 days. A single colony of bacteria growing in each of the agar plates was then sub-cultured in a 500 ml Erlenmeyer flask containing 100 ml of nutrient broth (NB) (Nutrient broth No.2, Lab M Limited) at 26 °C in the shake incubator at 155 rpm for another 3 days. 500 µl of the bacteria culture was then collected after 3 days of incubation and mixed with 500 µl of 40 % (v/v) glycerol in 2.5 ml cryovial (Thermofisher Scientific). This bacteria glycerol stock was then kept at -80 ºC to preserve it.

4.4.3 Preliminary study of cultivation on n-hexadecane

A preliminary study to evaluate the most efficient cultivation method using n-hexadecane growth substrate was tested using cultures of bacteria in different cultivation conditions, so that the best cultivation method could be employed in the subsequent adaptation studies.

Hydrocarbon (n-hexadecane) substrate was supplied using three different culture techniques: Hydrocarbon in liquid MM1 (HLM); Hydrocarbon mixed in MM1A agar (HMA); and Hydrocarbon surface -drops on MM1A agar (HDA) (Figure 4.2). HLM was prepared by adding 500 µl.L^{-1} of n-hexadecane in the liquid MM1, and HMA was prepared by adding 500 µl.L^{-1} of n-hexadecane in solid MM1 prepared (Section 4.3), before it solidified. In addition, HDA was prepared by adding 500 µl.L^{-1} of n-hexadecane in several drops on the solid MM1.

A single colony of NCIMB13064 bacterial strain as model was cultured in each of the HLM, HMA and HDA culture media and incubated in 26 °C and 155 rpm for 3 days. Growth measurements and observations were made according to colony forming units (CFU) that appear on the agar plate culture, or by the observation of turbidity and particulate bacterial cells in the liquid culture (Table 4.2). Any turbid liquid culture was sub-cultured in NA to reconfirm that the turbid solution and particulate matter present were bacterial cells.



(i) Hydrocarbon in liquid MM1 (HLM) | (ii) Hydrocarbon mixed in MM1A agar (HMA) | (iii) Hydrocarbon surface - drops on MM1A agar (HDA)

Figure 4.2 Three different cultivation method using n-hexadecane growth substrate in minimal media (MM1). Minimal media (MM1) was prepared according to protocol in Section 4.3.

4.4.4 Types of growth substrate

Strain NCIMB13064 (the bacterial model) was sub-cultured in 100 ml of liquid MM1 in a 250 ml Erlenmeyer flask and supplied with 50 µl (500 µl.L^{-1}) of hydrocarbon substrate either n-

hexadecane or n-hexane separately in triplicate. A control flask was prepared similarly without substrate. Observation of bacterial growth was carried out on the culture after 3 days of incubation by measuring the optical density at 600 nm on a UNICAM 8625 UV-VIS Spectrometer. OD readings were taken against deionised water.

4.4.5 Effect of growth substrate concentration

R. erythropolis NCIMB13064 was cultured in 100 ml liquid minimal media and supplied with 30 and 50 µl of n-hexadecane for concentrations of 300 $\mu l.L^{-1}$ and 500 $\mu l.L^{-1}$ and control (without substrate). Growth of bacteria in each substrate volume was monitored using methods of optical density measurement, and plate counts of colony forming unit (CFU). The bacterial suspension (2 ml) was taken every 3 hours and the growth was measured using a UV spectrophotometer at wavelength of 600 nm. At the same time, 50 µl of this bacterial culture was spread onto nutrient agar and incubated for 3 days for the colony count. A series of dilutions in Ringer's solution was made whenever required for a high growth sample. Agar plates with more than 300 CFU were neglected, and instead a plate from a diluted sample with less than 300 CFU was used for enumeration.

4.4.6 Growth monitoring of bacterial strains DSM311 and NCIMB13064

The *R. erythropolis* NCIMB13064 and DSM311 were cultured in 100 ml liquid (MM1) and added with n-hexadecane (for 300 $\mu l.L^{-1}$ and 500 $\mu l.L^{-1}$) and growth monitoring experiment was conducted with the same protocol described in Section 4.4.5.

4.4.7 Microscopic observation

R. erythropolis NCIMB13064 and DSM311 were cultivated separately in 10 ml of minimal media with 5 µl (500 $\mu l.L^{-1}$) of n-hexadecane and incubated for 3 days in 26 °C and 155 rpm. Then 10 % of formalin was added to the bacterial culture and left for 1 day to inhibit the bacterial growth. 5 µl of the inhibited bacteria were then transferred onto a glass slide and observed under a fluorescent microscope, model Nikon ECLIPSE Ci, equipped with QImaging software. Observations were made under normal light because the bacteria were from a pure culture and all the uniformly rod-shaped images observed were considered to be bacteria cells.

4.5 Results and discussion - Adaptation of pure bacterial strains

After three days of incubation, only 2 bacterial strains grew on the nutrient agar (NA) plates (Table 4.2), namely *R. erythropolis* NCIMB13064 and DSM311. No sign of growth were found in the NA plates of bacterial strains *R. erythropolis* NCIMB10422, NCIMB11148, NCIMB9905 and NCIMB9409. The hydrocarbon adaptation process was therefore carried forward only with the two viable strains.

Table 4.2 Observation of bacterial growth for pure cultures of *R. erythropolis* (different strains) in NA after 3 days of incubation in 26 °C. All bacterial strains were obtained as freezed-dried cultures.

Bacterial strain	Sign of bacterial growth
NCIMB13064	Presence of bacterial colonies
NCIMB10422	No colony formation
NCIMB11148	No colony formation
NCIMB9905	No colony formation
NCIMB9409	No colony formation
DSM311	Presence of bacterial colonies

4.5.1 Preliminary study of cultivation on n-hexadecane

The substrate supply study was conducted using 3 different methods, with n-hexadecane as the model substrate. In order to determine the best cultivation method, the presence of bacterial growth (*R. erythropolis* NCIMB13064) in each method was followed (Table 4.3). After 3 days of incubation, presence of suspended particulate matter (presumed to be bacterial cells) and culture turbidity were observed in the HLM culture, and some colonies of bacteria appeared in the HMA culture. However, bacterial colony growth was not seen in the HDA culture. These observations indicate that the HLM method allows bacteria to access the hydrocarbon efficiently, through the possible pathways postulated by previous studies (Noordman and Janssen, 2002; Hua and Wang, 2014). The pathways reported are: uptake of dissolved hydrocarbon (DH); direct contact of cells to large hydrocarbon drops (DCL); direct contact of cells to submicron-sized of hydrocarbon droplets (DCS); and biosurfactant-mediated uptake (BM). Usually, direct contact between hydrophobic compounds such as large hydrocarbon drops (DCL) and the hydrophobic region of a bacteria cell is prevented by the bacterial outer cellular membrane which is usually hydrophilic (Sikkema *et al.*, 1995). However, hydrocarbon-degrading bacteria overcome this barrier by adapting their outer membrane component, giving them an increased cell surface hydrophobicity (CSH). Consequently, adapted strains have higher CSH and are competent to uptake the hydrocarbon directly from droplets in the media (DCL). These adapted bacteria could also access the dissolved hydrocarbon (DH) and submicron-sized hydrocarbon droplets (DCS) as well, because the orbital shaker promotes high dissolution of hydrocarbon into the media, and allows dispersion of hydrocarbon into fine colloidal droplets in the aqueous media. Under the microscope, bacteria appear at the hydrocarbon: water interface. Physical dispersion of the hydrocarbon creates a greater surface

area and allows for more contact with the growing bacteria (Ławniczak *et al.*, 2011). The liquid culture method also allows the bacteria to produce biosurfactant (for BM), which lowers the interfacial tension between the immiscible surface of the hydrocarbon and water (Diniz Rufino *et al.*, 2014), facilitating adhesion of bacterial cells to the hydrophobic hydrocarbon (Mishra and Singh, 2012). The existence of the biosurfactants was assumed due to the appearance of a turbid milky white emulsion early in the growth period, which then developed into a more off-white opaque dispersion as the bacterial cells grew.

Table 4.3 Observation of growth of *R. erythropolis* NCIMB13064 according to the methods of cultivation on n-hexadecane.

Method of cultivation (substrate supply)	Observations	Probable substrate uptake pathways*
Hydrocarbon in liquid MM1 (HLM)	Presence of suspended particulate matters (PM) / bacterial cells and turbid culture. Sub-culturing of culture in NA confirmed the PM as bacterial cells, as they grew in the agar.	DH, DCL, DCS and BM
Hydrocarbon mixed in MM1A agar (HMA)	Presence of bacteria detected in bacterial colonies.	Restricted amount of DH
Hydrocarbon surface - drops on MM1A agar (HDA)	No bacterial colony formation	DCL, with poor asses to nutrients beneath the agar.

* DH: dissolved hydrocarbon; DCL; direct contact of cells to large hydrocarbon drops; DCS: direct contact of cells to submicron-sized of hydrocarbon droplets; BM: biosurfactant-mediated uptake. Observation was made after 3 days of culture in 26 °C and 155 rpm incubator.

However, routes for hydrocarbon uptake in hydrocarbon mixed in MM1A agar (HMA) were limited. The degree of growth in the HMA was less due to small amount of dissolved hydrocarbon (DH) that was available to bacteria (diffusion only), compared to HLM cultures (diffusion, dispersion and solubilisation of hydrocarbon). Moreover, zero growth was observed in HDA because the direct contact between the bacterial cell and hydrocarbon droplet was the only uptake mechanism, and generated relatively toxic concentrations. Also, the bacteria did not have good access to both the hydrocarbon on the agar surface, and the aqueous nutrients beneath the agar surface, at the same time. Therefore, it was concluded that subsequent adaptation experiments would be best conducted by adopting liquid cultivation such as the HLM method.

4.5.2 Types of growth substrate

Figure 4.3 shows growth of *R. erythropolis* NCIMB13064 in minimal media supplemented with different types of hydrocarbon, and reveals that significant differences in bacterial growth (optical density) ($p < 0.05$), one-way ANOVA). In addition, through a Post-hoc (Dunnette t-test) analysis (Table 4.4), use of n-hexadecane as growth substrate was confirmed to significantly increase ($p < 0.05$) the strain NCIMB13064 bacterial growth (OD: 1.129 ± 0.007) compared to the control (OD: 0.085 ± 0.009). However, addition of n-hexane (same amount) to the culture was found to bring no effect ($p > 0.05$) to the bacterial growth (OD: 0.084 ± 0.005).

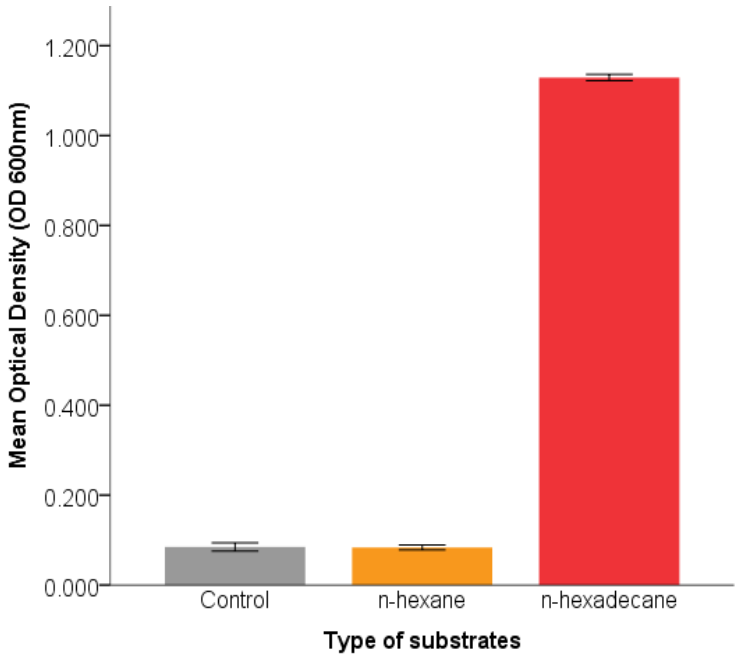


Figure 4.3 Growth of *R. erythropolis* NCIMB13064 (as optical density, OD^{600nm}) on two growth substrates after 3 days of incubation.

Table 4.4 Optical density for a culture of *R. erythropolis* NCIMB13064 in minimal media supplied with different growth substrates, and statistical analysis of the effect of substrate type on bacterial growth (in terms of optical density).

Statistical analysis	**Substrate**		
	Control	Hexane	n-hexadecane
Optical Density (Mean)	0.085 ± 0.009	0.084 ± 0.005	1.129 ± 0.007
Post Hoc test* (Significant value, p)		0.981	0.000

* Comparison of substrate with control

Furthermore, neither visual bacterial cell suspensions nor change in optical density was observed in the culture grown in n-hexane. Additions of different amounts of n-hexane (250 – 2000 µl.L^{-1}) to the culture was also carried out to find its optimum concentration, in case the initial concentration was either insufficient or excessive for bacterial growth. However, no visual signs of growth were observed at any of these concentrations (Figure 4.4) as shown by the fact that no increment of optical density took place in any of the culture flasks. The numerical results from varying the substrate concentration are shown in Figure 4.5.

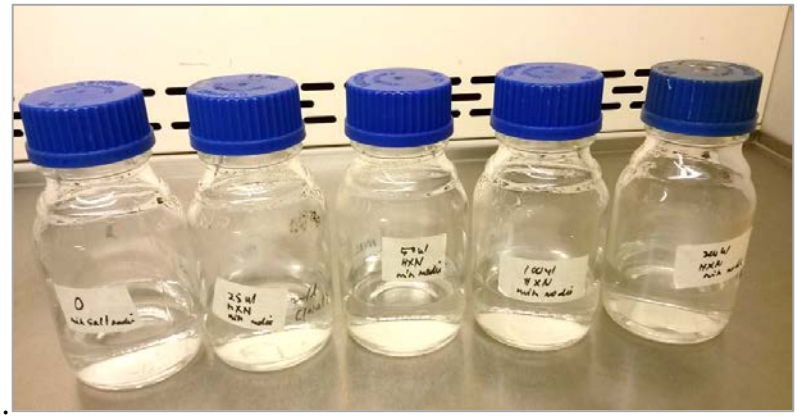


Figure 4.4 Culture of *R. erythropolis* NCIMB13064 in MM1 supplied with different n-hexane concentration (250 µl.L^{-1} to 2000 µl.L^{-1}) after 3 days of incubation.

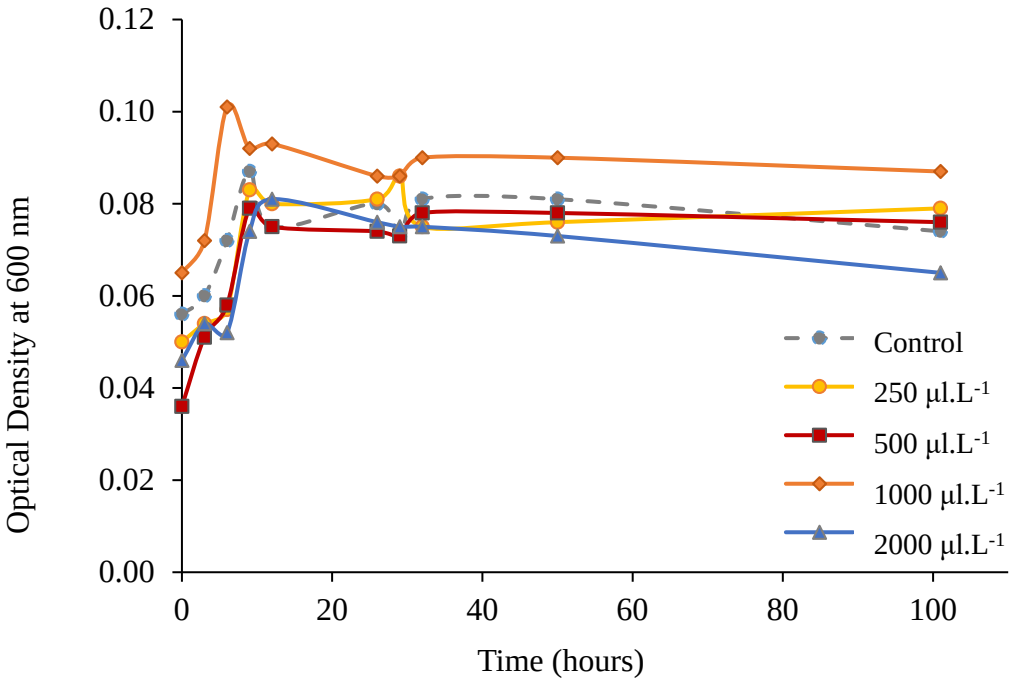


Figure 4.5 Growth of *R. erythropolis* NCIMB13064 in 100 ml of MM1 liquid medium and 250 to 2000 µl.L^{-1} of n-hexane growth substrate. Bacteria were enumerated for measurement of optical density at each time point using a spectrophotometer.

In contrast, there was strong evidence of growth in the culture of strain NCIMB13064 in higher amounts of n-hexadecane (Figure 4.6 and 4.7). The visual turbidity of the bacterial culture was observed to increase with increases in n-hexadecane concentration (Figure 4.6), as well as the optical density (Figure 4.7). The growth pattern (increase of OD) in Figure 4.7 indicates that the use of a suitable hydrocarbon, such as n-hexadecane, can successfully support bacterial growth, in contrast to n-hexane.

Figure 4.6 Cultures of *R. erythropolis* NCIMB13064 in MM1 supplied with different n-hexadecane concentrations (250 µl.L^{-1} to 2000 µl.L^{-1}) after 3 days of incubation.

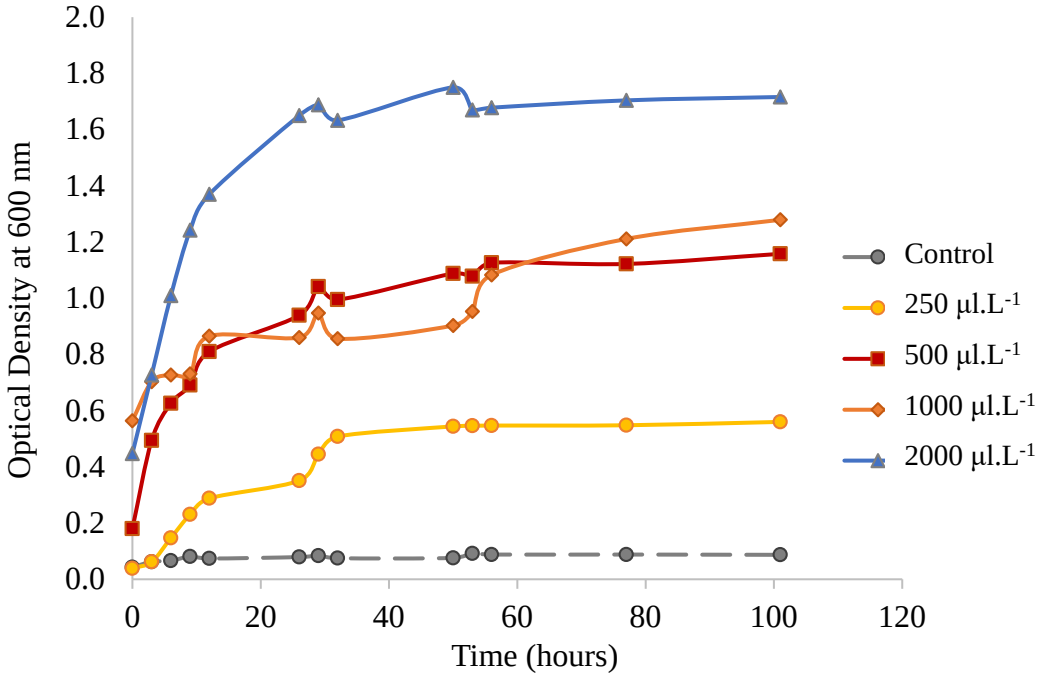


Figure 4.7 Growth of *R. erythropolis* NCIMB13064 in 100 ml of MM1 liquid medium and 250 to 2000 µl.L^{-1} of n-hexadecane growth substrate. Bacteria were enumerated at each time point using a spectrophotometer at 600 nm.

However, compared to a previous study, Stancu (2014) found that *R. erythropolis* (strain $IBBP_{o1}$ isolated from crude oil-contaminated soil) could grow on n-hexane. However, the cell surface hydrophobicity (CSH) of their adapted strain was found to be very low (29 %), which confirms that n-hexane was not an efficient growth substrate for the hydrophobic-adaptation of bacteria.

In contrast, *R. erythropolis (*strain DCL14) was found to utilize n-hexadecane well, and adapted into highly hydrophobic cells (CSH of 92 %) (de Carvalho *et al.*, 2009). Moreover, culture of strain DCL14 in shorter chain n-alkanes, such as n-hexane, produced very low CSH compared growth on longer chain hydrocarbons. This confirms that n-hexane is not an efficient substrate for initiating hydrophobic-adaptation, especially for strain NCIMB13064 which gave extremely poor growth on n-hexane compared to strains $IBBP_{o1}$ and DCL14. Therefore, the longer-chain hydrocarbon of n-hexadecane was confirmed as the most suitable growth substrate for bacterial strain NCIMB13064. In theory, n-hexadecane should be a suitable substrate to induce hydrophobic-adaptation of bacteria, due to its high octanol-water partition coefficient (log P_{ow}) of 8.8, compared to 3.50 of the n-hexane (Geok *et al.*, 2003). The high log P_{ow} of n-hexadecane shows that it has higher water repelling characteristics compared to hexane, which suggests that it might be a more favourable substrate for the bacteria to grow on. Furthermore, midsize n-alkanes (C10 to C18 in length) such as n-hexadecane, have been proven to be more readily available growth substrates than n-alkanes with either longer or shorter chains (Pepper *et al.*, 2014) including n-hexane (C6).

The most important finding was that a short-chain aliphatic alkane (<C10) such as n-hexane (Table 4.5) was toxic for the microbial species due to its higher water solubility, notably 9.50 $mg.L^{-1}$ compared to a very low value of 9×10^{-4} $mg.L^{-1}$ for n-hexadecane (C16). The n-hexane would therefore be more toxic because its high water solubility would allow it to dissolve more fully in the aqueous phase, and then to readily access the microbial cell membrane, and ultimately alter the membrane fluidity, causing the bacterial cell to lose its integrity (Tarradellas *et al.*, 1996; Pepper *et al.*, 2014). This means, bacterial cells would be more likely to be disrupted and would be less able to grow. These chemical properties explain why the *R. erythropolis* NCIMB13064 grew well in a substrate of n-hexadecane (C16 chain) but were inhibited from growing in n-hexane (C6). It was concluded that, in future experiments, the bacteria should be grown only in n-hexadecane as substrate, and not in hexane. Therefore, n-hexadecane was utilised to study the effect of substrate dosage on the growth of bacteria in subsequent experiments.

Table 4.5 Physicochemical properties of the hydrocarbons used as growth substrates for the adaptation of bacteria.

Substrate	Formula	Molecular weight ($g.mol^{-1}$)	Octanol-water partition coefficient (log P_{ow})	Solubility ($mg.L^{-1}$)
n-hexadecane	$C_{16}H_{34}$	226.44	8.80 [a]	9.00×10^{-4} [b]
n-hexane	C_6H_{14}	86.178	3.50 [a]	9.50 [c]

References: [a] Geok *et al.* (2003); [b] Stroud *et al.* (2007); [c] Sigma-aldrich (2016)

4.5.3 Effect of growth substrate concentration

The objective was to investigate different growth substrate concentrations which supported the greatest level of bacterial growth. A minimum concentration of substrate for good growth bacteria was sought in order to minimise the cost of producing the bacterial biomass that might be used subsequently as an adsorbent for estrogens in a full-scale process, particularly to improve the chance of it being a cheaper adsorbent material than activated carbon.

From Figure 4.7, the growth of bacteria for 500 $\mu l.L^{-1}$ and 1,000 $\mu l.L^{-1}$ of substrate showed very similar results, which indicates that 500 $\mu l.L^{-1}$ was an adequate substrate concentration to be investigated further. In addition, a substrate concentration of 2000 $\mu l.L^{-1}$ was not considered optimal because it add substrate cost compare to the 500 $\mu l.L^{-1}$ yet improve cell yield by less than 50 % (only a 0.5 OD increase).

The best substrate concentration was further investigated by culturing the strain NCIMB13064 in n-hexadecane at 0, 300 and 500 $\mu l.L^{-1}$ (Figure 4.8). Growth of the bacteria was found to increase rapidly from three days of incubation onwards. Significant increases ($p < 0.05$) in numbers of colony forming units (CFU) were found between the substrate dosages at day 3 to day 5 of the incubation period (Table 4.6). The highest bacterial growth was observed in the culture of strain NCIMB13064 in 500 $\mu l.L^{-1}$ of substrate dosage (n-hexadecane) compared to the 300 $\mu l.L^{-1}$ dose and the control. This confirms that the growth of strain NCIMB13064 was significantly affected by the dose of substrate added to the culture. Therefore, the alternative hypothesis is accepted.

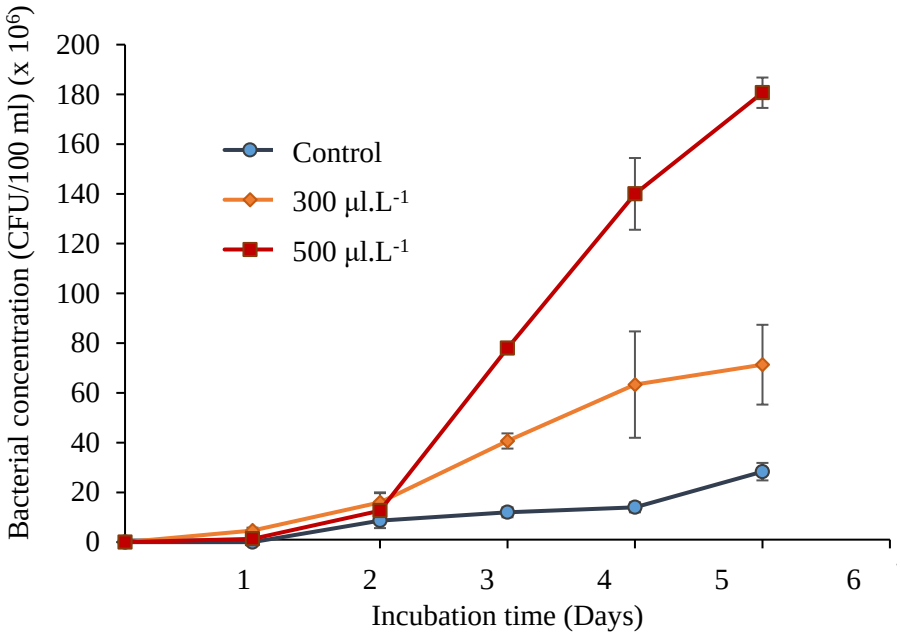


Figure 4.8 Growth of strain NCIMB13064 in 100 ml of liquid MM1 medium containing 300 µl.L^{-1} or 500 µl.L^{-1} of n-hexadecane growth substrate. The Control was prepared without substrate. Bacteria were enumerated at each time point point as CFU per 100 ml using NA plate.

Table 4.6 Mean colony forming units (CFU) of *Rhodococcus erythropolis* NCIMB13064 grown at different substrate (n-hexadecane) concentrations. Analysis of bacterial growth was made between the substrate dosages.

Substrate dosage	**Bacterial growth, CFU (10^6)**				
	Day 1	Day 2	Day 3	Day 4	Day 5
Control[1]	0.00^{a}	8.67^{a}	12.00^{a}	14.00^{a}	28.33^{a}
300 µl.L^{-1}	4.67^{b}	12.67^{a}	40.67^{b}	63.33^{b}	71.33^{b}
500 µl.L^{-1}	1.33^{a}	16.00^{a}	78.00^{c}	140.00^{c}	180.67^{c}

$^{a, b, c}$ Means that do not share a letter are significantly different ($p < 0.05$), but these comparisons are only valid within each day and not valid between days.

[1] Control: culture containing bacteria without substrate.

4.5.4 Relationship between CFU and optical density methods of enumeration

It should be noted that the growth of bacteria in the optimum substrate concentration study (Section 4.4.5) was measured using colony forming units instead of optical density. The reason for monitoring the bacterial growth in CFUs was to eliminate the possibility that hydrocarbon-degrading bacteria could have increased their hydrocarbon uptake during the cultivation by producing the biosurfactants, which could also have contributed a turbidity characteristics of the bacterial culture by producing emulsions of the growth substrate with similar optical properties as the bacterial suspensions (Section 2.9.1). This theory can also be supported by the observation that turbidity did not appear in the no-substrate control flask because the bacteria could not initiate the production of biosurfactants without the presence of n-hexadecane. The emulsified substrate molecules and biosurfactant might produce a milky appearance in the growth medium making the optical density reading a less reliable method of enumerating bacterial cells. A biosurfactant, possibly trehalose tetraesters (Figure 4.9) (Petrikov *et al.*, 2013) that was present in the culture of *R. erythropolis* using n-hexadecane was a cell-bound molecule. Therefore the amount of biosurfactant increased proportionally with bacterial growth; however, the turbidity does not represent the actual growth of cells directly (Figure 4.10). Furthermore, the bacterial cells were observed to begin aggregating as clumps after 4 to 5 days of the incubation. At this point, the aggregates would slightly reduce the scattering of bacteria relative to the same amount of biomass as single dispersed cells, leading to a lower optical density measurement. Thus, the measurement of CFUs was considered to be more reliable for accurate monitoring of bacterial growth.

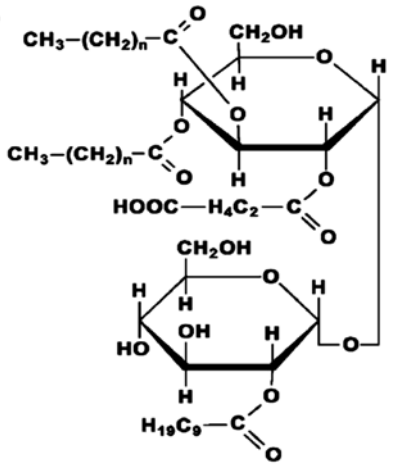


Figure 4.9 Structure of glycolipid biosurfactant trehalose tetraesters produced by *Rhodococcus* sp. bacteria during the cultivation of the bacteria with hexadecane growth substrate (Petrikov *et al.*, 2013).

The precise relationship between culture optical density and CFU is shown in Figure 4.10, and shows a good correlation with a linear regression of $R^2 = 0.8467$. This implies that the optical density is not perfectly proportional to CFU but is a fair estimate of it, and can serve as a good working estimate of bacterial growth (Section 4.5.2). However, the OD value cannot be used as an accurate parameter in a detailed investigation such as studies on bacterial growth phases, in which case CFU measurement is deemed to be a more reliable measurement of bacterial biomass for future experiments.

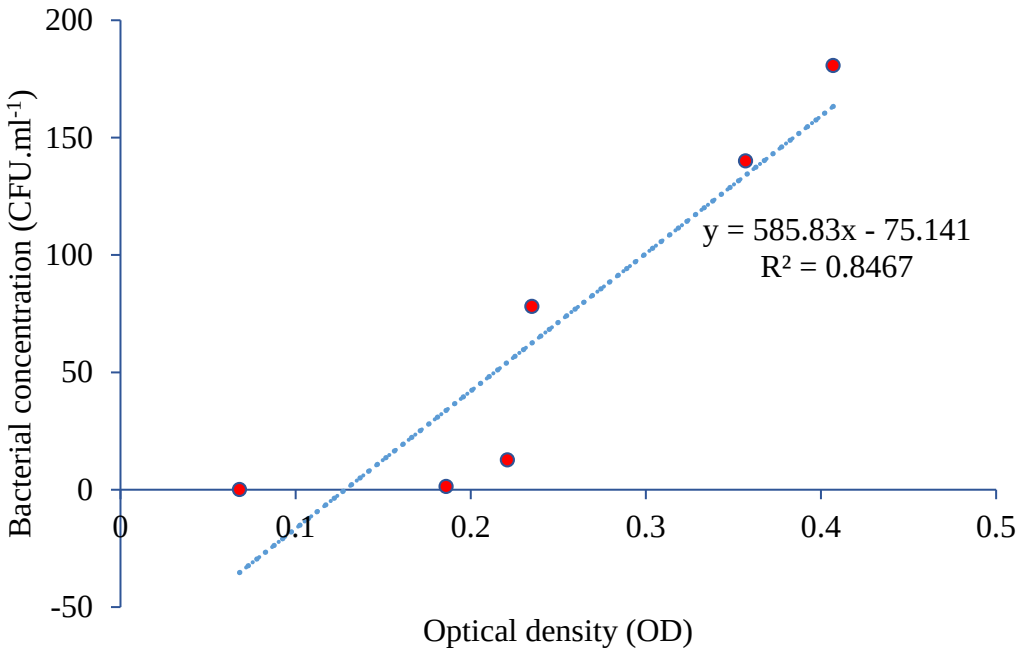


Figure 4.10 Relationship between bacterial concentration (CFU.ml^{-1}) and optical density (OD at 600 nm) for *Rhodococcus erythropolis* NCIMB13064 grown for 5 days with 500 µl.L^{-1} hexadecane.

4.5.5 Bacterial growth phase

Rhodococcus erythropolis NCIMB13064 and DSM311 were grown for five days, and daily enumeration of the CFU made using NA as described in Section 4.5.3. As the experiment proceeded severe aggregation appeared as predicted. Therefore further CFU measurement was not conducted after 5 days, due to the presence of bacterial clumps, and actual bacterial growth could not be measured either from optical density or CFU measurement. Similarly, the use of flow cytometer for total numbers of bacterial cells for growth monitoring was also not suitable because the bacteria cells could not be dispersed properly, even though bacterial dispersion techniques by vortex spinning, addition of surfactants, such as Triton X-100, 100, Tween 80 and sonication for up to 5 hours were carried out. Furthermore, the hydrophobic aggregated cells could severely block the flow cytometer machine, and therefore its use was avoided.

From the earlier CFU growth monitoring, strain NCIMB13064 gave its optimum growth at 500 µl.L^{-1} of substrate. Its exponential growth phase appeared to occur around day 3 to day 5 (Figure 4.11) and the stationary phase was presumed to occur after day 5 of incubation. Equally, measurements of CFU for bacterial strain DSM311 (Figure 4.12) were only conducted for 4 days due to the severe aggregation of bacterial cells. These cell aggregations occurred because hydrocarbon-degrading bacteria increase hydrocarbon uptake by producing extracellular polymeric substance (EPS), which was a sticky substance that bridges between the bacterial cells and hydrocarbon droplets, allowing direct access to the hydrocarbon by the bacterial cell (Section 2.9.2). The strain DSM311 was found to have similar trend of growth to that of strain NCIMB13064, with exponential growth phase between day 3 and day 5, and the stationary phase was also presumed to occur after day 5. Similar to strain NCIMB13064, its optimum growth substrate was also found to be 500 µl.L^{-1} of n-hexadecane.

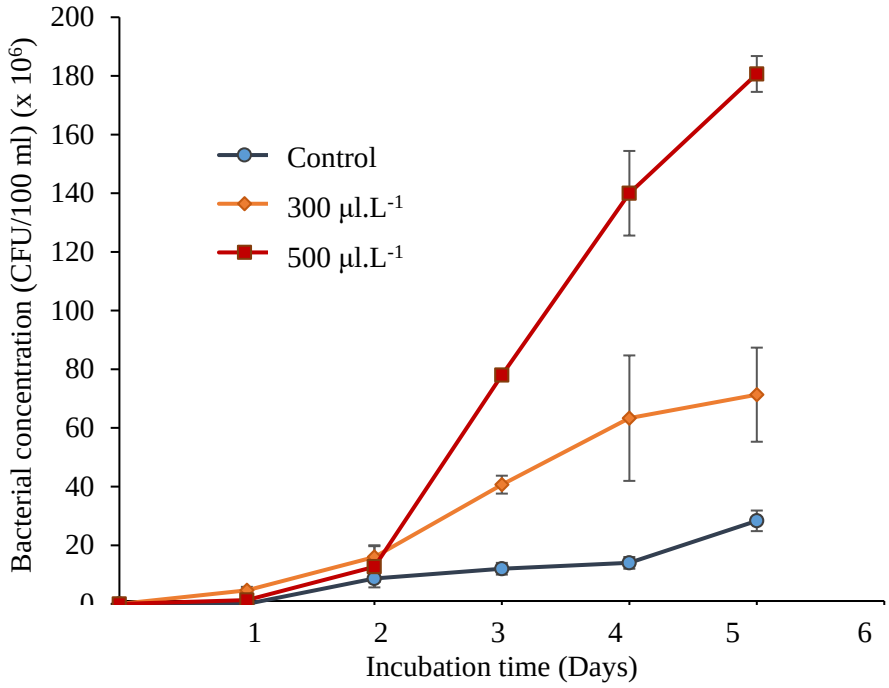


Figure 4.11 Growth curve of *Rhodococcus erythropolis* NCIMB13064 cultivated in liquid medium supplied with 300 µl.L^{-1} and 500 µl.L^{-1} of n-hexadecane growth substrate and control without substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates.

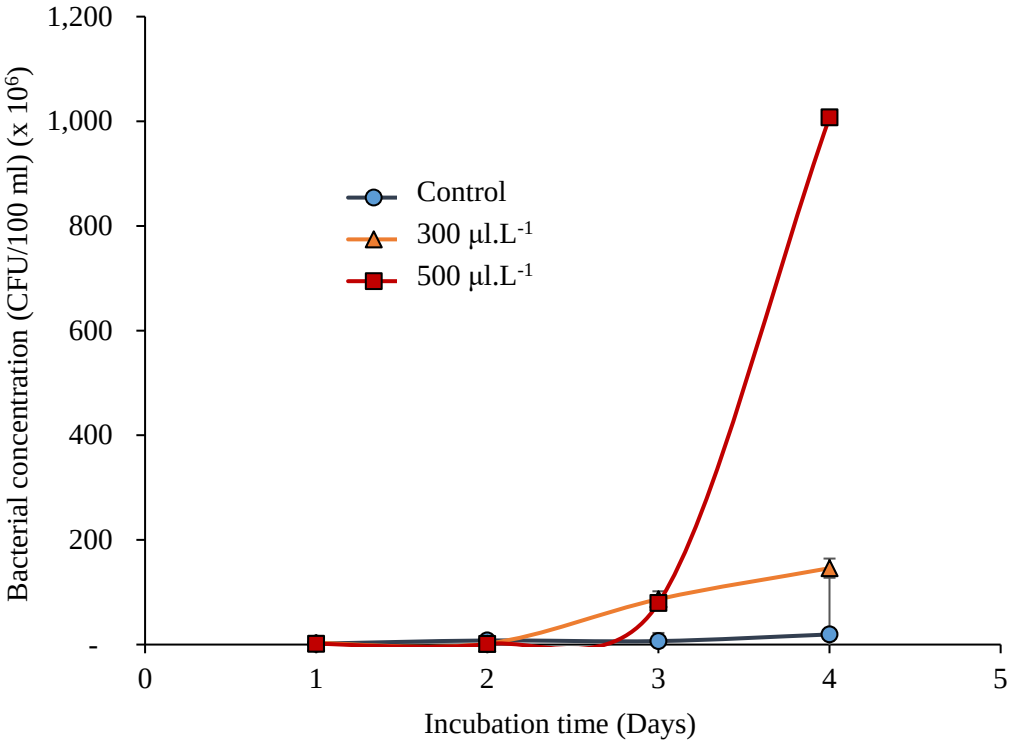


Figure 4.12 Growth curve of strain *Rhodococcus erythropolis* DSM311 cultivated in liquid medium supplied with 300 µl.L^{-1} and 500 µl.L^{-1} of n-hexadecane growth substrate and control without substrate. Bacteria were enumerated at each time point point as CFU per 100 ml using NA plates.

4.5.6 Morphology image for the adapted bacteria

Through morphological observations, both of the bacterial strains NCIMB13064 and DSM311 appeared as rod-shaped cells, which was expected as they were known to be *Rhodococcus erythropolis* (Figure 4.13). Moreover, size of bacterial cells strain NCIMB13064 and DSM311 were measured to be in the ranges of 1.5 - 2.5 µm and 1.0 - 2.0 µm, respectively. Strain DSM311 was observed to grow in large clusters, which was presumed to be related to the presence of EPS. However, this effect was less prominent in the strain NCIMB13064.

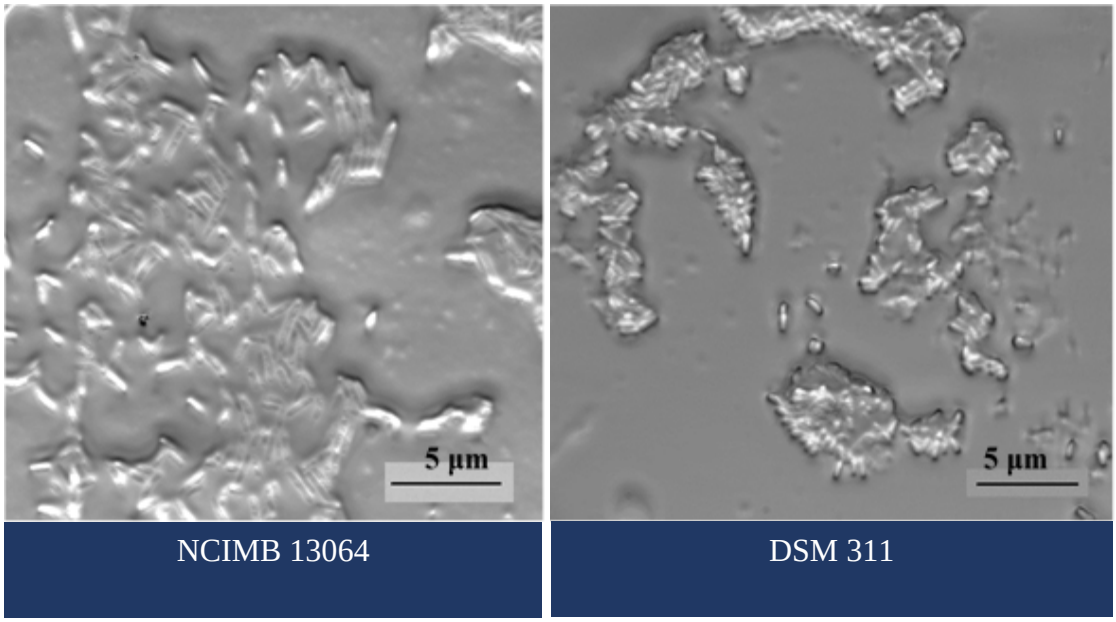


Figure 4.13 Images of the adapted *R. erythropolis* NCIMB13064 and DSM311 cultivation using MM1 media with n-hexadecane (500 µl.L^{-1}) growth substrate, observed under 1000 times magnification using fluorescent microscope.

4.6 Methodology - Isolation of bacteria from soil

Samples of diesel-contaminated soils were used to isolate hydrophobic microorganisms living in oily soil conditions and adapted to the hydrophobic environment it represents. The soils were collected from two points in Cockle Park farm, Morpeth. Placed in sample collection bags labelled as Soil 1 and Soil 2 which were kept for not more than 1 week at room temperature (22 ± 2 °C). The isolation of bacteria was conducted according soil-enrichment technique as summarised in Figure 4.14.

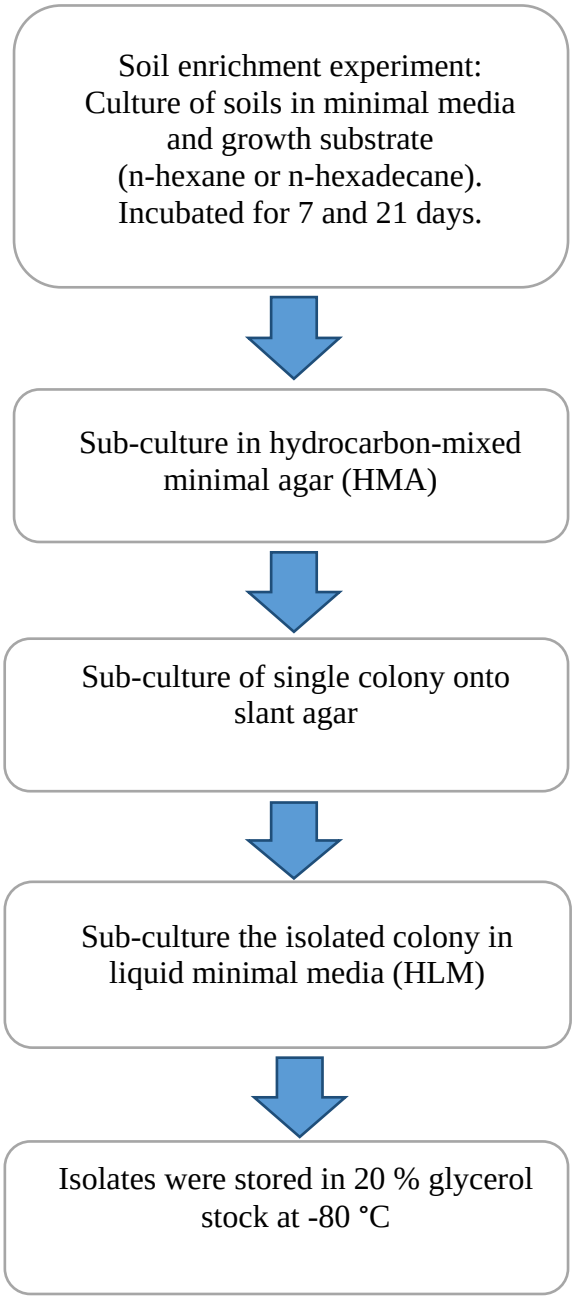


Figure 4.14 Experimental steps for the isolation of hydrophobic bacterial species from diesel-contaminated soil through soil enrichment technique.

The isolation process was carried out by culturing 50 g (field moist weight) of each soil sample in 100 ml of liquid minimal media (MM1) supplied with 500 µl.L^{-1} of n-hexane or n-hexadecane in 250 ml Erlenmeyer flasks at 26 ºC and 155 rpm in an orbital shaker incubator. After 7 and 21 days of incubation, samples of the enrichment culture were taken using a sterilized inoculation loop, and sub-cultured (streaked) in a hydrocarbon-mixed agar (HMA) containing n-hexadecane or n-hexane, similar to the cultivation technique in Section 4.4. The HMA was used in this experiment even though it was not the best medium for efficient hydrocarbon uptake, however this culture technique was suitable for separation of single bacterial colonies capable of direct growth on the hydrocarbon. The streaked agar cultures were incubated in 26 ºC for 5 days to obtain visible colonies. All non-identical (visually) single colonies that grew on the agar plate were then picked and sub-cultured in slant agar tubes prepared beforehand with the same solid MM1 media and substrates (4.3). The cultures were incubated in 26 °C for 5 day, and pure bacterial colonies which appeared in the slant agar were then sub-cultured in liquid MM1 with substrates of either n-hexane or n-hexadecane for 3 days. 1 ml of each bacterial culture was then preserved in 20 % glycerol stock at -80 °C and all isolates were labelled. The experimental work is summarised in Figure 4.15.

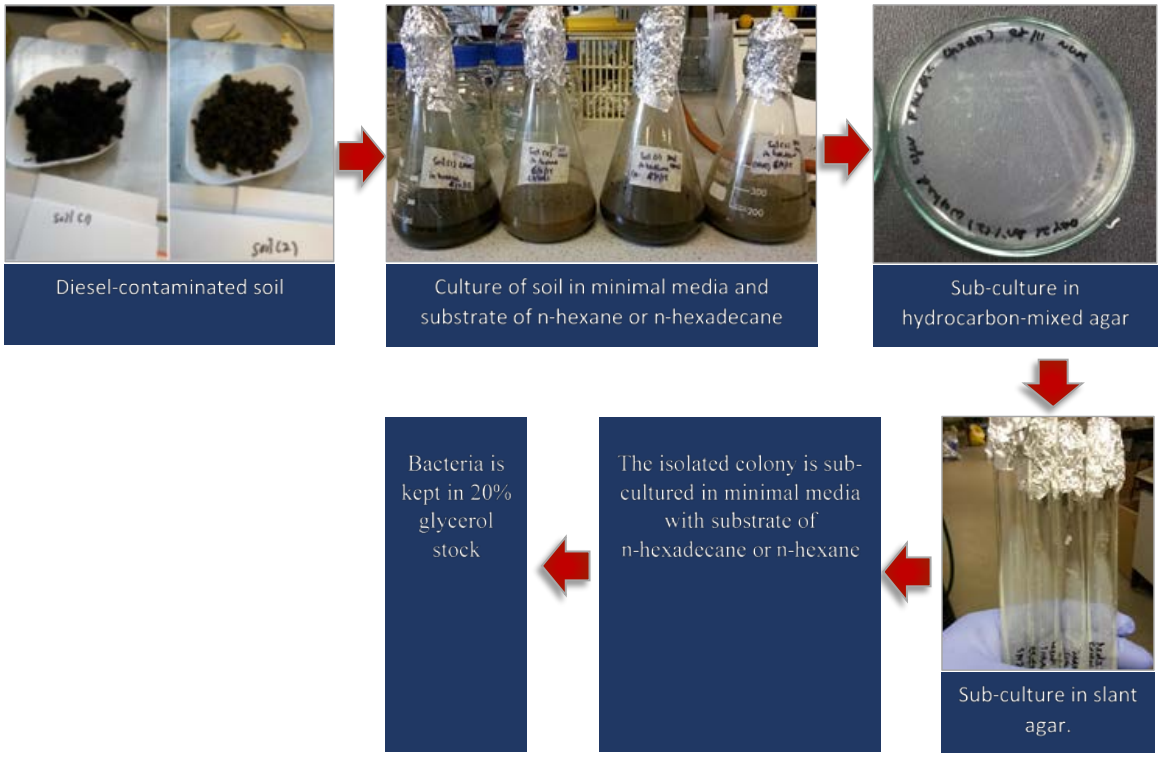


Figure 4.15 Experimental steps for the isolation of hydrophobic bacterial cells from diesel-contaminated soils using soil-enrichment technique.

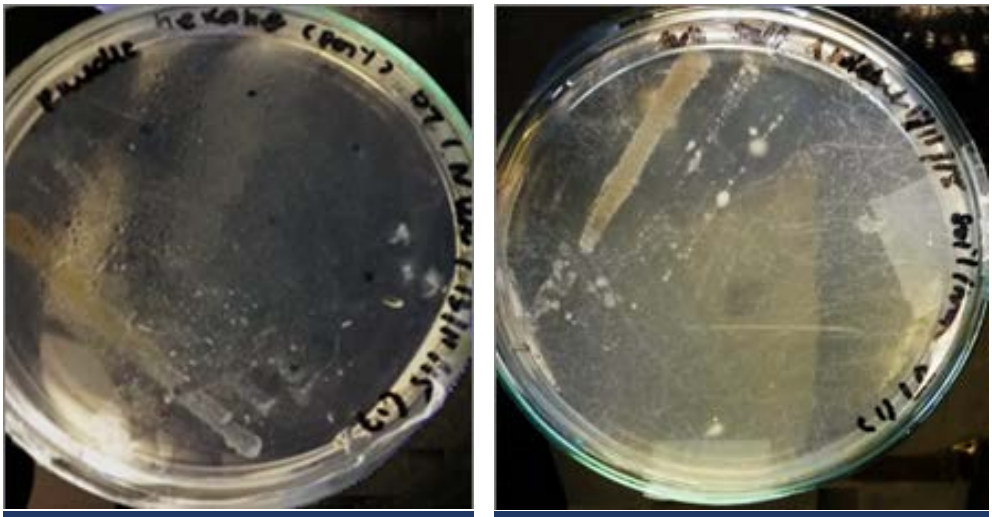
Further investigations for optimum substrate dosage and growth monitoring were carried out for the soil-isolates strains using protocols similar to the pure bacterial strains (Section 4.4.6.).

4.7 Results and discussion - Isolation of bacteria from soil

Using soil-enrichment technique, five potentially different strains of bacteria were successfully isolated. Three strains were isolated after 7 days of soil enrichment and another two were isolated after 21 days. Details of the findings are described and discussed in the subsequent sections.

4.7.1 Types of substrate

Growth of bacterial colonies were found in soil enrichment cultures supplied with n-hexadecane substrate, but no sign of growth was found using n-hexane substrate (Figure 4.16). These results are similar to those in the adaptation of pure bacterial strains (Section 4.5.2), which indicates that only n-hexadecane was found to be suitable growth substrate. Bacterial strains isolated after 7 days of enrichment were labelled as soil-isolates PD2-1, PD2-2 and PD2-3, whereas the 21 days-isolated strains were recorded as SD1-1 and SD2-1 (Table 4.7).



(i) Sub-culture of soil in n-hexane HMA

(ii) Sub-culture of soil in n-hexadecane HMA

Figure 4.16 Culture of diesel-contaminated soils in hydrocarbon-mixed agar (HMA) with (i) n-hexane and (ii) n-hexadecane substrates, for the separation of single colonies of new bacterial strains.

Table 4.7 Bacterial strains isolated from diesel-contaminated soils using enrichment culture added with hydrocarbon substrates in 7 or 21 days of incubation period.

Isolate code	Growth substrate	Soil-enrichment incubation period
PD2-1	n-hexadecane	7 days
PD2-2	n-hexadecane	7 days
PD2-3	n-hexadecane	7 days
SD1-1	n-hexadecane	21 days
SD2-1	n-hexadecane	21 days

4.7.2 Optimum concentration of growth substrate

The optimum substrate concentration was determined for each of the soil-isolates by monitoring growth of the isolates in minimal media supplied with different n-hexadecane concentrations (0 - 500 µl.L^{-1}). Bacterial concentration, as numbers of the bacterial colonies (CFU), growing in each of the culture were analysed statistically using one-way ANOVA (Table 4.8).

Table 4.8 Growth (CFU per 100 ml) of the soil-isolate bacterial strains in MM1 supplied with different n-hexadecane growth substrate concentrations after 5 days of incubation.

Substrate concentration (hexadecane)	Bacteria growth (CFU) after 5 days incubation				
	PD2-1	PD2-2	PD2-3	SD1-1	SD2-1
Control (no substrate)	60^{b}	1,000^{c}	200^{b}	60^{c}	0^{b}
300 µl.L^{-1}	31,200^{a}	8,000^{b}	24,200^{a}	2,990^{b}	2,950^{a}
500 µl.L^{-1}	29,710^{a}	56,000^{a}	32,800^{a}	7,980^{a}	4,140^{a}

$^{a, b, c}$ Means that do not share a letter are significantly different ($p < 0.05$), but these comparisons are only valid within each strain and not valid between strains.

All of the soil-isolates were found to have better growth in the minimal media supplemented with n-hexadecane compared to control (contained the inoculation culture of bacteria in minimal media without substrate) after 5 days of incubation. The bacterial growth in both of 300 µl.L^{-1} and 500 µl.L^{-1} concentrations were determined to be significantly ($p < 0.05$) higher than controls. This indicates that n-hexadecane was a suitable carbon source that can be accessed by all of the bacterial strains, because they had been exposed to it during the enrichment process and they were considered to have successfully undergone induction of the alkane hydroxylase enzyme (Tebyanian *et al.*, 2013; Chiciudean *et al.*, 2017). Moreover, the

isolated bacteria were also predicted to be hydrophobic strains as they had survived in hydrophobic environments (diesel saturated soil).

In theory, higher substrate dosage should enhance the bacterial growth, unless an excessive amount has inhibited it. Therefore, additions of higher concentrations of n-hexadecane (500 $\mu l.L^{-1}$) into the culture significantly improved growth of strains PD2-2 and SD1-1 compared to 300 $\mu l.L^{-1}$. However, isolates of strains PD2-1, PD2-3 and SD2-1 were found to give no statistically similar growth at 300 $\mu l.L^{-1}$ and 500 $\mu l.L^{-1}$ of n-hexadecane (Table 4.8). Therefore, to maintain the same culture conditions, and without compromising their growth efficiencies, all of the bacterial strains were subsequently grown on 500 $\mu l.L^{-1}$ of n-hexadecane in future experiments.

4.7.3 Growth phase of the soil-isolate bacteria

Results from growth of the soil-isolate bacterial strains PD2-1, PD2-2, PD2-3, SD1-1 and SD2-1 in minimal media supplemented with different n-hexadecane concentrations including controls (no substrate) are presented in Figure 4.17 - Figure 4.21.

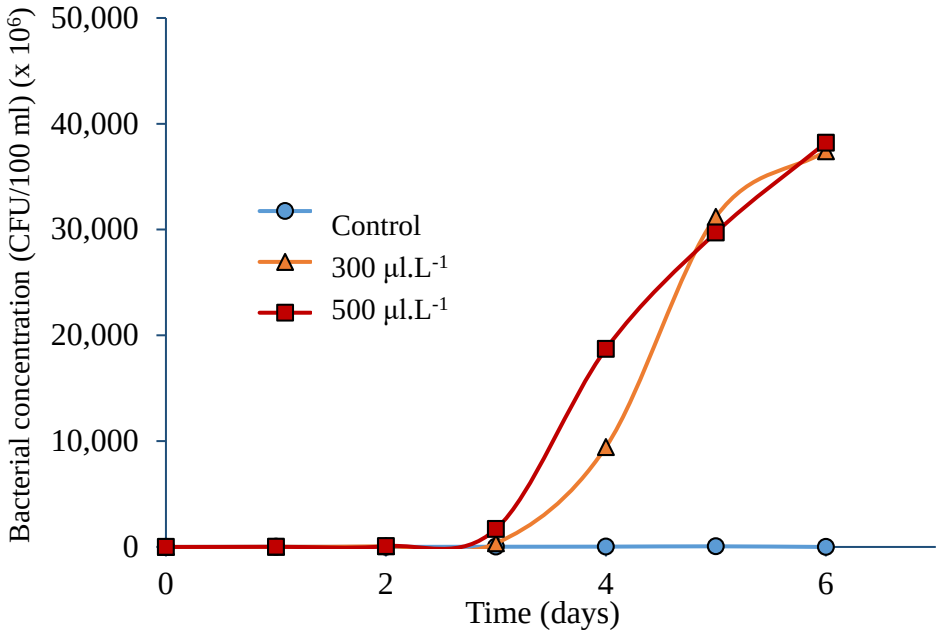


Figure 4.17. Growth curve of soil isolate PD2-1 in 100 ml of MM1 liquid medium and n-hexadecane growth substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates.

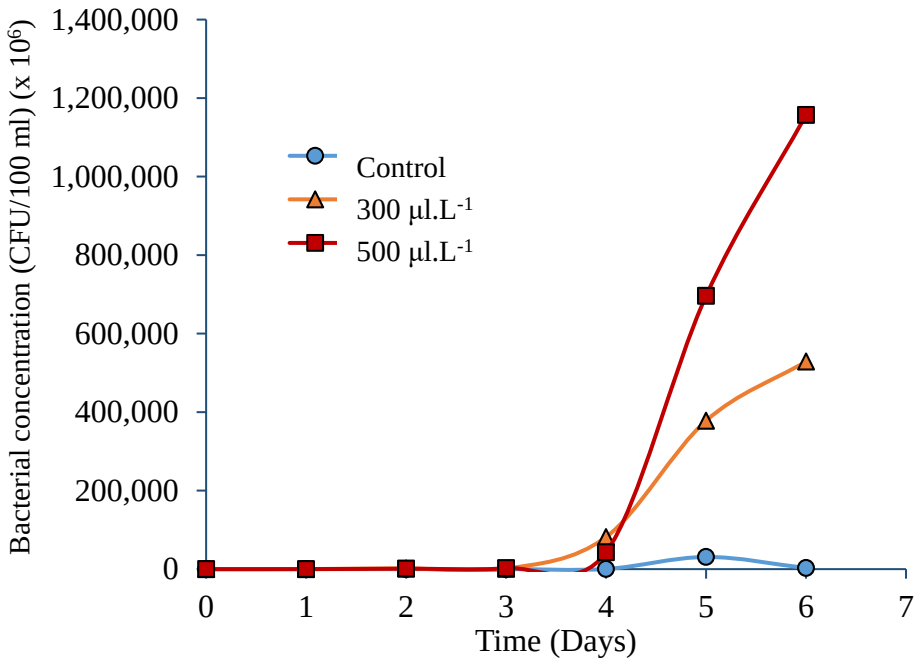


Figure 4.18 Growth curve of soil isolate PD2-2 in 100 ml of MM1 liquid medium and n-hexadecane growth substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates.

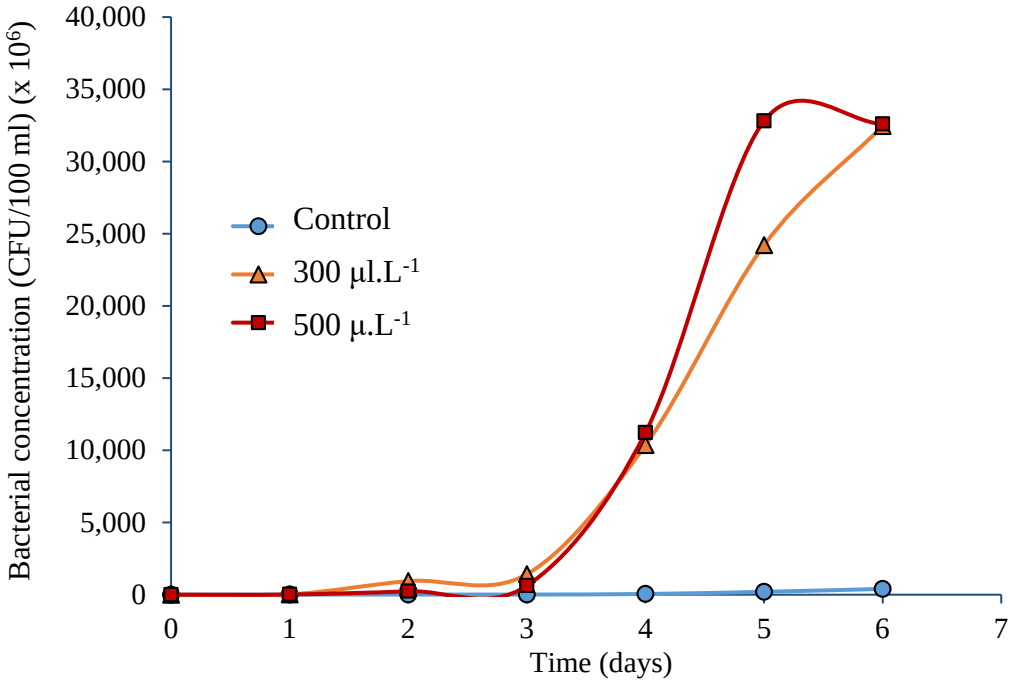


Figure 4.19 Growth curve of soil isolate PD2-3 in 100 ml of MM1 liquid medium and n-hexadecane growth substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates.

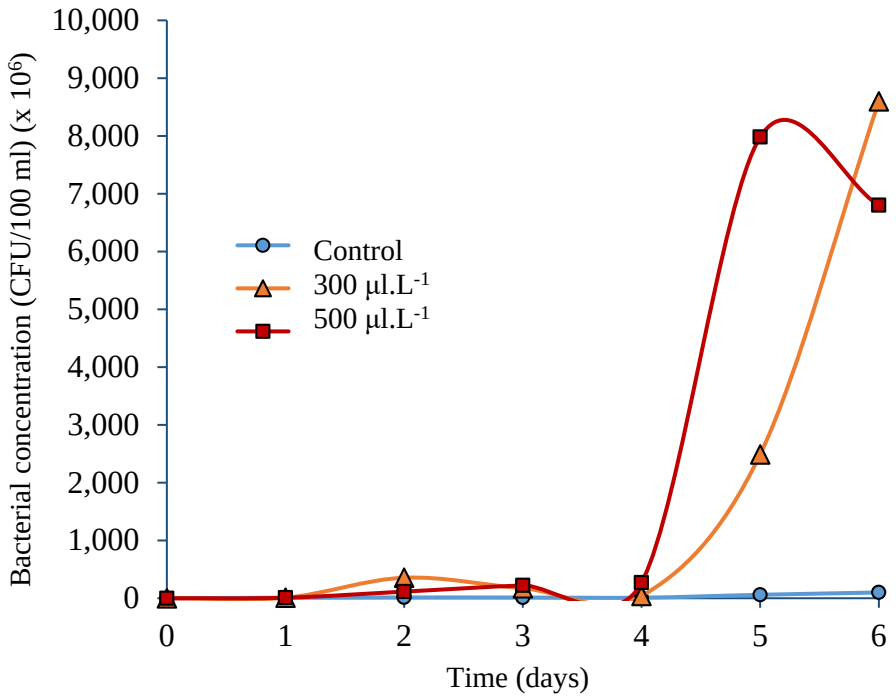


Figure 4.20 Growth curve of soil isolate SD1-1 in 100 ml of MM1 liquid medium and n-hexadecane growth substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates

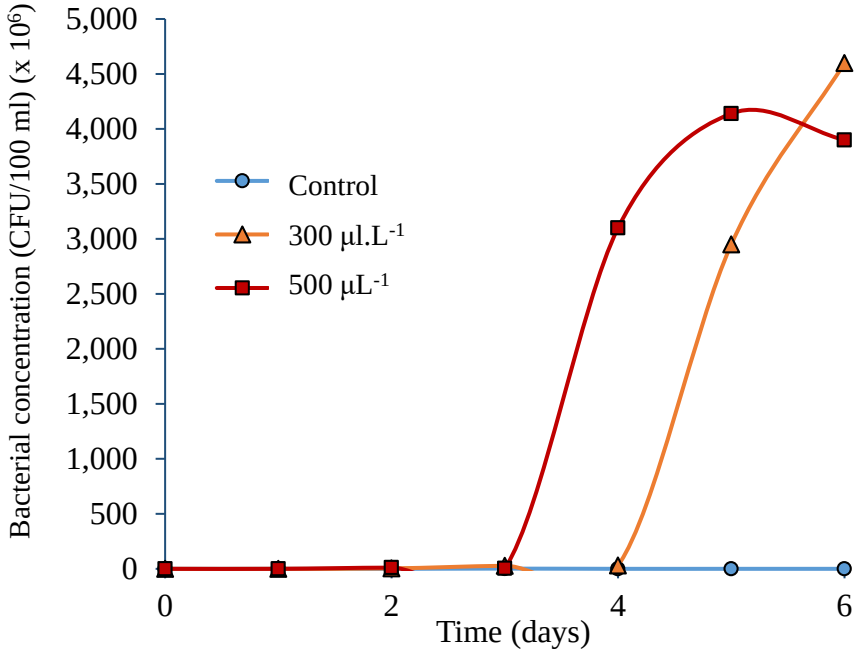
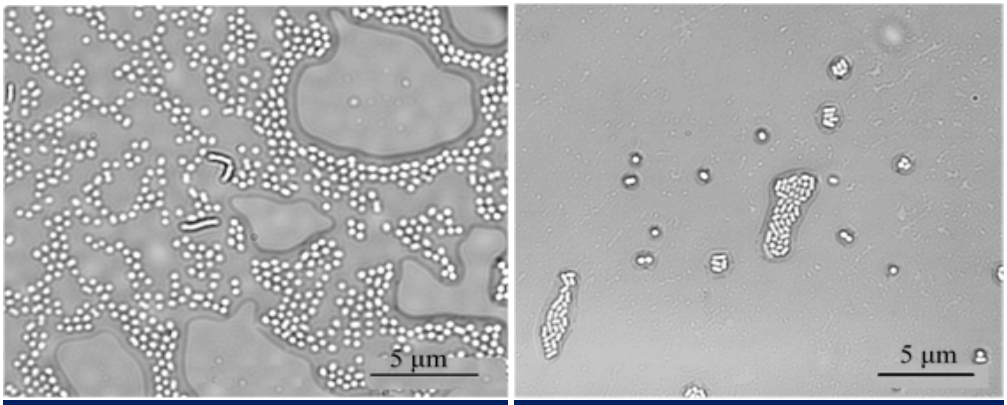


Figure 4.21 Growth curve of soil isolate SD2-1 in 100 ml of MM1 liquid medium and n-hexadecane growth substrate. Bacteria were enumerated at each time point as CFU per 100 ml using NA plates.

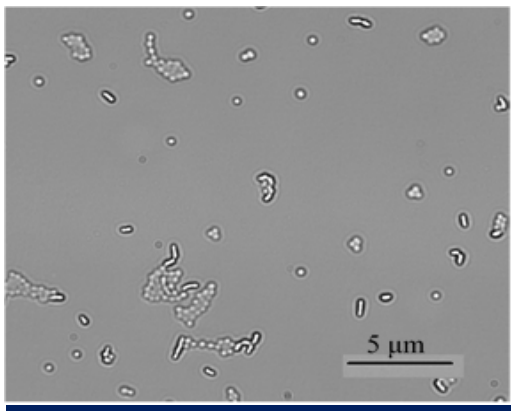
According to the shape of the growth curves for the soil-isolate bacteria, at day 5 of incubation, all of the bacteria appeared to be either at the middle or end of the exponential phase. Therefore, in subsequent experiments, all of the soil-isolate bacteria were either grown for 5 days to achieve exponential growth phase cells, or grown for 10 days to achieve stationary phase cells, as biomass produced at these two different growth states was evaluated as a potential wastewater treatment process for estrogen removal (Chapter 6).

4.7.4 Morphology of soil-isolate bacteria

Microscopic images of the soil-isolates bacteria observed under 10,000 times magnification using a fluorescence microscope (under normal light) are shown in Figure 4.22 and Figure 4.23.



(i) Soil-isolate strain PD2-1 (ii) Soil-isolate strain PD2-2



(iii) Soil-isolate strain PD2-3

Figure 4.22 Images of soil-isolate bacterial strains: (i) PD2-1 (ii) PD2-2 and (iii) PD2-3 isolated after 7 days of cultivation using MM1 media with n-hexadecane (500 µl.L^{-1}) growth substrate, observed under 1000 times magnification using fluorescent microscope.

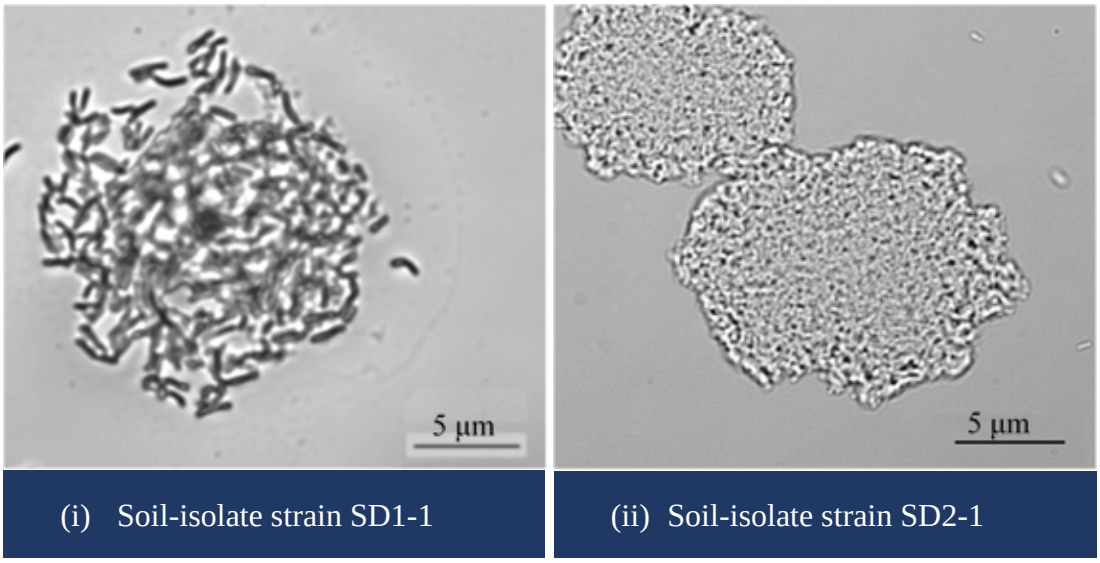


Figure 4.23 Images of soil-isolates bacterial strains: (i) SD1-1 and (ii) SD2-1 that isolated after 21 days of cultivation using MM1 media with n-hexadecane hexadecane (500 µl.L^{-1}) growth substrate, observed under 1000 times magnification using fluorescent microscope.

The soil-isolate bacterial strains of SD1-1 and SD2-1 isolated after 21 days of enrichment were observed to grow in clumps (Figure 4.3), however, in contrast, discrete cell growth was observed in the cultures of strains isolated after 7 days of enrichment (PD2-1, PD2-2 and PD2-3) (Figure 4.22). The clumping bacterial growth that observed under the microscope was presumed to occur due to the presence of extracellular polymeric substance (EPS) (Section 4.5.5). Initially, the presence of EPS during the hydrophobic bacterial adaptation does not seem to make sense because EPS is typically a highly hydrated hydrophilic polysaccharide (Liu *et al.*, 2008). However, EPS can also exhibit hydrophobic properties (Flemming and Wingender, 2010), which due the presence of methyl-linked polysaccharides, and acetyl groups in the EPS molecular structure. This indicates that aggregation of cells in n-hexadecane cultures could be due to the presence of hydrophobic EPS that facilitates the hydrocarbon uptake (Kim *et al.*, 2002; Tzintzun-Camacho *et al.*, 2012).

In addition, the soil-isolates of SD1-1 and SD2-1 underwent soil-enrichment in n-hexadecane for 21 days, 3 times longer than PD2-1, PD2-2 and PD2-3 (7 days). This longer exposure may also have produced a more hydrophobic bacteria, which might be more suitable to be employed in the removal of estrogens from wastewater. Further investigations on the hydrophobicity of the adapted bacterial strains is reported in Chapter 5.

4.8 Conclusion

R. erythropolis strains NCIMB13064 and DSM311 were found to have a tolerance and able to grow on hydrocarbon substrate, and culture of solid and liquid minimal media with n-hexadecane as sole carbon were the most efficient cultivation conditions.

Type of substrate was considered to significantly affect the adaptation process as the adapted bacteria grew well on the highly hydrophobic substrate (n-hexadecane) in preference to n-hexane which has a lower octanol-water partition coefficient.

Substrate concentration significantly affected the level of bacterial growth, with lower concentrations (less than 300 $\mu l.L^{-1}$) deemed to be limiting on growth, but this was not true for all strains as higher concentrations (500 $\mu l.L^{-1}$) did not always improve growth compared to lower concentrations (300 $\mu l.L^{-1}$). Therefore an optimum concentration of 500 $\mu l.L^{-1}$ of n-hexadecane was identified as a suitable growth substrate for the hydrophobic-adaptation process.

Overall, the main objective (Section 1.3 (1)) of obtaining hydrophobic biomass through the adaptation of pure bacterial strains and soil-bacterial isolates using a hydrophobic compound of hydrocarbon, i.e. n-hexadecane, as growth substrate has been successfully achieved.

Chapter 5 Characterisation of the isolated and adapted bacterial strains

5.1 Introduction

A study of the physicochemical characteristics of the adapted strains of *R. erythropolis* NCIMB13064 and DSM311, as well as the new strains isolated from soil (Chapter 4), was conducted according to workflow in Figure 5.1. In theory, the adapted *R. erythropolis* NCIMB13064 and DSM311 were expected to exhibit higher cell surface hydrophobicity (CSH) compared to the parent strains. Furthermore, the new soil-isolate strains were expected to show strong hydrophobic characteristics immediately after isolation from soil, since they had been exposed to hydrophobic growth substrates for up to 7 or 21 days during the isolation process. Theoretically, this could generate high CSH after adaptation, making them potentially ideal bacterial adsorbents for estrogen removal.

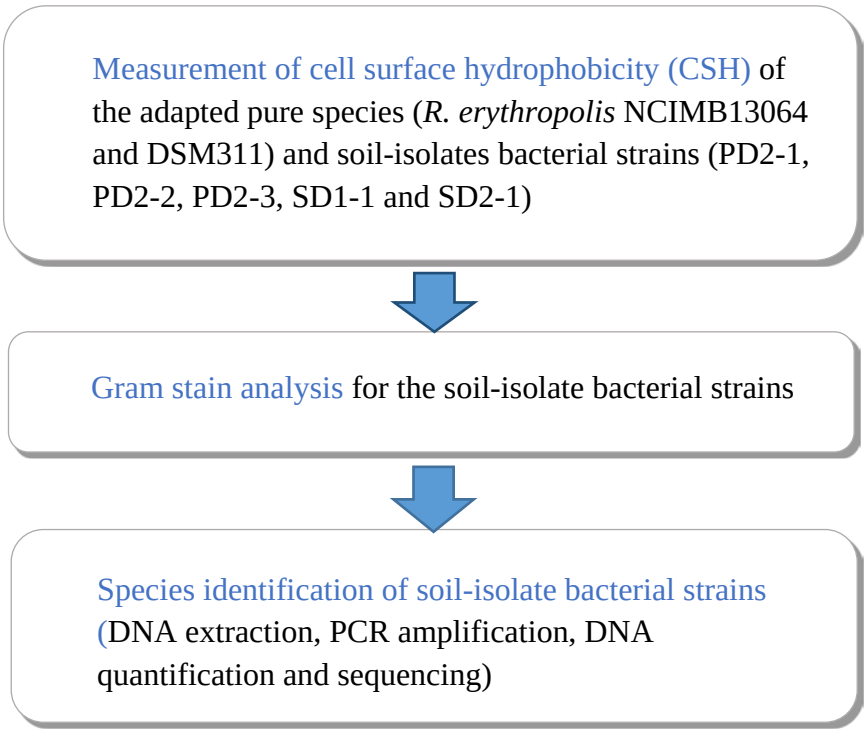


Figure 5.1 Workflow of experimental analysis conducted on the bacteria strains.

5.2 Aim

The aim of this study was to investigate the effect of adaptation on pure commercial strains of bacteria and new bacterial strains isolated from soil using a hydrophobic growth substrate, and to identify the new isolated species.

5.2.1 Objectives

In order to accomplish the aim, the following objectives were developed:

1. To study the effect of growth substrate adaptation of the selected bacteria on their CSH characteristics.
2. To study the CSH profile during the batch cultivation of the bacteria.

5.2.2 Hypotheses

The alternative hypotheses that were proposed related to the objectives are;

1. The adapted and new isolated bacterial strains acquire higher CSH compared to their parent strains after the adaptation process.
2. The CSH of bacterial strains increases throughout the bacterial growth cycle, particularly from the exponential to the stationary phases.

5.3 Methodology

5.3.1 Cell surface hydrophobicity (CSH) measurement

Cell surface hydrophobicity (CSH) of bacteria can be measured by the microbial adherence to hydrocarbon (MATH) technique (Rosenberg, 2006) or contact angle (Section 2.10). Unfortunately, the contact angle technique requires a smooth, uniform surface of bacteria, which is usually prepared by filtering bacterial cells through a membrane filter (Oliveira *et al.*, 2001). This procedure requires a drop of water to be placed on this uniform layer of bacteria so that the contact angle between the axisymmetric shape of the water droplet and the bacterial layer can be measured. However, this procedure is not possible for bacterial cells that exhibit an aggregation effect producing a non-uniform thickness of cells (Figure 5.2). Therefore this method was not used in the current study, and the MATH technique was deemed to be more reliable. In addition the MATH technique is both simple and rapid technique.

The CSH of the bacterial strains was measured according to MATH technique, and was evaluated at days 3, 5 and 10 of incubation during batch growth. Day 3 of incubation represents the beginning of bacterial growth (the end of lag phase), day 5 is the middle of exponential phase, and day 10 represents the stationary phase of bacterial growth, as determined in Chapter 3. The measured values of CSH of the bacterial strains were compared to *E.coli* as the negative control. *E.coli* was chosen as the negative control because it is typically found in wastewater which carries various pollutants, including estrogens, and would therefore receive only low exposure to hydrophobic substrates in the environment.

Phosphate urea magnesium (PUM) buffer was prepared before the experimental work by diluting 22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4.7H_2O$ in distilled water into 1.0 litre solution (Rosenberg *et al.*, 1980). All bacterial strains of NCIMB13064, DSM311,

PD2-1, PD2-2, PD2-3, SD1-1 and SD2-1 were cultivated separately in 10 ml of minimal media (MM1) (Section 4.3) and added with 5 µl of n-hexadecane (500 $\mu l.L^{-1}$) as the growth substrate in 20 ml glass universal tubes. The bacteria were cultivated in triplicate for 3, 5 and 10 days of incubation. Bacterial cells were harvested at the end of the incubation period and centrifuged at 4,000 g for 10 minutes, before being washed twice with PUM buffer to remove the residual n-hexadecane. The washed cells were then reconstituted in PUM to an initial optical density (A_0) between 0.4 - 1.0 at a wavelength of 600 nm using the UNICAM 8625 UV-VIS Spectrometer.

1.2 ml of the culture solution was then taken out and added with 200 µl of new n-hexadecane reagent in a glass tube. The culture solution and hydrocarbon mixture was vortexed at full speed for 2 minutes and left at room temperature for 1 hour for the partitioning of the aqueous and oil phases. Because speed can affect the mixing process, the vortex speed was maintained at a uniform level in each CSH measurement assay. 2 ml of the aqueous phase was carefully extracted using a needle and its optical density was measured and recorded as A_1. Based on the recorded A_0 and A_1 data, the percentage of the bacterial cell adhesion to the hydrocarbon was calculated according to Equation 2.3 (Section 2.10) for cell surface hydrophobicity. The measurements were repeated for all of the bacterial strains prepared in triplicate.

5.3.2 Gram stain characterisation

Gram stain analysis was conducted for each bacterial strain to differentiate between Gram positive and Gram negative types. This identification could provide general information on changes in bacterial morphology. Gram detection assays were carried out according to the Gram staining protocol (Smith and Hussey, 2005).

All of the bacterial strains were cultivated in 10 ml of minimal media (Section 4.3) supplied with 5 µl of hexadecane (500 $\mu l.L^{-1}$) and incubated for 3 days. A drop of sterile deionized water was placed on top of a microscope glass slide and an appropriate amount of bacteria colony was dispensed from the agar plate using the inoculating loop onto the water drop, spread out as a thin layer biofilm. Heat smearing was carried out by passing the slide through a flame for a few seconds. The crystal violet dye was applied on top of the smear and left for 1 minute and washed with water to remove any remaining stain which was unattached the cells. Gram Iodine was applied for 1 minute and then washed away. A decolorizing process with 95 % ethanol solution was conducted afterwards and washed with water after 1 minute. The final stain of Safranin was then applied to the smear, washed with water and air-dried before being observed under a wide field fluorescence microscope fitted with a colour camera (Leica DM6 - upright).

5.3.3 DNA Extraction and PCR amplification

This experiment was only conducted for the soil-isolate bacterial strains PD2-1, PD2-2, PD2-3, SD1-1 and SD2-1 in order to identify their species. These bacterial strains were cultured for 3 days in liquid MM1 (Section 4.3) and n-hexadecane growth substrate (500 $\mu l.L^{-1}$) , and then DNA extraction was carried out utilizing a FastDNA^{TM} SPIN Kit for Soil according to the manufacturer's protocols (MP Biomedicals, UK). The extracted DNA products were stored at -80 ºC and thawed prior to the PCR amplication process.

The extracted DNA was amplified by PCR with universal primer pair P8FpL (5'-AGTTTGATCCTGGCTCAG-3') and P806R (5'- GGACTACCAGGGTATCTAAT-3'). A negative control of sterile water was also prepared for the PCR process. The chemicals for the experiment, 17.75 µl nuclease-free water, 2.5 µl buffer, 0.5 µl dNTP, 0.25 µl enzyme and 1 µl each of primers P8FpL and P806R (McCabe *et al.*, 1996) were all supplied by Sigma-Aldrich. All of the chemicals were added to a 2 ml PCR sterile tube and mixed. The same mixture content was replicated in 6 PCR tubes, and then 2 µl of each DNA sample (5 bacterial strains), as well as a 2 µl of sterile water (control) were added separately to the mixture. Samples and control were amplified in in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) with the following conditions: 1) initial denaturing step at 94 ºC/ 4 min, 2) 35 cycles of denaturation (94 ºC/ 60 s), annealing (58 ºC/ 45 s) and extension (72 ºC/ 60 s) and 3) final extension at 72 ºC/ 7 min. The amplified DNA of the PCR product was stored at -80 °C.

5.3.4 Detection of DNA fragments size, quantification, and sequencing

Agarose gel electrophoresis analysis was carried out to investigate the bacterial DNA fragments sizes. One litre of 50X TAE buffer (Omega Bio-tek, Inc) was prepared beforehand. Agarose gel (1.5 %) was prepared by dissolving 1.5 g of agarose powder (A9539, Sigma Aldrich) in 100 ml TAE 50X buffer, prepared in a 250 ml Duran bottle in a microwave oven at 100 °C for 3 minutes. Nancy dye (20X) 20 ml (Sigma-Aldrich) was added to the agar mixture at 50°C and it was then poured into the casting tray and left for 30 minutes to allow it to solidify. The solidified agar was then placed in the electrophoresis chamber containing the TAE buffer.

Each of the amplified DNA sample (5 µl) (Section 5.3.3) was added to each clean 2 ml PCR tube containing 2 µl buffer. A DNA ladder solution (7 µl) (Invitrogen E-gel DNA ladder, Thermo Fischer Scientific) and DNA/buffer mixture (7 µl) were added separately into the agar well. The process was repeated for all DNA samples in separate wells. A negative control was added without the DNA sample. The electrical voltage was set at 94V for 90 minutes to allow the DNA fragments to travel in the agar matrix. After 90 minutes, the agar was carefully taken out from the chamber and placed in the UV E-Gel imager (Invitrogen, Thermo Fischer

Scientific). A photograph of the fragment band on the agar was captured for DNA fragment size identification.

The amplified PCR product was then purified (Qiagen MinElute kit, Qiagen) according to the protocol provided by the manufacturer. Qubit™ dsDNA HS assay kit was used to quantify DNA by using the Qubit 2.0 Fluorometer, and concentration of the DNA samples can be calculated using Equation 4.1:

$$\text{Concentration of samples } (ng.ml^{-1}) = \text{QF value} \times (200/X) \qquad \text{(Equation 4.1)}$$

Where the QF value is the value given by the Qubit 2.0 Fluorometer, and X is the volume of sample in microliters, added to the assay tube. All of the purified DNA samples were then adjusted according to the conditions required by the Sanger sequencing service provider (DBS Genomics, Durham University). 20 µl of each sample was added to a single tube and sent to Durham University together with the same PCR primers. Information about the samples such as DNA fragment size, concentration and primer details were provided together with the sample packages.

Data on DNA nucleotide sequence were analysed in Snapgene software and aligned to each other using CLUSTALX software. Phylogenetic analysis was conducted with MEGA software, to construct distance-based tree.

5.4 Results and discussion - cell surface hydrophobicity

The CSH investigations carried out were; CSH of pure *R. erythropolis* NCIMB13064 and DSM311 (compared to after adaptation); and comparison of the CSH values of all strains of pure adapted *R. erythropolis* strains, soil-isolates strains and *E. coli*.

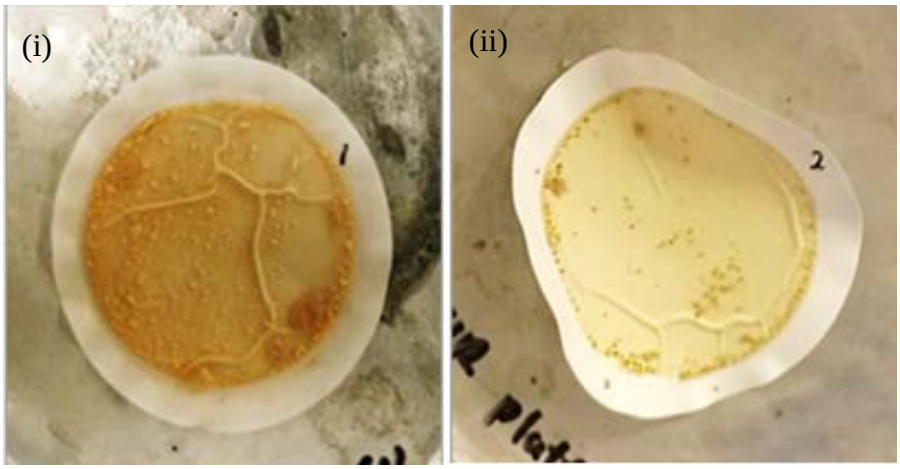


Figure 5.2 Filtered bacterial cell of (i) soil-isolate strain SD2-1 and (ii) *R. erythropolis* DSM311 cultured in liquid MM1 and n-hexadecane growth substrate.

5.4.1 Hydrophobicity of the pure strain of R. erythropolis and soil-isolates bacteria

CSH values for the adapted bacterial strains measured at different incubation times. According to Figure 5.3, the CSH of the bacterial strains adapted with n-hexadecane vary with incubation time, with continuous increments in its level with longer incubation times for the bacterial strains DSM311, NCIMB13064, SD2-1 and PD2-1. However, a different pattern of changes in CSH was found in SD1-1, PD2-3, and for the negative control strain of *E. coli*, where the CSH value increased between day 3 to day 5 of incubation and then declined towards the end of the incubation period. This indicates that the moderate (60 - 65 %) CSH values obtained at day 5 of incubation had fallen by day 10 to 13 - 43 %, causing the SD1-1 and PD2-3 strains to lose most of their hydrophobicity characteristics. The reduction of CSH in these strains is believed to happen due to the cells having actually entered the death phase (Figure 5.4).

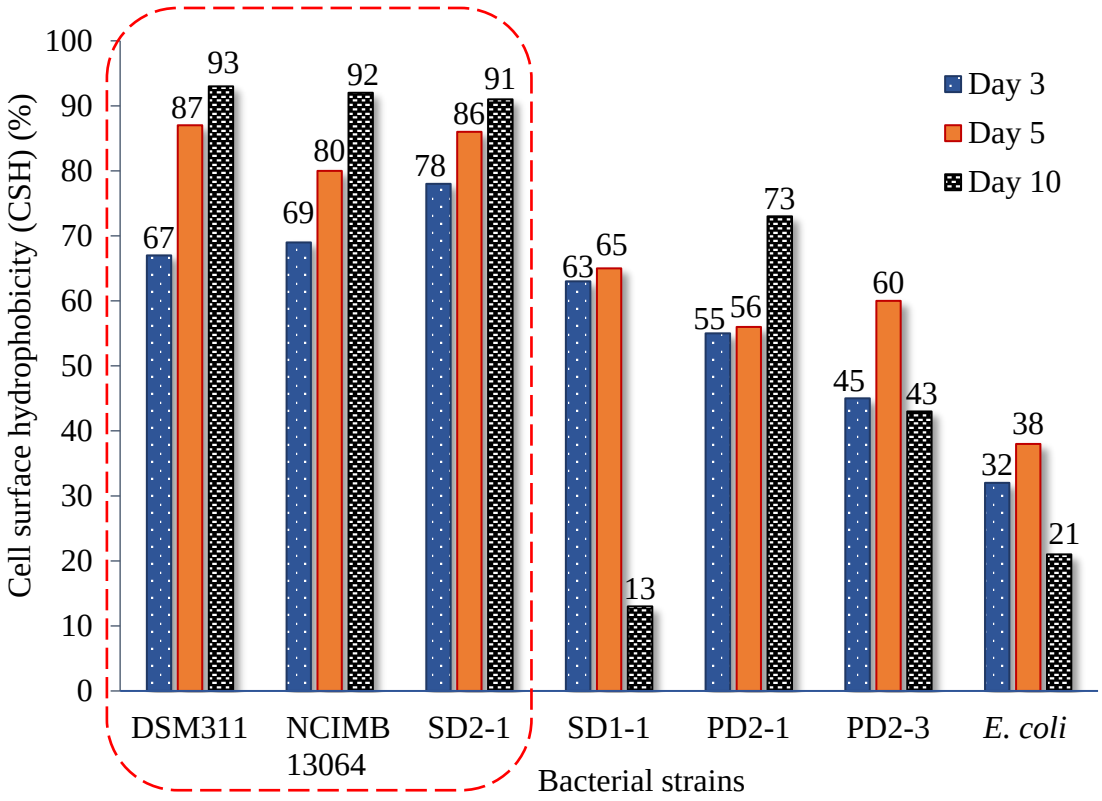


Figure 5.3 Cell surface hydrophobicity of adapted pure *R. erythropolis* strains, soil-isolate bacterial strains and *E. coli* at 3, 5 and 10 days of incubation using the MATH hydrophobicity measurement technique. Broken red line indicates the most hydrophobic strains.

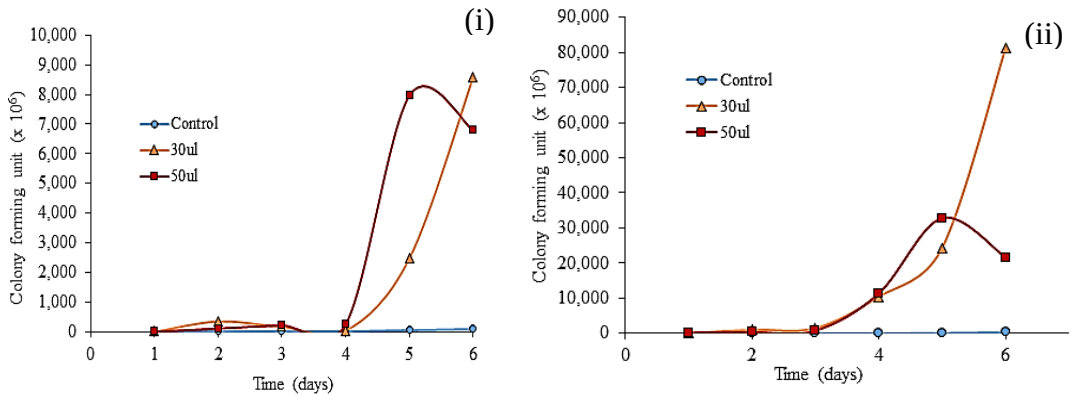


Figure 5.4 Growth phase of: (i) SD1-1 and (ii) PD2-3 during the adaptation process. Cultivation in liquid MM1 supplied with different volumes of n-hexadecane growth substrate (figures extracted from Chapter 3).

It is suggested that strains that continuously increased their CSH values from day 3 to day 10 (DSM311, NCIMB13064, SD2-1) had successfully undergone adaptation (Figure 5.3) and developed into highly hydrophobic cells. More specifically, *R. erythropolis* DSM311 achieved 67 % of hydrophobicity after 3 days, which increased to 87 % at day 5, and achieved the highest CSH among all of the bacteria, finally reaching 93 % on day 10. Similar increments of CSH were observed in the strain NCIMB13064, with 69 % CSH measured on day 3, increasing to 92 % on day 10. The soil-isolate strain SD2-1 developed a CSH of 78 % at day 3, which increased to 91 % by day 10. Therefore, the latter three strains were selected for further investigation as they showed the best CSH and were considered the most promising strains on that basis for the removal of the hydrophobic estrogen contaminants from water. In comparison, *E. coli* as the negative control had very low CSH values, between 21 to 32 %, confirming it to be a hydrophilic strain (Mitra *et al.*, 2005) which should not have the capability of removing estrogen efficiently.

The bacterial adaptation process was found to successfully improve CSH value of the NCIMB 13064, DSM 311 and SD2-1 strains, which became highly hydrophobic as the incubation time increased. Presumably, the longer incubation times allowed the bacteria adapt to the hydrophobic conditions (environmental stress) by altering their membrane lipid component and subsequently the hydrophobicity of the cell surface increased, as proposed by Baumgarten *et al.* (2012). So, further investigations was carried out to compare the CSH values between the adapted and parent strains of NCIMB13064 and DSM311. In addition, CSH development through the growth cycle for the strain SD2-1 was also observed.

5.4.2 Effect of adaptation on the CSH of pure bacterial species R. erythropolis

The CSH values for the adapted strain DSM 311 (DSM311-A) and its parent strain (DSM311-P) were measured between day 3 to day 10 to investigate effect of adapting the bacteria in minimal media and n-hexadecane growth substrate. (Table 5.1 and Figure 5.5).

Table 5.1 Paired t-test analysis of cell surface hydrophobicity of *R. erythropolis* DSM311 comparing the adapted cells and the parent strain.

Samples	Cell surface hydrophobicity (CSH) (%)		
	Day 3	**Day 5**	**Day 10**
DSM311-P	85.67[a]	87.00[a]	87.33[b]
DSM311-A	67.33[b]	87.00[a]	92.67[a]

[a,b] Means that do not share a letter are significantly different ($p < 0.05$). Comparisons are only valid within each day and not valid between days.

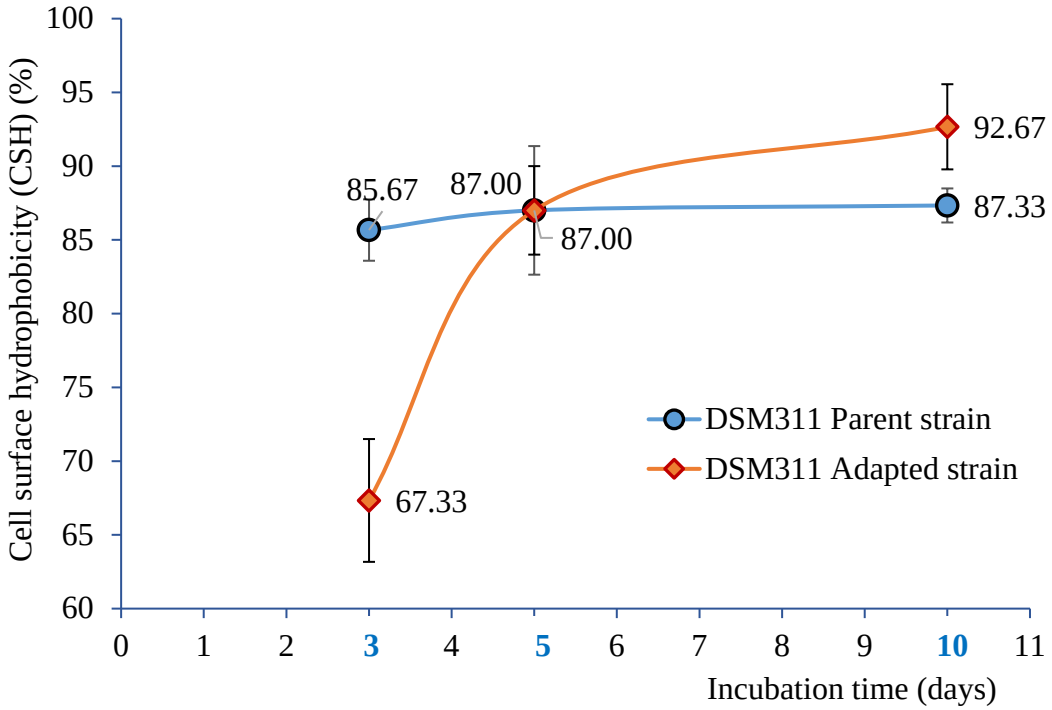


Figure 5.5 Cell surface hydrophobicity of the hydrocarbon-adapted *R. erythropolis* strain DSM311 (cultivated in liquid MM1 and n-hexadecane as growth substrate) compared to the parent strain cultivated in nutrient broth (NB).

Based on the measurement of CSH values of the adapted DSM311 (DSM311-A) and its parent strain (DSM311-P) shown in Figure 5.5, a significant difference in CSH ($p < 0.05$) was present at day 3 of the incubation period. The CSH of DSM311-A was found to be 67.33 %, which was

lower than that of DSM311-P at 85.67 ± 2.08 %. This was an unexpected result, because the adaptation of bacterial cells with hydrocarbon was expected to increase their hydrophobicity instead of reducing it. However, the CSH of DSM311-A was then observed to increase from 67.33 ± 4.16 % to 87.00 ± 3.00 % at day 5, and reached 92.67 % at the end of the 10 days incubation, which was significantly higher than the value for DSM311-P at 10 days. The lower CSH of DSM311-A compared to DSM311-P at day 3 could be best explained by assuming that the bacterial cell was still at the stage of adapting to the hydrophobicity of the n-hexadecane, and its CSH was not yet fully developed, or some other factors in the different growth media (MM1 and NB) were influencing the hydrophobicity. However, as the incubation time increased, DSM311-A had already adapted well to the hydrophobic conditions and achieved higher CSH at day 5 (87%) and day 10 (92.67 %). In comparison, as predicted, the DSM311-P showed an almost constant CSH for the whole incubation period, with an increase of only 1.66 %, from 85.67 to 87.33 %. Statistically, there was a significant increase in CSH ($p < 0.05$) in *R. erythropolis* strain DSM311 after 10 days of the adaptation process, to a value of 92.67 ± 2.89 % CSH compared to 87.33 ± 1.15 % CSH for parent strain (DSM311-P).

A very similar pattern of CSH development through the growth cycle was observed for the *R.erythropolis* strain NCIMB13064 (Figure 5.6). According to the analysis in Table 5.2, the initial CSH of the adapted strain (NCIMB13064-A) increased considerably from 69.33 % at day 3 to 92 % after 10 days of incubation. Even though the percentage of CSH of the NCIMB13064-A was initially lower than its parent strain (NCIMB13064-P) between days 3 to 5, it increased to a significantly higher level than the parent strain at day 10. In summary, both of the adapted strains DSM311-A and NCIMB-A had a significantly higher CSH ($p < 0.05$) than their parent strains once they reached stationary phase (day 10 of incubation). According to these results, the null hypothesis can be rejected and the adaptation of pure bacterial strains with n-hexadecane growth substrate was shown to significantly increase the CSH of the bacterial cells.

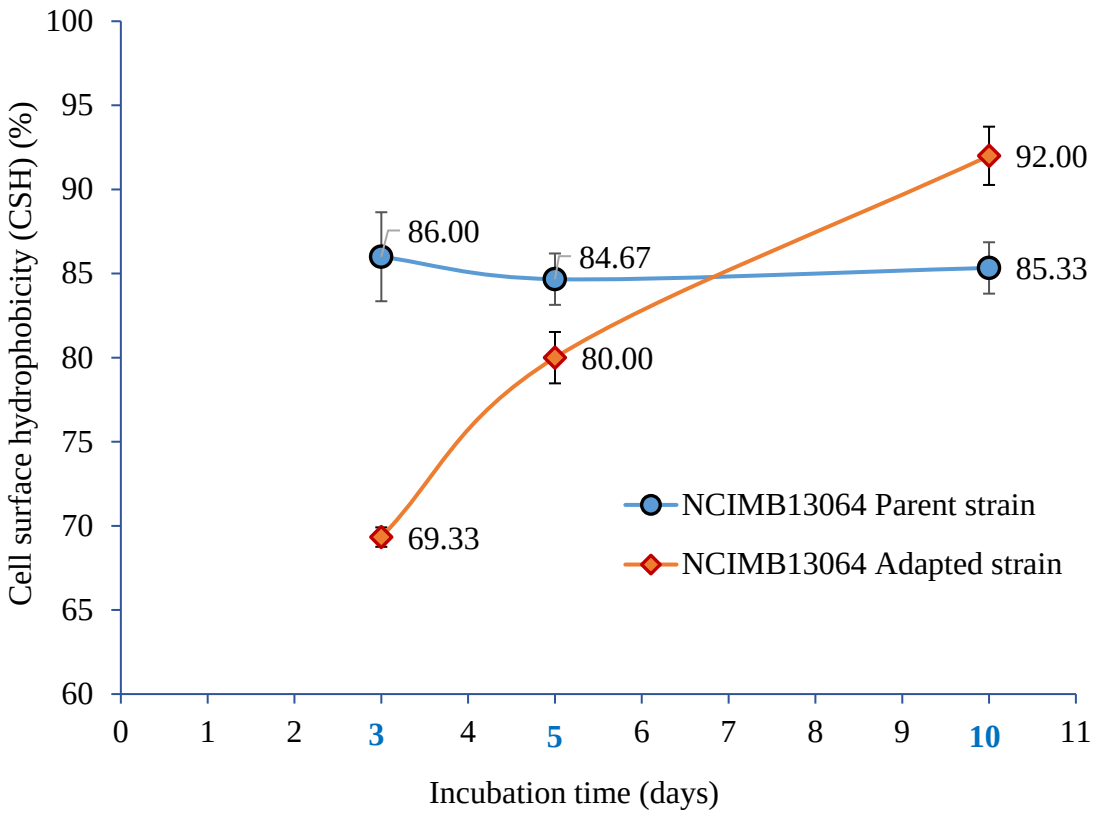


Figure 5.6 Cell surface hydrophobicity of the hydrocarbon-adapted *R. erythropolis* strain NCIMB13064 (cultivated in liquid MM1 and n-hexadecane as growth substrate) compared to the parent strain cultivated in liquid broth.

Table 5.2 Paired-t-test analysis of cell surface hydrophobicity of *R. erythropolis* strain NCIMB13064 comparing the adapted cells and the parent strain.

Samples	Cell Surface Hydrophobicity (%)		
	Day 3	**Day 5**	**Day 10**
NCIMB13064-P	86.00[a]	84.67[a]	85.33[a]
NCIMB13064-A	69.33[b]	80.00[a]	92.00[b]

[a, b] Means that do not share a letter are significantly different ($p < 0.05$). Comparisons are only valid within each day and not valid between days.

Table 5.3. Adaptation of pure bacterial strains (*Rhodococcus erythropolis*) in minimal media and hydrocarbon growth substrates. Comparison of the current and previous studies.

Experimental design/ result	Present study	de Carvalho *et al.* (2009)	Stancu (2014)
Bacterial species	*R. erythropolis* strain DSM311	*R. erythropolis* strain DCL14	*R. erythropolis* strain IBB_{PO1}
Growth substrate	n-hexadecane	n-hexadecane	n-hexane
Concentration of growth substrate	500 μlL^{-1} (0.05 %)	1 %	5 %
CSH by MATH	92.67 %	> 90 %	29 %

When compared to a similar experiment conducted by de Carvalho *et al.* (2009) (Table 5.3), the CSH of the adapted strains in the current study was found to be similarly high (> 90 %). However, the concentration of n-hexadecane growth substrate utilised in the current study was 20-fold lower than the amount used in that research, which indicates the adaptation process in current study was possibly more efficient. In contrast, a very poor CSH (29 %) was obtained by Stancu (2014), which suggests n-hexane is not an effective substrate to trigger physiological adaptation and the development of high levels of hydrophobicity. This result can be related to the discussion in Section 4.5.2, where n-hexane with its higher water solubility was found to inhibit the growth of *R. erythropolis* strain DSM311. However, it was used successfully as the growth substrate for *R. erythropolis* IBB_{PO1} by Stancu (2014), which suggests that their strain was more hydrophilic and possibly not suitable for estrogen removal. In conclusion, the adapted *R. erythropolis* obtained in the current study was found to be a highly hydrophobic strain obtained and could be cultured more economically on much lower levels of growth substrate (n-hexadecane) compared to previous research, and represents an ideal candidate strain for effective estrogen removal.

5.4.3 Comparison of CSH for soil-isolate bacteria at different incubation periods

CSH values of the most hydrophobic soil-isolate bacterium (SD2-1) obtained from the soil enrichment technique identified in Section 5.4.1, was measured at day 3, day 5 and day 10 of cultivation to investigate changes in hydrophobicity related to incubation time (Figure 5.7). Statistical analysis of CSH value by one-way ANOVA and Tukey analysis (Table 5.4) revealed that the CSH of SD2-1increased significantly ($p < 0.05$) with incubation time, starting from 78 ± 4.36 % at day 3 and increasing to 86 ± 3 % at day 5, with further enhancement of CSH to

91.33 ± 2.31 % after 10 days of incubation. According to the results, the null hypothesis can be rejected, and it was confirmed that a significant increase in CSH took place when cultivating the SD2-1 in minimal media supplied with n-hexadecane growth substrate for a longer incubation periods (3 – 10 days).

Table 5.4 Profile of cell surface hydrophobicity for the soil-isolate bacterium strain SD2-1 grown in n-hexadecane growth substrate.

Soil-isolate bacterium	Cell Surface Hydrophobicity, CSH (%)		
	Day 3	Day 5	Day 10
Strain SD2-1	78.00[a]	86.00[ab]	91.33[b]

[a,b] Means that do not share a letter are significantly different ($p < 0.05$)

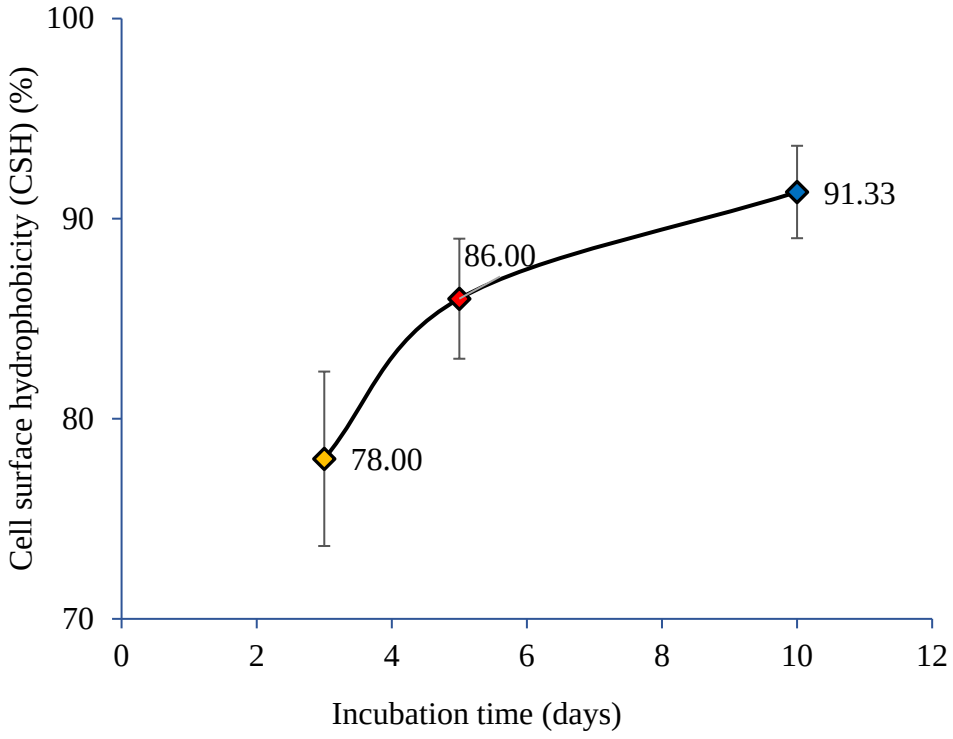


Figure 5.7 Cell surface hydrophobicity of soil-isolate bacteria (SD2-1) at different incubation times.

5.5 Results and discussion – Gram stain

Analysis of bacteria for Gram stain status was conducted to distinguish between groups of bacteria based on different cell wall constituents. The identification of bacterial Gram classification was accomplished by observing the colour of the bacterial cells in microscopic

images (Budin *et al.*, 2012). Gram-positive bacteria were identified by the presence of violet cells and Gram-negative species with pink cells. This is because gram-positive bacteria have thick layer of peptidoglycan in their cell walls that could retain the crystal violet stain, surviving the decolourization process. Meanwhile, Gram-negative bacteria have thin layer of the peptidoglycan that allows for decolourization by the alcohol, and this colourless cell was later stained pink due to the application of safranin dye.

According to Figure 5.8, all of the isolated bacteria PD2-1, PD2-2, PD2-3, SD1-1 and SD2-1 were Gram-positive, which suggests they have the ability to maintain cell viability at a higher concentrations of organic compounds (n-hexadecane growth and enrichment substrate) than Gram-negative strains (De Carvalho *et al.*, 2004). This explains why all of the isolated bacteria were Gram-positive.

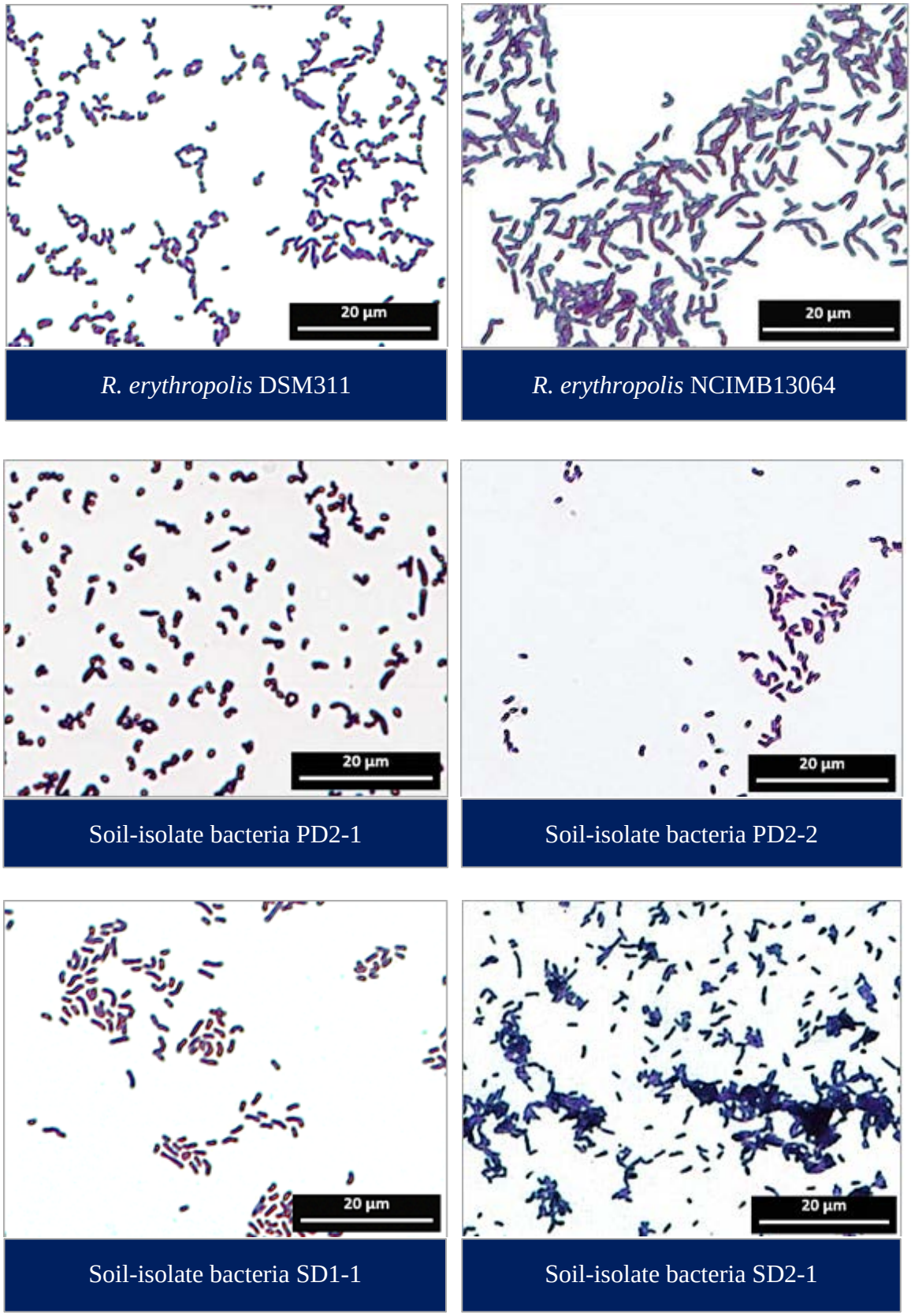


Figure 5.8 Microscopic images from bacterial Gram stain analysis of pure *R. erythropolis* (conducted as verification) and soil-isolates bacteria.

5.6 Results and discussion – bacterial identification

5.6.1 DNA fragment detection and Quantification

Viewing of agarose gel electrophoresis samples (Figure 5.9) confirmed that all of the bacteria have approximately 700 - 800 DNA fragments. The concentrations of the PCR-amplified DNA as quantified by the Qubit 2.0 Fluorometer and determined using Equation were between 6,400 – 15,860 ng.ml^{-1} (Table 5.5). This information concerning the DNA fragments and quantification was passed to Durham University together with the DNA products and primers used in the PCR amplication for species detection by Sanger Sequencing.

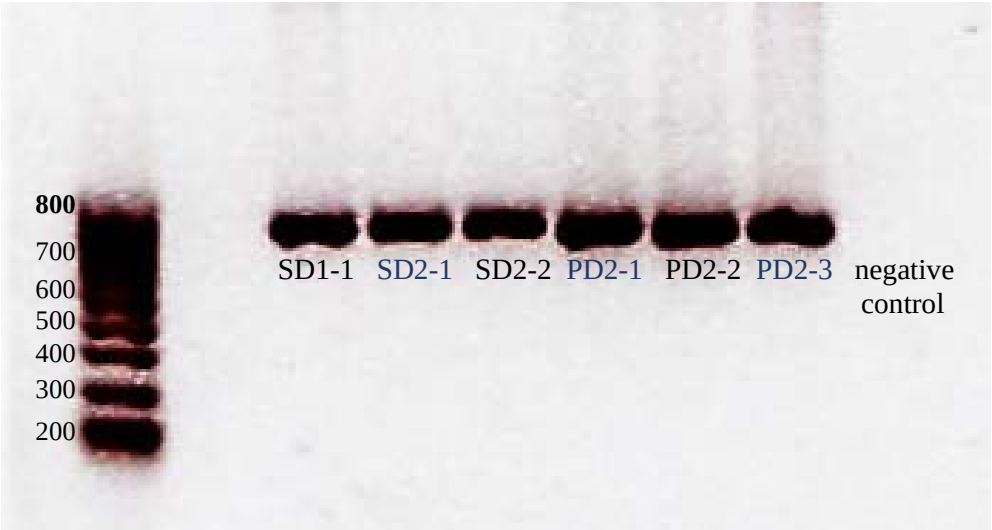


Figure 5.9 Identification of DNA fragments size in gel electrophoresis technique.

Table 5.5 DNA quantification for soil-isolates bacterial strains.

Strains	QF value*	DNA concentration (ng.ml^{-1})
PD2-1	36.7	7,340
PD2-2	72.2	14,440
PD2-3	79.3	15,860
SD1-1	72.6	14,520
SD2-1	32.0	6,400

*QF value was quantified by the Qubit 2.0 Fluorometer and DNA concentration was determined by using Equation 4.1.

5.6.2 Sequencing

The DNA nucleotide sequences and chromatogram results obtained from Durham University were analysed in Snapgene software. Nucleotide sequences were aligned to each other using CLUSTALX software and overlapping chromatogram peaks and bases were identified and corrected. The final corrected DNA sequences of each bacterial strain (Appendix 1) was then run in the Nucleotide Basic Local Alignment Search Tool (BLAST) NCBI and significant matches for genes were obtained. The species detected for each bacterial strain according to the BLAST library are as in Table 5.6. In addition, phylogenetic analysis was conducted with MEGA software and distance-based tree was conducted (Appendix 2).

Table 5.6 Significant species detected based on sample DNA sequencing and database sequences in the BLAST library.

Bacteria	Significant species (%)
SD1-1	99 % *Rhodococcus erythropolis*
SD2-1	99 % *Tsukamurella tyrosinosolven* 100 % *Tsukamurella pulmonis* 100 % *Tsukamurella pseudospumae*
PD2-1	99 % *Rhodococcus erythropolis*
PD2-2	99 % *Rhodococcus erythropolis*
PD2-3	99 % *Rhodococcus erythropolis*

The soil-isolate strain SD2-1, previously determined to have the highest value of CSH was found to belong to the bacterial genus *Tsukamurella*, and the other isolated bacteria (PD2-1, PD2-2, PD2-3 and SD1-1) were *Rhodococcus erythropolis* species. *Tsukamurella* sp. and *Rhodococcus erythropolis* are bacteria which contain mycolic acid. This is a component present in the outer layer of the cell membrane (Onaka *et al.*, 2011; Safaei *et al.*, 2017), and it creates a highly impermeable outer barrier membrane (Onaka *et al.*, 2011), which contributes to the high hydrophobicity of *R. erythropolis* and *Tsukamurella* sp. This shared characteristic is a major reason these genera have the capability to be isolated and adapted using hydrophobic compounds as growth substrates (e.g. n-hexadecane).

5.6.3 Comparison of CSH of the soil-isolate bacteria with previous studies

Adaptation of the genera of *Tsukamurella* sp. using hydrocarbon of n-hexadecane has been studied previously (Tebyanian *et al.*, 2013; Chiciudean *et al.*, 2017), however these publications have several shortcomings.

Table 5.7 Comparison of main parameters from past studies and present study for the adaptation of *Tsukamurella* sp. using n-hexadecane growth substrate.

Bacterial species	**Amount of n-hexadecane (v/v)**	**CSH (%)**	**Reference**
T. tyrosinosolvens	2.5 %	29	Tebyanian *et al.* (2013)
T. carboxydivorans	1.0 %	No record	Chiciudean *et al.* (2017)
T. tyrosinosolvens / T. pulmonis/ T. pseudospumae	0.05% (500 $\mu l.L^{-1}$)	91	Current study

A bacterial isolate from a petroleum reservoir soil, *T. tyrosinosolven*, has been found to be the most active hydrocarbon degrading-species for n-hexadecane among other isolates (Tebyanian *et al.*, 2013). Nevertheless, the CSH of this bacterium was found to be only 29 %, suggesting it is not is not a particularly hydrophobic strain, and this presumably occurred because a very high amount (2.5 % v/v) of n-hexadecane was added as the bacterial growth substrate during its cultivation. This high concentration might have inhibited the bacterial growth sufficiently to cause cell death, and the presence of dead cells might have influenced the measurement of CSH. A similar effect took place in the SD1-1 and PD2-3 strains, which showed a drop in CSH upon entering the death phase, compared to the high CSH (91 %) of *Tsukamurella* strain SD2-1 in the present study (Section 5.4.1). Unfortunately, no measurement of CSH was made during the adaptation and investigation of n-hexadecane biodegradation by *T. carboxydivorans* species (Chiciudean *et al.*, 2017) so the theory of cell death affecting CSH remains speculative.

5.7 Conclusion

The adaptation of pure bacterial strains of *R. erythropolis*, NCIMB13064 and DSM311, to generate higher hydrophobicity after culturing in n-hexadecane (Chapter 4) has successfully produced adapted strains with high CSH. Based on the statistical analysis of the CSH levels of DSM311, this adapted strain was found to acquire higher CSH compared to its parent strain, proving that the hydrophobic adaptation had been successfully achieved. The CSH changes

support the assumption previously made in Chapter 4, where adapted DSM311 and NCIMB13064 strains were suspected of altering the properties of their membrane lipid to become saturated, as a protection mechanism against the toxicity of hydrocarbons and one which finally develops greater hydrophobic characteristics in the cell.

In addition, the SD2-1 bacterial strain isolated from diesel-contaminated soil was also found to be a highly hydrophobic bacterium, with CSH at a similar level to *Rhodococcus erythropolis* DSM 311 and NCIMB13064 strains (90 - 93% CSH). This strain was identified as *Tsukamurella* sp., a Gram-positive bacteria, according to Sanger sequencing analysis. Both *Tsukamurella* sp. and *Rhodococcus erythropolis* have previously been found to contain mycolic acid which creates a highly impermeable outer barrier membrane. In other words, in addition to altering the properties of its membrane lipid, these bacterial strains also have the ability to come into contact with free phase n-hexadecane without suffering toxicity effects. Based on the significant differences in CSH of the adapted and parent strains, these findings support the hypothesis that the adapted and new isolate bacterial strains acquire higher CSH after the adaptation process compared to their unadapted parent strains.

In addition, the CSH of the adapted *R. erythropolis* strains DSM 311 and NCIMB13064, and *Tsukamurella* sp. SD2-1 was also found to increase with the bacterial growth phase, that the CSH of stationary phase bacteria being higher than that of exponential phase. In theory, longer incubation period allows the bacteria undertake further adaptation with each successive generation. However, other soil-isolate bacteria exhibited reductions in CSH towards the end of the incubation period and finally lost most of their hydrophobic characteristics.

Bacterial strains obtained in the current study with the highest CSH levels, were the adapted *R. erythropolis* strains DSM311 (93 %) and NCIMB (92 %), and the soil-isolate bacterium *Tsukamurella* sp. SD2-1 (91 %). These strains were considered to have high potential for use as novel adsorbents and degraders capable of removing estrogens from municipal wastewater by a new tertiary treatment process. *Tsukamurella* sp. SD2-1 is the only high CSH strain to have been isolated from soil in the current study, whereas several *R. erythropolis* strains were isolated from soil, notably PD2-1, PD2-2, PD2-3 and SD1-1 but have lower CSH. The latter strains were deemed to be less favourable for further investigation as potential bacterial adsorbents in the subsequent estrogen removal studies (Chapter 6).

In summary, the objective (Section 1.3 (2)) of investigating the CSH profile of the bacteria has been met, and the findings support the hypothesis (Section 1.4) that the CSH of the pure bacteria increases after the adaptation, and the CSH varies during different phases of bacterial growth.

Chapter 6 Estrogen Removal in Batch Reactors

6.1 Introduction

Estrogens have been reported to cause adverse impacts on aquatic organisms, effects being observed at trace level of part per trillions and higher. These effects have been suggested to take place at Environmental Quality Standards of 0.4 $ng.L^{-1}$ for E2 and predicted no-effect concentration (PNEC) of 3.6 $ng.L^{-1}$ for E1 (European Commission, 2016).

In municipal wastewaters, the presence of estrogens has been detected at various concentrations, determined by factors such as source of the wastewater as well as sampling season. They can be present at concentrations below the limit of quantification by GC-MS (Qiang *et al.*, 2013) up to concentrations as high as 3050 $ng.L^{-1}$ (E1) (Pessoa *et al.*, 2014). A mean concentration of approximately 184 $ng.L^{-1}$ of E1 has been calculated for the influents of municipal wastewater treatment plants (WTP) based on a comparison of case studies in the literature (Section 2.2.1). However, due to the inefficient removal of estrogens by conventional WTP, an average of 88.51 $ng.L^{-1}$ of E1 is found in their effluents. To achieve higher estrogen removal, therefore, advanced treatments such as adsorption and biodegradation have been proposed as tertiary processes for WTP. Some of the previous adsorption studies evaluated the use of adsorbent materials, including activated carbon (Ifelebuegu *et al.*, 2006), and activated sludge (Ren *et al.*, 2007b). The adsorption of estrogens in these studies was reported to take place due to the electrostatic interaction between the adsorbent materials and the estrogen molecules. In addition, the adsorption process is also mediated by hydrophobic interactions (Margot *et al.*, 2013b; Hartmann *et al.*, 2014; Ifelebuegu *et al.*, 2015; Jiang *et al.*, 2017). Apart from adsorption, biodegradation has also been identified as an efficient mechanism involved in estrogen removal from wastewater, through work on activity of activated sludge and pure bacterial strains (Yoshimoto *et al.*, 2004; Larcher and Yargeau, 2013). However, no previous research has been carried out using hydrophobic bacteria adapted specifically for use as an adsorbent material for the purpose of estrogen removal.

In the present study, several hydrophobic-adapted strains of *R. erythropolis* and soil-isolate bacteria were successfully obtained in the microbiological study (Chapter 4 and Chapter 5), and were evaluated as potential adsorbents for estrogen removal. According to the CSH assays conducted in Chapter 5, values of the cell surface hydrophobicity (CSH) of the bacteria were found to vary, with some of them exhibiting highly hydrophobic characteristics. The highly hydrophobic strains were presumed to be most competent in establishing interactions with the

hydrophobic estrogen compounds, either through adsorption or biodegradation process, and were assessed further in this chapter for these characteristics through batch reactor studies.

6.1.1 Aim

To gain a better understanding of the effects of adapting bacteria into hydrophobic strains, in terms of their effectiveness in removing estrogen, via adsorption as well as biodegradation processes.

6.1.2 Objectives

To address this aim, the following objectives were set:

1. To examine correlations between the CSH values of bacterial strains and their performance in adsorbing estrogen from wastewater using batch reactors.
2. To determine the optimum dosage of bacterial biomass for effective estrogen removal.
3. To determine the optimum contact time for the estrogen removal process.
4. To evaluate the performance of estrogen removal by live bacteria allowing for biodegradation and adsorption, and inhibited bacteria where the sole mechanism was adsorption.
5. To understand whether harvesting bacterial biomass from different phases of the bacterial batch growth cycle can affect the estrogen removal performance.

6.1.3 Hypotheses

1. The CSH of the bacterial strains is correlated with their estrogen removal efficiency.
2. The degree of estrogen adsorption from wastewater is proportional to quantity of bacterial biomass used.
3. The performance of estrogen adsorption in batch reactors increases with the contact time applied.
4. The adsorption of estrogen onto bacterial biomass is affected by the stage of bacterial growth phase that the biomass was harvested.

6.1.4 Experimental work plan

The estrogen removal processes were conducted in batch reactors utilizing synthetic wastewater as the sample medium, and estrone (E1) as the model pollutant due to its hydrophobicity and the high concentration found normally in municipal wastewater. A summary of the experimental plan is shown in Figure 6.1.

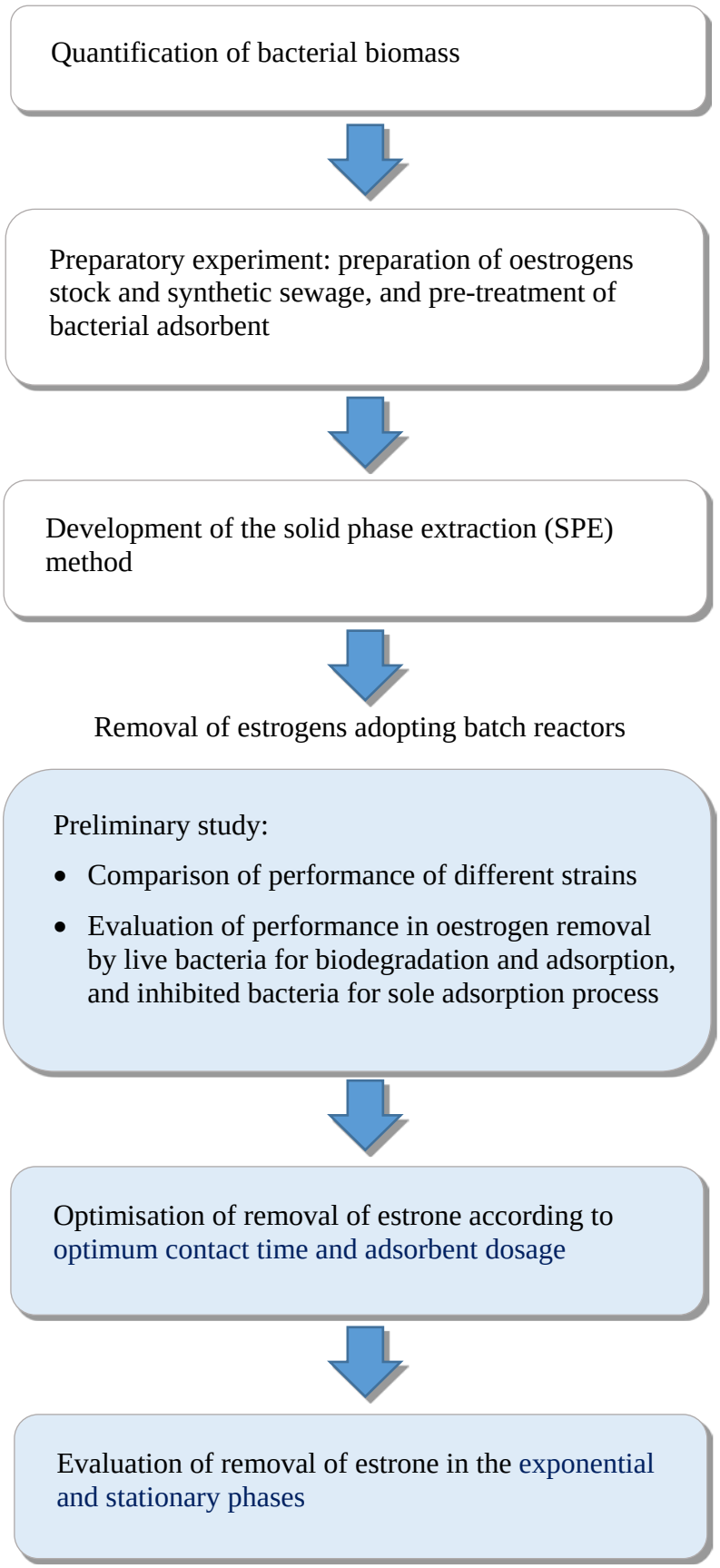


Figure 6.1 Flow sheet of the work plan evaluating removal of estrogens using of hydrocarbon-adapted bacteria.

Experiments to understand how estrogen removal involved both adsorption and biodegradation process are summarised in the Figure 6.2.

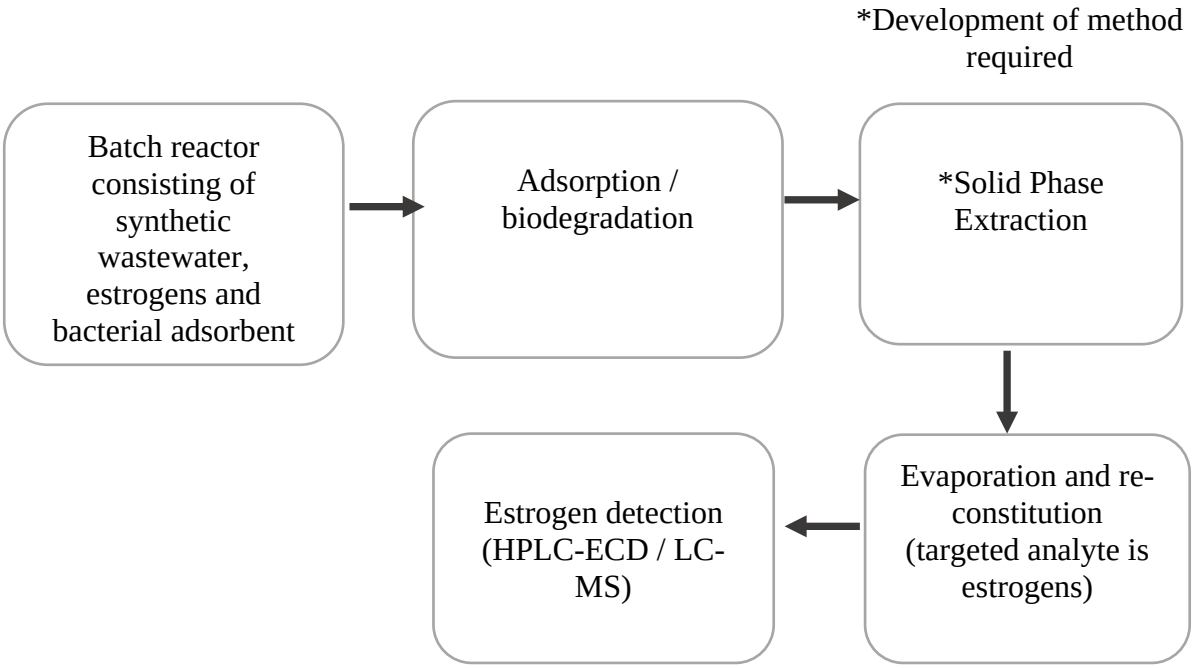


Figure 6.2 Process of estrogen removal adopting adsorption and biodegradation processes in batch reactors.

6.2 Ratio of biomass: estrogen ratio on estrogen removal

The amount of bacterial biomass added to the batch reactor had to be fixed at a suitable amount, measured in biomass weight. To assign an appropriate ratio of biomass to estrogen, the amount of bacteria was quantified in weight because measurement in terms of colony forming units (CFUs) through the agar-plating method would have limited the quantification method to live bacterial cells only, whereas the total adsorption has components contributed from both live and dead bacterial cells. In addition, the quantification of bacteria in terms of the total number of cells using the flow cytometry method was not applicable due to the natural aggregation of the hydrophobic bacterial cells in aqueous media.

Comparable ratios of the weight of bacteria to estrogen present in the water were evaluated with reference to the literature. During assays optimising of removal of estrone, an identified volume of the bacterial culture, representing 2.5 mg of dry weight bacteria cells was added to 500 ml of synthetic wastewater containing 200 $ng.L^{-1}$ of estrone. The mass ratio (w/w) of bacterial biomass to estrogen was fixed at 25,000:1.

6.2.1 Quantification of bacterial biomass

Volume of the bacterial culture representing for 2.5 mg of biomass dry weight was determined at day 5 of growth incubation period. Before the measurement of weight, the bacteria were cultivated beforehand, where a single colony of each strain was cultivated in 10 ml of minimal media with 5 µl of n-hexadecane as growth substrate, and incubated for 5 and 10 days at 26 °C and 155 rpm, in triplicate. Each 10 ml bacterial culture was filtered through a 0.2 µm cellulose nitrate membrane filter (11407-47-ACN; Sartorius) in a sterile vacuum magnetic filtration unit (Sentino® funnel; Pall laboratory, UK). The membrane filter had previously been dried overnight at 105 °C before filtration. The filters, containing the harvested bacterial cells were then dried in the same oven overnight and then cooled in a silica desiccator. The final constant weight of the biomass which represented 10 ml of culture was recorded, and the volume required for 2.5 mg of biomass weight was calculated from the results.

6.3 Preparation and pre-treatment process

Several preliminary experimental studies were conducted before the estrogen removal process was quantified. These were the preparation of estrogen stock and synthetic wastewater, thermal pre-treatment of the bacterial adsorbent, and the development of a method for the concentration of estrogen before analysis. Details of this experimental work are described in the following sections.

6.3.1 Preparation of estrogen stock and synthetic wastewater

A 100 $mg.L^{-1}$ of estradiol stock solution was prepared by diluting E2 powder (99 %) purchased from Sigma Aldrich in a solvent mixture of 50:50 acetonitrile to methanol in a sterile amber bottle. This was kept for up to 3 months at -20 °C in the freezer. A working solution of 1 $mg.L^{-1}$ was then prepared by diluting the stock in an aqueous solution of 10 % acetonitrile in sterilised bottle kept for up to 1 week at 4 °C. The same protocol was applied for the preparation of estrone (E1) and estriol (E3) stocks separately.

A synthetic wastewater sample was prepared according to the OECD Synthetic Sewage specifications (Pholchan *et al.*, 2008) using the chemicals listed in Table 6.1. The chemicals were dissolved in deionised water to 1 litre of solution, and mixed using a magnetic stirrer for homogenisation before being autoclaved. The sterile synthetic sewage stock was kept at 4 °C for not more than 1 week. It was diluted 100-fold with natural bottled spring water (Nestle Pure Life Bottled Water), containing typical wastewater levels dissolved minerals but no chlorine, for use in the estrogen removal experiments.

Table 6.1 Composition of the synthetic wastewater according to OECD Synthetic Sewage specifications.Composition of the synthetic wastewater according to OECD Synthetic Sewage specifications.

Reagent	Amount ($g.L^{-1}$)
$CaCl_2.2H_2O$	1.6
$MgSO_4.7H_2O$	0.8
K_2HPO_4	11.2
NaCl	2.8
Urea	12.0
Meat extract	44.0
Peptone	64.0

6.3.2 Pre-treatment of the bacterial adsorbent biomass

The current study employed an inhibition technique to denature the bacterial enzymes (to stop biodegradation processes) without altering the morphological and hydrophobicity characteristic of the bacterial cells, which are essential for the adsorption process.

In general, inhibition processes are classified as either Chemical or Physical treatments (Huang and Yeung, 2015). In chemical treatment, denaturing of enzymes can be achieved by using alcohol, however this can also lead to dehydration of the bacterial cell and serious shrinkage of the cell structure (Chao and Zhang, 2011). The chemicals can penetrate the cell membrane and may change the morphology and hydrophobicity of the adapted strains. Therefore, alcohol is not suitable for the inhibition of bacteria in the current study.

In contrast, Physical inhibition, namely thermal pre-treatment can denature the bacterial enzymes whilst preserving the cell morphology (Ren *et al.*, 2007b). However, the temperature of inhibition is a critical factor to be considered, and 80 °C for 30 minutes is normally selected because it preserves cell morphology, whereas a temperature of 121 °C does not (Lunsman and Lick, 2005).

The adapted and isolated bacterial strains NCIMB13064, DSM311, SD1-1, SD2-1, PD2-1, PD2-2 and PD2-3 were cultivated in 25 ml universal tubes containing 10 ml of liquid MM1 (Section 4.3) and 5 µl of n-hexadecane, and incubated at 25 °C at 155 rpm for five days. At the end of the incubation period, one set of the bacterial cultures were thermally inactivated in an

oven at 85 °C for 1 hour. Subsequently, this thermal inhibition step was increased to 85 °C instead of the 80 °C, to avoid reductions of temperature during the process of loading samples into the oven and the duration of the inhibition process was increased to 1 hour instead of 30 minutes so as to ensure that all bacterial cells had been inhibited properly (no viable cells were seen on agar plate growth checks). 50 µl of the thermally treated bacterial culture was then sub-cultured in nutrient agar (NA) purchased from Lab M Ltd. This was done to check that all of the bacteria had been successfully inhibited and that no signs of growth were seen after three days of incubation (25 °C). A separate set of bacterial cultures was to be used without thermal pre-treatment as live bacteria cells to allow biodegradation process to contribute to the overall estrogen removal process.

6.4 Development of the estrogen quantification method

Solid phase extraction (SPE) was adopted before the measurement of estrogens, for the purposes of removing any interfering substances present in the wastewater, and to concentrate the low levels of estrogen ($ng.L^{-1}$) from a high volume of sample into 1 ml of sample containing substantially higher concentrations that could be detected by LC-MS and HPLC-ECD. The wastewater concentration of estrone was initially set at two different concentrations, 1 $mg.L^{-1}$ which was used in the preliminary experiments, and 200 $ng.L^{-1}$ which was used in the optimisation study. The high initial concentration of estrone was used in the preliminary study to facilitate the effective monitoring of its removal when the quantification assay was still under development, and the lower concentration was adopted for the optimisation experiments to investigate the efficiency of removal at levels close to the actual concentrations found in municipal wastewater. An appropriate SPE cartridge capacity required for the high initial concentrations of estrogen was determined in preliminary tests to ensure the quantification process was not affected by poor recovery from the SPE cartridges.

6.4.1 Development of the estrogen quantification method for high estrogen concentrations (part per thousand)

To determine the appropriate cartridge that would produce optimum recovery, further development of the method was conducted. Capacity of the cartridge was calculated according to the amount of analyte it could bind, given the percentage of analyte recovery.

50 ml of 1 $mg.L^{-1}$ E2 stock was added to 50 ml of deionised water for an initial concentration of 1 $mg.L^{-1}$ in 250 ml Duran bottles, in quadruplicate. Solutions which contained a total of 50 µg of E2 were vortexed to homogenise estrogen content in the water. Then, 50 ml of solution from each of two bottles were loaded into two Oasis PRiME HLB 3 cc /60 mg cartridges

(duplicate). Meanwhile, 1ml of solution from each of another 2 bottles was loaded into another 2 cartridges. A washing process was then carried out using 3 ml of 10 % methanol to remove substances other than estrogen from the water sample, followed by the elution of the targeted E2 analyte with 5 ml of acetonitrile: methanol solvent at a ratio of 90:10. The solution was then evaporated at 60 °C in a RapidVap Vertex^{TM} evaporator before being reconstituted in 1 ml of 10 % acetonitrile. Protocols for the SPE method are summarised in Figure 6.3 (i). The extracted estrogen solution was then filtered using a 0.22 μm syringe filter (SF13PTFE022NS; Stratlab Ltd., UK) into HPLC amber vials and analysed via HPLC-ECD (Section 6.8). The amount of estrogen recovered from the SPE was calculated by analysing the area of the E2 chromatogram peak provided by the HPLC-ECD compared to the standards that were prepared in the range of 1 - 50 μg.ml^{-1}.

6.4.2 Development of the estrogen quantification method for low estrogen concentrations (part per trillion)

500 ml of 200 ng.L^{-1} samples of E1, E2 and E3 mixtures (carrying 100 ng of each estrogen) in synthetic wastewater were loaded into a larger capacity OASIS HLB 6 cc /200 mg SPE cartridge to determine its recovery rate. The SPE elution was conducted according to the protocol shown in Figure 6.3 (ii).

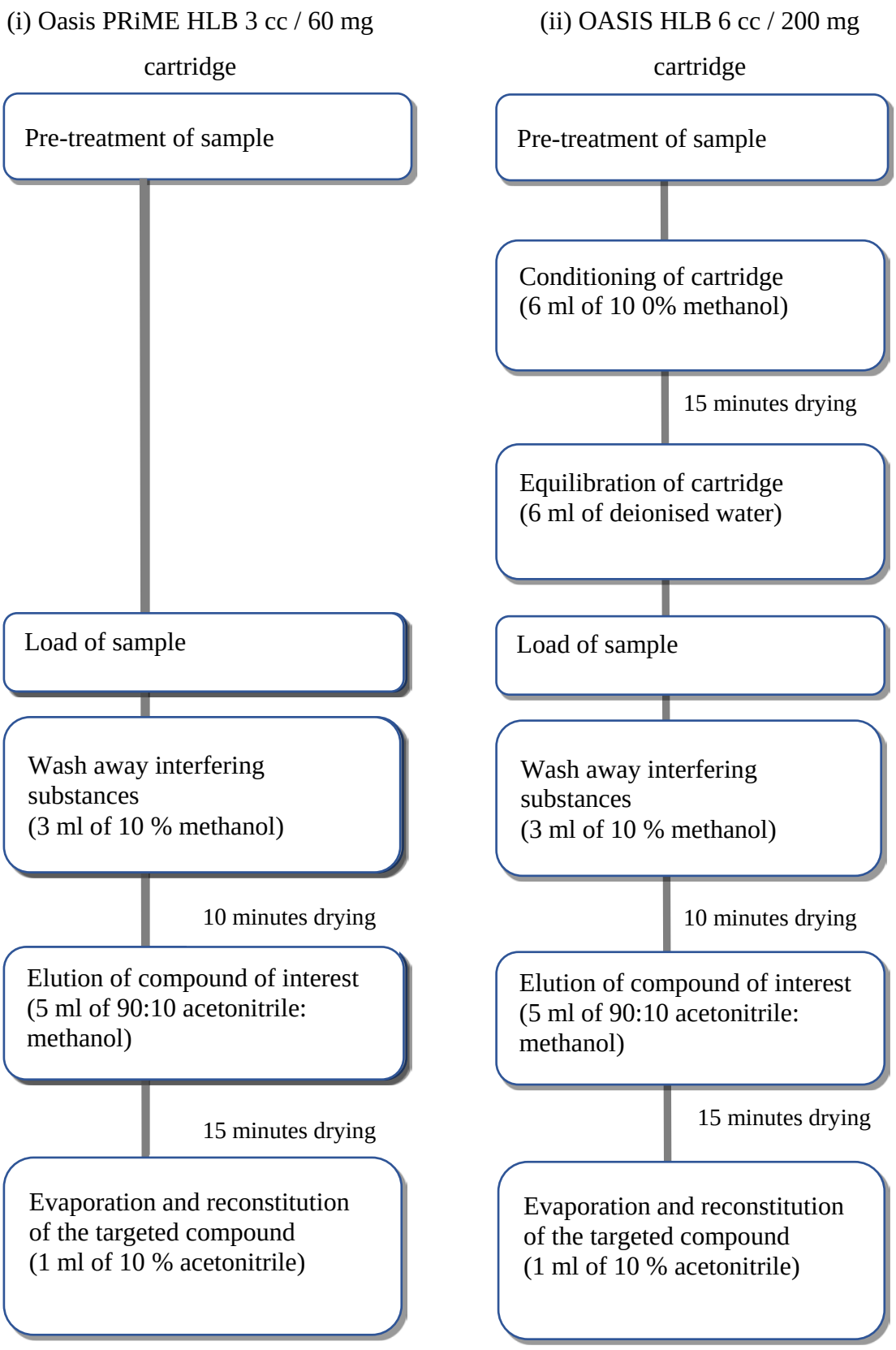


Figure 6.3 Solid phase extraction protocol (i) Oasis PRiME HLB and (ii) Oasis HLB cartridge

6.5 Preliminary estrogen removal experiments (part per thousand)

6.5.1 Evaluating the performance of bacterial strains in removal of estradiol

A preliminary experiment was conducted mainly to evaluate some of the most efficient bacterial strains (based on CSH value) which were expected to produce a high degree of estrogen removal. These shortlisted bacterial strains were then utilised in more extensive estrogen removal assays.

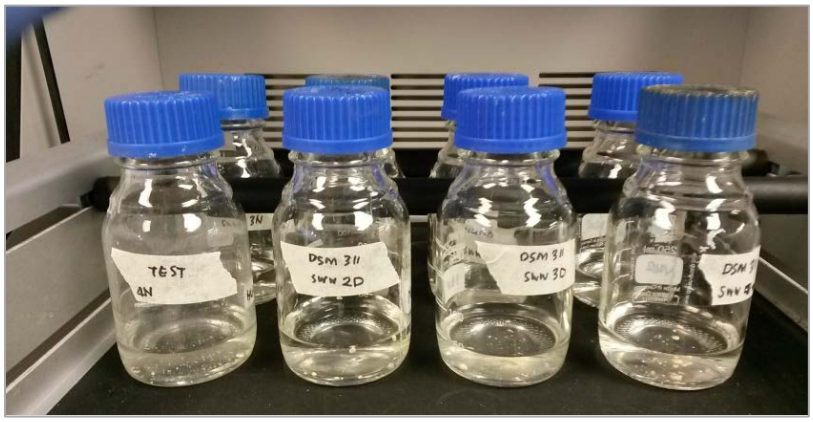


Figure 6.4 Batch reactors for the preliminary experiment to evaluate the most efficient bacterial strains for use in subsequent work. The 50 ml samples contained 1 mg.L^{-1} of 17β-estradiol (E2) in synthetic wastewater.

2.5 mg samples of each bacterial biomass of strains NCIMB13064, DSM311, PD2-1, PD2-3, SD1-1, SD2-1 as well as *E. coli*, as a calculated volume from the stock biomass (Section 6.2) was added to 50 ml of synthetic wastewater containing 1 mg.L^{-1} of E2. The batch reactors were agitated for 24 hours at 20 °C in an orbital incubator as 155 rpm. 1 ml of each sample was loaded into an Oasis PRiME HLB cartridge 3 cc/60mg and SPE was conducted according to the protocol shown in Figure 6.3 (i) before analysis by HPLC-ECD (Section 6.8).

6.5.2 Evaluating the performance of bacterial strains in removal of estrone

Similar preliminary experiment was further conducted except E1 was used as estrogen model and all bacterial strains had previously been inhibited before the estrogen removal process.

6.5.3 Comparison of estrone removal by live and inhibited bacteria

The above experiment was repeated using live bacterial strains (no thermal pre-treatment) (LB) to compare the performance in estrogen removal of live (both adsorption and biodegradation processes) and thermally inhibited (adsorption process only) bacteria (IB). The tops of reactors containing live bacterial cells were left open and the incubator was wedged open to allow for oxygen supply. All samples were tested in duplicate.

6.6 General adsorption process (part per trillion)

This experiment was conducted using estrogen at a lower concentration of 200 ng.L^{-1}, which is near to the average amount present in real wastewater as determined in the literature (Section 2.2.1). Batch reactors with high volumes (500 ml) were employed (Figure 6.5).

Figure 6.5. Batch reactors for optimisation of estrogen removal via adsorption process. The 500 ml samples contained 200 ng.L^{-1} of estrone in synthetic wastewater.

A 200 ng.L^{-1} of E1 in 500 ml of synthetic wastewater in 1.0 litre of amber bottle was prepared by adding 100 µl of 1.0 mg.L^{-1} of estrogen stock (Section 6.3.1). 5.0 mg of adsorbent was added to 500 ml of synthetic wastewater containing 200 ng.L^{-1} of E1. The batch reactors were agitated for 24 hours at 20 °C in an orbital incubator as 155 rpm. A volume of 500 ml of each sample was loaded into an Oasis PRiME HLB cartridge 6cc/200mg and SPE was conducted according to the protocol shown in Figure 6.3 (ii) before analysis by HPLC-ECD (Section 6.8).

6.6.1 General quantification process for estrone concentrations (part per trillion)

The final concentrations of estrone in batch reactors were measured after completion of 24-hour reaction process. Adsorbents were removed from the wastewater by centrifugation at 4,000 rpm and 500 ml of each supernatant was loaded into the OASIS HLB 6cc 200 mg SPE cartridge according to the protocol described in Figure 6.3 (ii) before analysis by HPLC-ECD (Section 6.8). The larger capacity SPE cartridge of OASIS HLB 6cc 200 mg was used compared to the preliminary study due to the higher volume of sample (500 ml) employed.

6.7 Optimisation process

Optimisation of the estrogen removal process was conducted by determining the optimum contact time and adsorbent dosage.

6.7.1 Contact time

The experiment was carried out using a parallel method, lower concentrations of estrogen and high reactor volumes (500 ml) were employed. A serial method (removing multiple time-course samples from the same bottle) was impractical because the volume of all aliquots taken from a reactor in interval enumeration must not exceed 1 % of the total, as recommended by the Organisation for Economic Co-operation Development (2000). The amount of 1 % from 500 ml at a concentration of 200 $ng.L^{-1}$ would carry an extremely small amount of estrogen, below the limit of detection of the instrument, and therefore the removal process could not have been monitored properly. Therefore, this optimisation study used the parallel method, and the SD2-1 bacterial strain was employed as the adsorbent model.

The bacterial strain SD2-1 was cultivated for 5 days and then was thermally pre-treated (Section 6.3.2). Estrone (E1) was then added at a concentration of 200 $ng.L^{-1}$ in a 500 ml of synthetic wastewater contained in a batch reactor of 1 litre amber bottle. A 2.5 mg (9.0 ml bacterial culture) of the thermally pre-treated SD2-1 culture was added into the reactor. A series of 18 triplicate reactors were incubated at 20 °C and 250 rpm for 10 minutes, 30 minutes, 1 hour, 6 hours, 12 hours and 24 hours of adsorption. After completion of the adsorption process, the concentration of estrone was then quantified (Section 6.6.1).

6.7.2 Effect of bacterial adsorbent dosage

The concentration of the inhibited bacterial strain SD2-1 adsorbent was varied in a range from low to high amounts of biomass (0.3 to 10 $mg.L^{-1}$). The biomass quantities were achieved by adding 0.6, 1, 3, 6, 9, 12, 15 and 18 ml volumes of biomass sample (Table 6.1). These different masses of bacterial adsorbent were added to identical 1 litre amber bottles containing 500 ml of synthetic wastewater and 200 $ng.L^{-1}$ of E1, and agitated at 250 rpm in an incubator at 20 °C. After 24 hours, final concentration of E1 was measured according to Section 6.6.1.

6.7.3 Exponential vs Stationary bacterial growth phase performance

The highest CSH of bacterial strains SD2-1, DSM 311 and NCIMB13064, were collected while at their exponential and stationary growth phases, which were previously identified as taking place on days 5 and 10 respectively. Volumes of biomass representing 5.0 mg of the exponential and stationary phase bacterial adsorbent was determined beforehand (Table 6.8). The bacterial cultures were thermally pre-treated (Section 6.3.2) and added to batch reactors containing 200 $ng.L^{-1}$ of E1 in 500 ml of synthetic wastewater. Each set of exponential and stationary phase reactors were prepared in triplicate. The reactors were agitated in an incubator at 250 rpm at 20 °C for 24 hours and estrogen was quantified as in Section 6.6.1.

Table 6.2 Volume of bacterial strain SD2-1 representing different concentration of biomass adsorbent.

Biomass concentration ($mg.L^{-1}$)	Volume (ml)
0.0	0.0
0.3	0.6
0.5	1.0
1.6	3.0
3.3	6.0
5.0	9.0
6.6	12.0
8.3	15.0
10.0	18.0

6.7.4 Comparison of estrone removal by live and inhibited bacteria

Removal of estrone by the stationary growth phase of live bacterial strains (LB) was also measured to compare the performance with thermally inhibited bacteria (IB). 10 $mg.L^{-1}$ of bacterial adsorbent strains NCIMB13064, DSM311 and SD2-1 were added to the batch reactors containing 200 $ng.L^{-1}$ of E1 in 500 ml of synthetic wastewater. Each set of LB and IB reactors were prepared in triplicate and agitated in an incubator at 155 rpm (allow for biodegradation process) and 250 rpm (adsorption only) respectively at 20 °C for 24 hours and final concentration of estrone was quantified as in Section 6.6.1.

6.8 Estrogen quantification

6.8.1 Measurement of estrone in HPLC-ECD

The concentration of estrone (E1) in the liquid phase of the reactors containing synthetic wastewater was purified and extracted using SPE, was determined by employing an HPLC with electrochemical detection (ECD). In the HPLC system, a Thermo Scientific HPLC UltiMate 3000 RS Pump, UltiMate 3000 RS autosampler and column compartment were used. A reverse phase UHPLC column was used (Accucore C18), with dimensions 100 mm× 21 mm and 2.6 μm particle size (Thermo Scientific). Ninety microliters of estrone standard mixtures or samples in the isocratic profile mobile phase of 62:38 (v/v) of solvent A:B were injected in the column at a flow rate of 0.4 $ml.min^{-1}$ for 5 minutes. Solvent A was water-acetonitrile (95:5, v/v) with 0.1 % formic acid, and Solvent B was acetonitrile-water (95:5) with 0.1 % formic acid buffer. The UHPLC column was connected to the electron capture detection (ECD) chamber of an

UltiMate 3000 ECD-3000RS Electrochemical Detector. Boron-doped cell potential was set at 1800 mV. The concentration of E1 was then determined based on its peak area in the HPLC-ECD chromatogram (Section 6.9.3).

6.8.2 Degree of estrogen removal

Degree of estrogen removal was calculated using Equation 6.1.

$$\frac{C_0 - C_e}{C_0} \times 100\% = \text{Removal of estrogen by the adsorbent (\%)} \qquad \text{(Equation 6.1)}$$

Where C_0 is the initial concentration, C_e is the final concentration of estrogen (after removal treatment) (Ifelebuegu *et al.*, 2015).

6.9 Results and discussion

6.9.1 Growth yields from bacterial cultures and soil-isolates

Biomass production was measured in 10 ml cultures grown on liquid MM1 (Table 4.1) after 5 days of incubation for each bacterial strain, and the results summarised in Table 6.3. From a statistical analysis using One-way ANOVA and Tukey pairwise comparisons, it was determined that there were no significant differences in the biomass weight between all of the adapted bacterial strains. However, when compared to *E. coli*, all of the bacterial strains were found to exhibit significantly lower growth yield ($p < 0.05$). The objective of this experiment was to determine the volume of bacterial culture representing 2.5 mg of biomass adsorbent so that the appropriate volume could be added to each batch reactor in the adsorption experiments. The biomass weight obtained from 10 ml of each bacterial culture was then converted into a volume representing 2.5 mg biomass (Table 6.4).

Table 6.3. Dry-weight of hydrophobic-adapted bacterial strains biomass measured after 5 days of incubation for the exponential growth phase cells.

Biomass yield for different bacterial strains (mg / 10 ml)[1]							
DSM311	NCIMB13064	SD1-1	SD2-1	PD2-1	PD2-2	PD2-3	E.coli
3.95[b]	3.55 [b]	3.00 [b]	2.80 [b]	3.35 [b]	2.85 [b]	4.80 [b]	13.75 [a]

[a,b] Means that do not share a letter are significantly different ($p < 0.05$).

[1] The Liquid MM1 was used for the adapted bacterial strains and nutrient broth for the *E.coli.* The initial test showed that *E.coli* could not grow on n-hexadecane growth substrate. Data not presented.

Table 6.4 Volume of bacterial cultures representing 2.5 mg of biomass adsorbent when harvested at day 5 of incubation.

Strains	Volume of bacterial culture (ml)
DSM311	6.0
NCIMB	7.0
SD2-1	8.9
SD1-1	8.3
PD2-1	7.9
PD2-2	8.7
PD2-3	5.2
E. coli	1.8

6.9.2 Development of SPE method for the preliminary experiments

A preliminary trial with loadings of different volumes, e.g. 1 ml and 50 ml of synthetic wastewater containing 1 $mg.L^{-1}$ estrogen were tested to determine the capacity of the SPE cartridge (Oasis PRiME HLB 3 cc /60 mg cartridge) in order to assess its capacity in subsequent experiments. In practice, it was decided that either a reactor sample of 50 ml that carries 50 µg of analyte could be loaded into the cartridge or, alternatively, a lower volume of 1 ml (carrying 1 µg) that represents 2 % of the total sample could be used, provided the recovery rate is within 71 - 95 % (Fayad *et al.*, 2013).

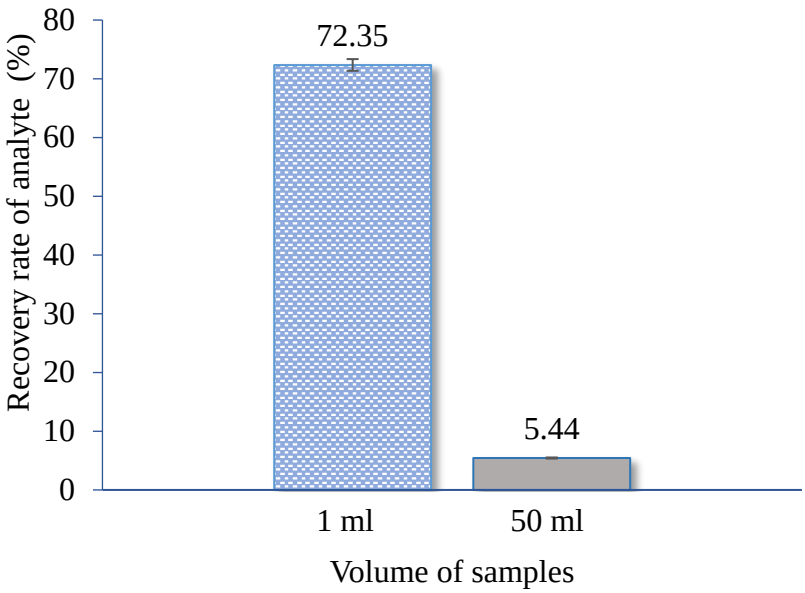


Figure 6.6 Recovery rate for different volumes of samples carrying 17β-estradiol (E2) analyte loaded into the SPE cartridge of the Oasis PRiME HLB 3 cc / 60 mg. The E2 concentration was the same in both samples.

Results for analyte recovery rates using the 3 cc / 60 mg SPE cartridge of Oasis PRiME HLB are shown in Figure 6.6. A volume of 50 ml (carrying 50 µg of estrogen) was found to produce an extremely low average recovery rate of 5.44 ± 0.10 %, presumably because the column became overloaded. In contrast, a good recovery rate of 72.35 ± 5.16 % was obtained by loading a 1 ml sample which carried 1µg of analyte. The latter recovery rate was in the range recommended (Fayad *et al.*, 2013). Therefore, the volume of samples that would be needed for analysis was set at 1 ml, as this was compatible with the capacity of the cartridge, and was utilised in the preliminary experiment comparing the performance of the bacterial strains.

6.9.3 SPE method development for the optimisation experiment

A higher capacity of cartridge (OASIS HLB 6 cc / 200 mg) was employed for the optimisation experiment, and its recovery rate for 100 ng of estrogens was determined. Figure 6.7 displays a typical chromatogram from the HPLC-ECD system, with peaks identified for each estrogen compound, in which E3 eluted first at a retention time of 1.4 minutes, followed by E2 at 3.8 minutes and lastly E1 at 5.6 minutes. The specific retention times were identified from separate runs of each estrogen as individual calibration standards.

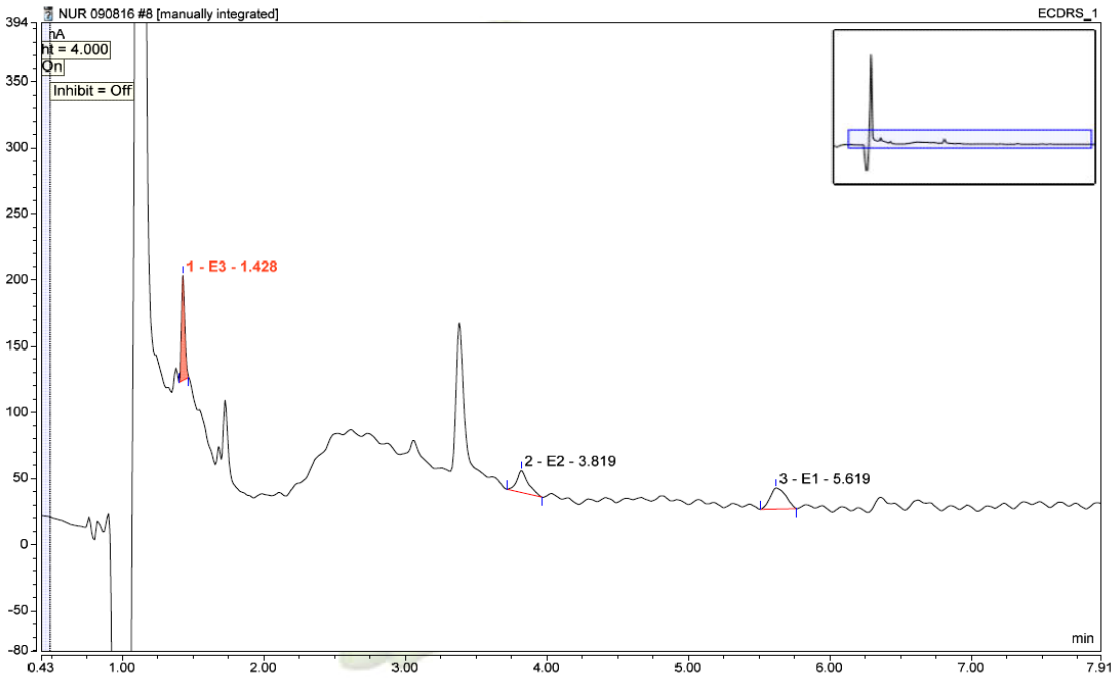


Figure 6.7 Chromatogram showing E1, E2 and E3 peaks observed in the HPLC-ECD system.

Areas of the identified peaks of E1, E2 and E3 were calculated and compared against the areas of a 6-point standard curve in the range 20 ng to 150 ng. Using the response derived from standards, the concentration of estrogens was determined and the average recovery rate for 100 ng of analyte was found to be 81 % for E1, 94 % for E2 and 67 % for E3 (Figure 6.8). Therefore, the OASIS HLB 6cc 200 mg cartridge was considered to be a better extraction tool for E1 and E2 in the optimisation study than the Oasis PRiME HLB 3 cc/ 60 mg SPE cartridge. Nevertheless it was suggested as less suitable for E3 due to its lower percentage of recovery.

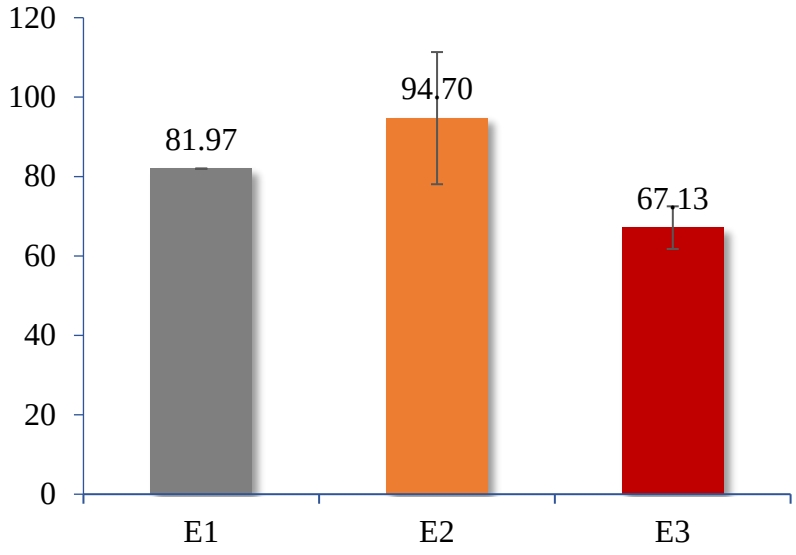


Figure 6.8. Recovery rate of 500 ml of 200 ng.L^{-1} sample, containing 100 ng of estrogen compound in the OASIS HLB 6 cc 200 mg SPE cartridge.

6.9.4 Preliminary evaluation of estrogen removal performance by bacterial strains

This preliminary study was conducted using the adapted bacterial strains collected after 5 days of incubation. The purpose of this study was to investigate relationship between the cell surface hydrophobicity (CSH) of the bacteria and their performance in removing estrogens. The first estrogen removal assay was conducted using E2 due to its high hydrophobicity. The adapted bacteria and *E.coli* were all added to the reactors without being thermally pre-treated in order to give a preliminary evaluation on their performance in removing estrogen.

Figure 6.9 presents results of final concentrations of E2 and E1 after the estrogen removal treatment. Degree of estrogen removal was calculated using Equation 6.1 (Section 6.8.2). All value were reported as measured after the SPE extraction and were not adjusted for loses during SPE. Strains of PD2-1 and NCIMB13064 were thought to completely degrade and adsorbed all of the E2, however 0.2 $mg.L^{-1}$ and 0.44 $mg.L^{-1}$ of E1 were also found in the reactors respectively. The presence of E1 considered that E2 has been partially degraded into E1, thus, complete removal of estrogens was eventually not achieved. The high estrogen removal in reactor containing NCIMB13064 correlates with its high CSH. However, bacterial strains DSM311 and SD2-1, which were also found to have high CSH values (Section 5.4.1) reduced the E2 level to 0.29 $mg.L^{-1}$ and 0.16 $mg.L^{-1}$, respectively. Nevertheless, high amounts of E1 were detected in the reactors, and this does not represent efficient performance in terms of total estrogen removal. Poor total estrogen removal efficiency was observed for almost all of the bacterial strains except NCIMB13064 and PD2-1. As expected from the CSH results, *E.coli* showed the lowest degree of total estrogen removal, probably due to its hydrophilic characteristics (CSH of 38 %). *E. coli* was selected as control as it is always presents in municipal wastewater and does not have the capability to degrade estrogens.

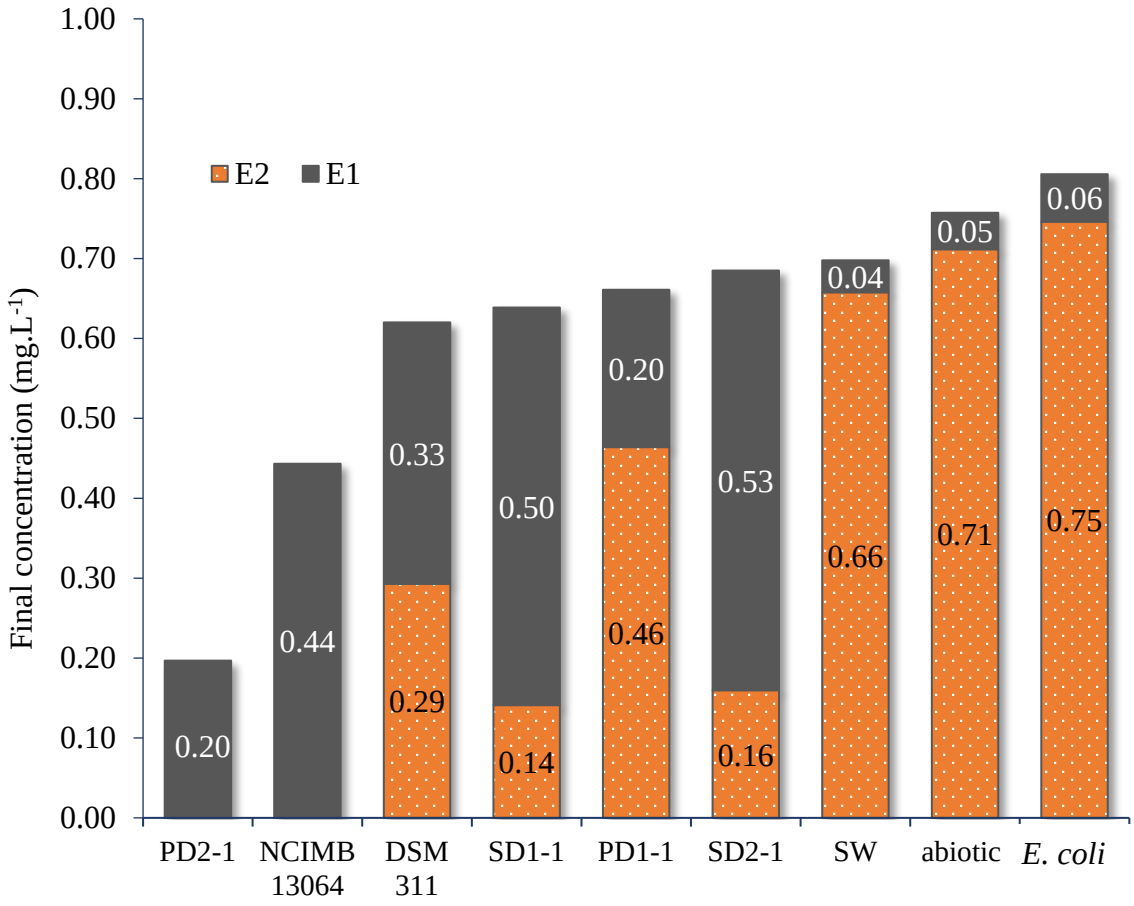


Figure 6.9 Final concentrations of estrogen (E2 and E1) after combined biodegradation and adsorption processes with live hydrocarbon-adapted bacterial strains compared to E.coli from initial concentration of 1.0 mg.L^{-1} of E2 in 1 ml samples.

Interestingly, 0.76 mg.L^{-1} final concentration, or 76 % recovery, of estrogen was detected in the abiotic control sample, and this was slightly higher than the cartridge recovery rate of 72.35 % obtained in the method development (Section 6.9.2). This confirms that reduction in the final concentration of estrogens in the samples containing bacterial adsorbents were due to the bacterial activity, and there was negligible interference from glassware adsorption, soluble organic matter adsorption, or photo-degradation.

Unfortunately, the conversion of E2 to E1 seen in this study, and reported in several other biodegradation studies (Yu *et al.*, 2013; Fernández *et al.*, 2017) made the quantification of bacterial performance relatively complicated. Therefore, the evaluation of estrogen removal in subsequent assays was conducted by employing E1 as the model estrogen compound since it has the same aqueous solubility as E2, at 13.0 mg.L^{-1} (Silva *et al.*, 2012; Adeel *et al.*, 2016).

6.9.5 Evaluating the performance of bacterial strains in removing estrone

Further experiments on the removal of estrone (E1) were carried out, focussing on removal mechanism of adsorption, by utilising thermally pre-treated biomass to inhibit biodegradation processes (Section 6.5.2). Final concentrations of E1 in the liquid phase after 24 hours of adsorption are shown in Figure 6.10 and the percentage of E1 removal presented in Figure 6.11. The removal process taking place in these reactors is considered to be mainly attributed to the adsorption process, due to the thermal inhibition of the bacterial cells. The biodegradation process was considered to be absent due to the denaturing of the bacterial enzymes in thermal pre-treatment at 85 °C. In addition, the reactor contents were odourless at the end of the experiment, in contrast to the strong odour which was usually found after the biodegradation/adsorption removal process with live cells, due to the decomposition of organic matter by the live bacteria (Garcha *et al.*, 2016).

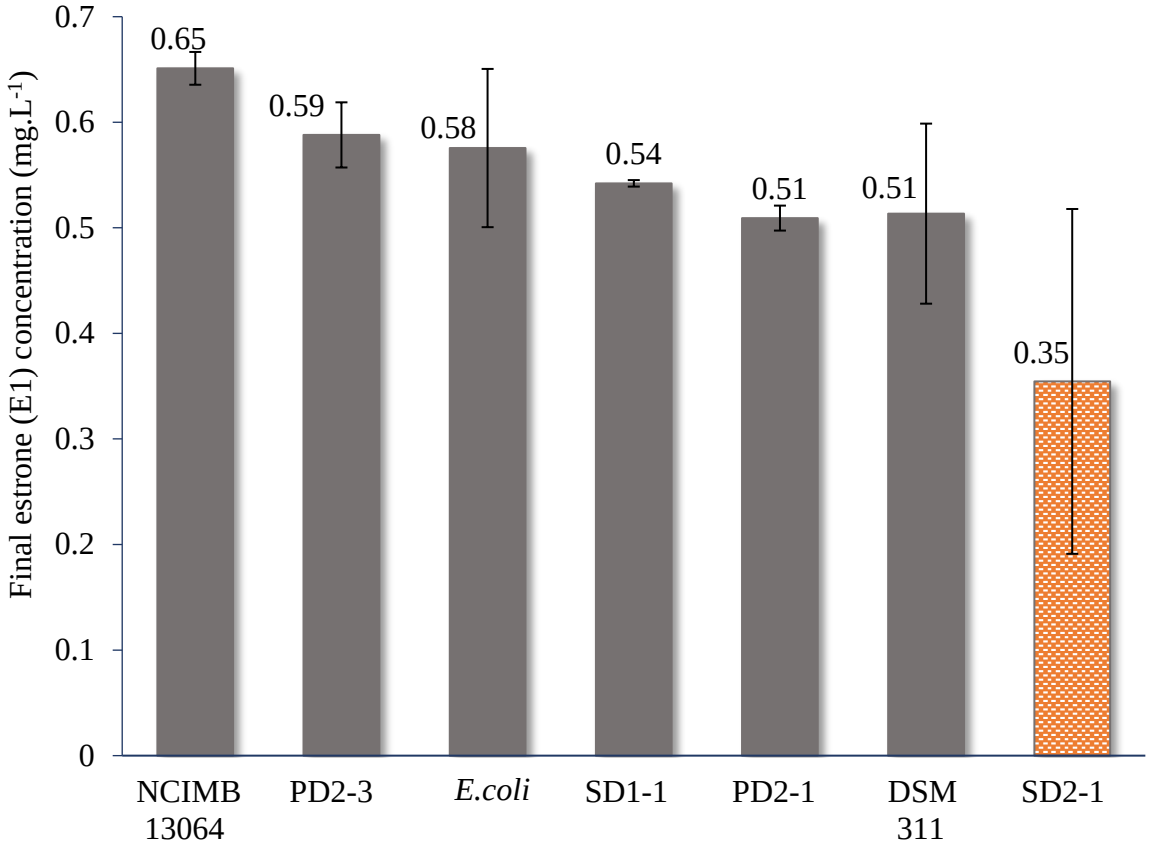


Figure 6.10 Final concentrations of E1 in the liquid phase after the adsorption process. The adsorbents were thermally inhibited hydrophobic-adapted bacterial strains, the reactor contained synthetic wastewater with an initial E1 concentration of 1 $mg.L^{-1}$.

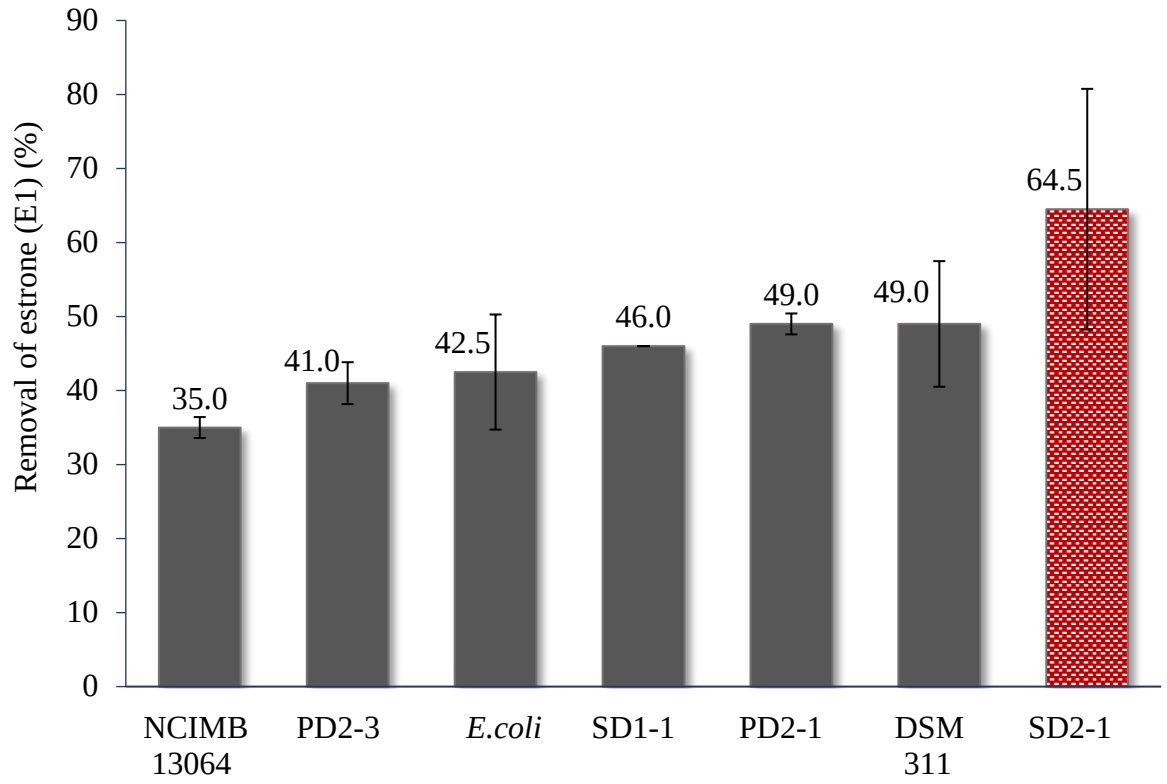


Figure 6.11 Degree of E1 removal in the liquid phase after the adsorption process. The adsorbents were thermally inhibited hydrophobic-adapted bacterial strains, the reactor contained synthetic wastewater with an initial E1 concentration of 1 $mg.L^{-1}$.

Throughout this experiment, the SD2-1 bacteria was found to produce the highest estrone (E1) removal of 64.5 ± 16.3 % in comparison to the other adapted bacterial strains shown in Figure 6.11, with final concentration of 0.35 $mg.L^{-1}$ remaining in the aqueous phase after treatment (Figure 6.10). Nevertheless, results of one-way analysis of variance (ANOVA) shown in Table 6.5 demonstrate that no significant difference ($p > 0.05$) E1 removal efficiency was found between the bacterial strains. The test however, could discriminate between strains with strong or weak estrone removal ability.

Table 6.5 Analysis of degree of estrone (E1) removal in the adsorption process using inhibited hydrophobic-adapted bacterial strains.

	Estrogen Removal (%)						
Bacterial strains	NCIMB 13064	PD2-3	*E.coli*	SD1-1	PD2-1	DSM311	SD2-1
Estrone (E1) removal (%)	35^{b}	41^{ab}	43^{ab}	46^{ab}	49^{ab}	49^{ab}	64.5^{a}

[a.b] Means that do not share a letter are significantly different ($p < 0.05$).

As noted above, the SD2-1 strain produced higher E1 removal than the other bacterial strains, and therefore it was considered to be the most efficient bacterial strain in removing estrogens. This was followed by strain DSM311, at 49 % removal. These two higher rates of removal correlated with the high cell surface hydrophobicity (CSH) values of 87 % for DSM311 and 86 % for SD2-1, (Section 5.4.1). Based on the previous CSH experiments, the SD2-1, DSM311 and NCIMB13064 strains had been predicted to be the best candidate bacterial strains for the removal of estrogen due to their high CSH recorded in the range of 78 – 87 %. Surprisingly, NCIMB13064, with a CSH of 80 %, was shown to produce the poorest estrogen removal, which was even lower than that of *E. coli* which had the lowest CSH of 38 %. It is thought that this strain may have lost its hydrophobicity during the experimental work, and a thorough investigation of this was conducted and is discussed in Section 6.9.9.

6.9.6 Comparison of estrone removal by the Live and Inhibited bacteria

In addition to the adsorption process carried out using the inhibited bacteria (IB), estrone removal adopting live bacterial cells (LB) was also conducted.

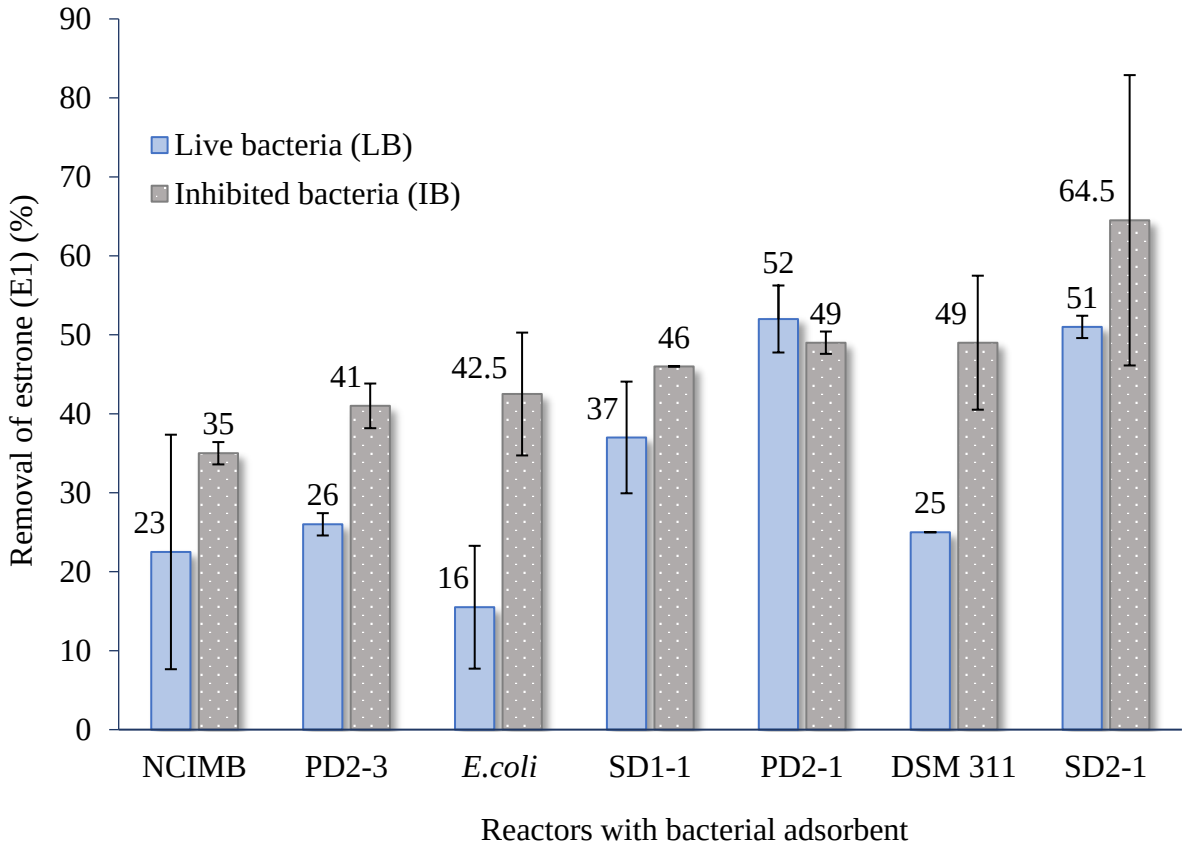


Figure 6.12 Comparison of estrone removal in reactors containing live bacteria (LB), or thermally inhibited bacteria (IB). All strains (except *E.coli*) had been adapted to generate hydrophobic strains using hydrocarbon growth substrate. Performance levels were compared with *E. coli*.

In theory, the actual process that could take place in the live bacteria (LB) reactor is suggested to be a combination of biodegradation and adsorption processes. Live bacterial cells have the ability to degrade the estrone compound and at the same time adsorb estrone molecules onto their highly hydrophobic cell surface. Nevertheless, according to the results of the analysis shown in Figure 6.12, the degree of estrone removal by strains in the LB reactor were actually lower than those in the Inhibited Bacteria (IB) reactor containing thermally pre-treated cells. The lower removal of estrone indicates that some interfering factor must have existed in the LB reactor, preventing the effective estrone removal by adsorption, because both biodegradation and adsorption should have taken place in the LB reactor and given higher removal than the IB reactor (adsorption processes only). This assumption of the presence of an interfering factor is supported by a number of publications in which either the adsorption or the biodegradation of estrogen has been reported to decline rapidly due to the presence of surfactants in wastewater (Zhang and Zhou, 2005; Kaczorek *et al.*, 2008; Koh *et al.*, 2008; Kaczorek and Olszanowski, 2011). Similarly, biological surfactants, known as biosurfactants, were considered to be the main inhibition factor that reduced the estrogen removal performance in the LB reactor in the present study. As discussed in Section 4.5.2, biosurfactants production were observed during the adaptation of the live bacteria with n-hexadecane growth substrate. Biosurfactants were known to be present in this culture due to the rapid emulsification of the hexadecane. In addition, the presence of biosurfactants was also observed as the formation of high turbidity in the LB reactors after the removal reactions were completed, compared to the IB reactor (Figure 6.13). The live bacteria in the LB reactor continued to grow during the removal process, using nutrients from the wastewater and residuals of hydrocarbon may have been carried over from the original bacterial culture, or from carbon sources stored intracellularly, and this led to the production of biosurfactants. In theory, additional amounts of glycolipid biosurfactant in the reactor will have increased the solubility of estrogen, in a process similar to the increasing solubility of hydrocarbon substrate for bacterial growth during the adaptation process. This eventually may have reduced the hydrophobicity of the estrogen and prevented its attachment to the hydrophobic bacterial cells, causing the reduction of the adsorption process due to less physical contact in LB reactor (Kaczorek *et al.*, 2008; Kaczorek and Olszanowski, 2011).

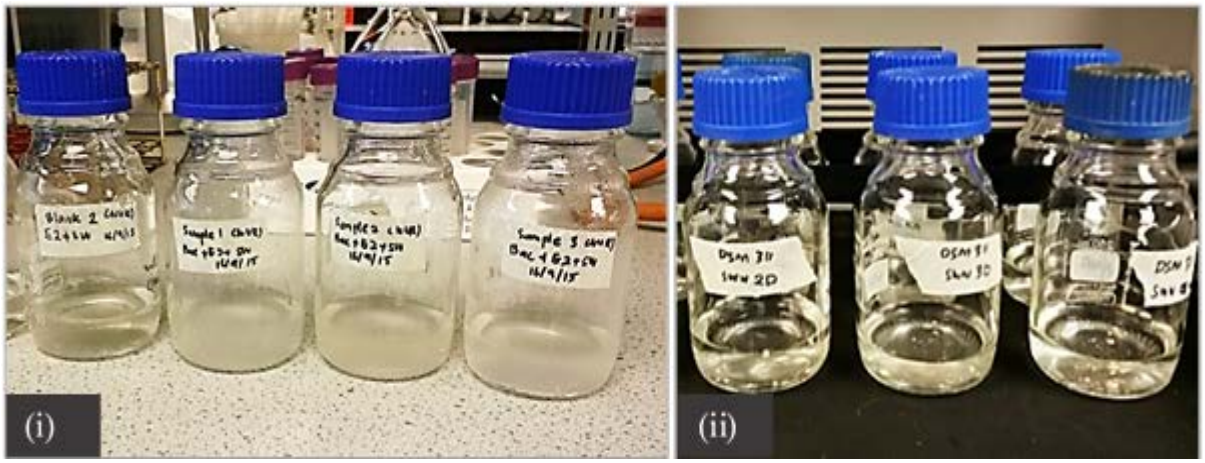


Figure 6.13. (i) The presence of biosurfactant in the reactor with live bacteria (LB) compared to the control (bottle on the left) and (ii) the clear solutions in reactors with inhibited bacteria (IB)

In addition to having lower levels of adsorption due to the presence of biosurfactants, removal of estrone via biodegradation in the LB reactor was inefficient because it is likely that none of the bacterial strains were good estrogen-degraders. *R. erythropolis* has been reported to have a moderate estrogen degrading ability in compared to other *Rhodococcus* genera such as *R. equi* and *R. rhodochrous* (Larcher and Yargeau, 2013). Moreover, it has also been shown to have a lower biodegradation performance, at only 10 % the added estrogen, compared to 47 % when a co-substrate was added (O'Grady *et al.*, 2009). In contrast, *E. coli* has been shown to be capable to synthesizing a natural conjugated estrogen, such as glucuronide, converting it into the active form estrogens in wastewater, thus bring out the harmful effect (Duong *et al.*, 2011). Because E1 is an unconjugated estrogen compound that *E.coli* could not degrade, and *E. coli's* low CSH should restrict the binding of E1, *E.coli* was expected to have the poorest performance in removing estrone in the LB reactor, which is what was observed (Figure 6.12).

In addition, relatively poor estrone removal (by biodegradation) occurred in all LB reactors because the bacteria had been previously grown in minimal media containing n-hexadecane growth substrate. Induction of estrogen degradative enzyme was suspected to not occur during the short-term (24 hour) exposure assays compared to 5 day period of adaptation that gave induction of cell hydrophobicity when cells were grown on n-hexadecane. Therefore biodegradation of estrone by the bacterial strains was difficult in the absence of the enzyme, as they could not use estrone for growth substrate (Li *et al.*, 2018).

These factors explain the lower estrogen removal produced in the LB reactor compared to the IB reactor. It may indicate that hydrophobic bacteria adapted using n-hexadecane may not be suitable for employment in the biodegradation process, but perform efficiently in estrone removal through an adsorption process due to their high CSH. In this light, further possible enhancements of removal performance were investigated through optimisation processes, and are presented in subsequent sections.

6.9.7 Optimisation of contact time

A study of the optimisation of estrogen removal was conducted using an initial concentration of 200 $ng.L^{-1}$ estrone (E1), which is close to the actual amounts present in real wastewater influent. According to the results shown in Figure 6.14, the concentration of E1 in the batch reactors containing thermally inhibited bacteria (IB) was found to rapidly decline from the initial concentration of 200 $ng.L^{-1}$ to 59.79 ± 1.75 $ng.L^{-1}$ after only 10 minutes of contact, and then continued to decline slowly over the next hour, finally reaching its minimum of 47.62 ± 1.37 $ng.L^{-1}$ after 24 hours of contact time. Statistical analysis using one-way ANOVA indicated that there was a significant effect ($p < 0.05$) of adsorption contact time on the concentration of E1, which was found took place in 30 minutes contact time (Table 6.6). However, the final values of concentration were found to gradually decrease only slightly until the end of incubation (24 hours) without significant differences. This indicates that the adsorption of E1 reached its equilibrium after 30 minutes and was complete after 24 hours.

Table 6.6. Final concentration of estrone (E1) in the liquid phase after the adsorption process according to contact time.

	Final concentration ($ng.L^{-1}$)						
Contact time (hours)	0.00	0.17	0.5	1.0	6.0	12.0	24.0
Bacterial strain SD2-1	200^{a}	59.79^{b} ± 1.75	51.17^{c} ± 4.47	48.04^{c} ± 1.97	51.60^{c} ± 0.02	52.79^{c} ± 1.43	47.62^{c} ± 0.96

[a, b, c] Means that do not share a letter are significantly different ($p < 0.05$).

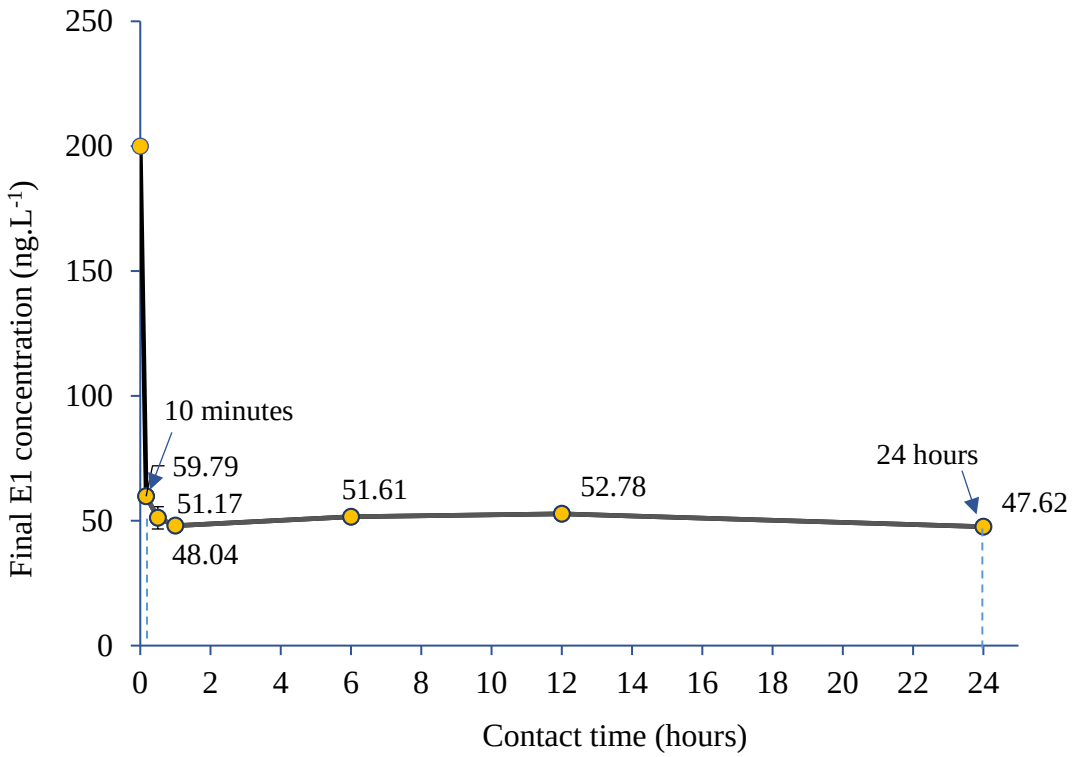


Figure 6.14 Effect of contact time on estrone (E1) removal using 2.5 mg (5 mg.L^{-1}) of SD2-1 bacterial adsorbent and an initial estrone concentration of 200 ng.L^{-1}

Generally, the adsorption of E1 has been reported to be a spontaneous process which achieves its equilibrium in less than one hour (Ren *et al.*, 2007b; Racz and Goel, 2010), or more than 1 hour (Zhang *et al.*, 2012; Hartmann *et al.*, 2014; Zheng *et al.*, 2016), and in some adsorption experiments, tests were only conducted over 24 hours (Hemidouche *et al.*, 2017). Consequently, contact time is found to have a significant effect on the efficiency and equilibrium of the adsorption process, although the effect may vary depending on other factors such as type of adsorbent as well as the experimental set-up.

6.9.8 Optimisation of adsorbent dosage

Further optimisation of the estrone (E1) removal process was conducted by investigating the effect of adsorbent dosage. The amount of *Tsukamurella* sp. SD2-1 bacterial adsorbent added was varied and the contact time was fixed at 24 hours. The objective of this study was to find an optimum dosage of biomass in order to achieve the best E1 removal performance at realistic, environmentally relevant E1 levels. The bacterial adsorbent dosage was varied between 0.0 – 10.0 mg.L^{-1}, which the highest dosage of 10.0 mg.L^{-1} contains 5.0 mg of adsorbent added to 500 ml of synthetic water.

Table 6.7. Final concentration of estrone (E1) in the liquid phase after 24 hours of the adsorption with different dosages of *Tsukamurella* sp. SD2-1 bacterial adsorbent.

Adsorbent dosage ($mg.L^{-1}$)	Final concentration of estrone (E1) ($ng.L^{-1}$)								
	0	0.3	0.5	1.6	3.3	5.0	6.6	8.3	10.0
Bacterial strain SD2-1	168.5^{a} 0	41.44^{b} ± 2.34	31.77^{bc} ± 0	27.11^{bc} ± 8.02	19.35^{cd} ± 5.33	18.88^{cde} ± 0.39	8.95^{def} ± 2.22	4.82^{ef} ± 2.49	3.08^{f} ± 3.08

$^{a, b, c, d, e, f}$ Means that do not share a letter are significantly different ($p < 0.05$).

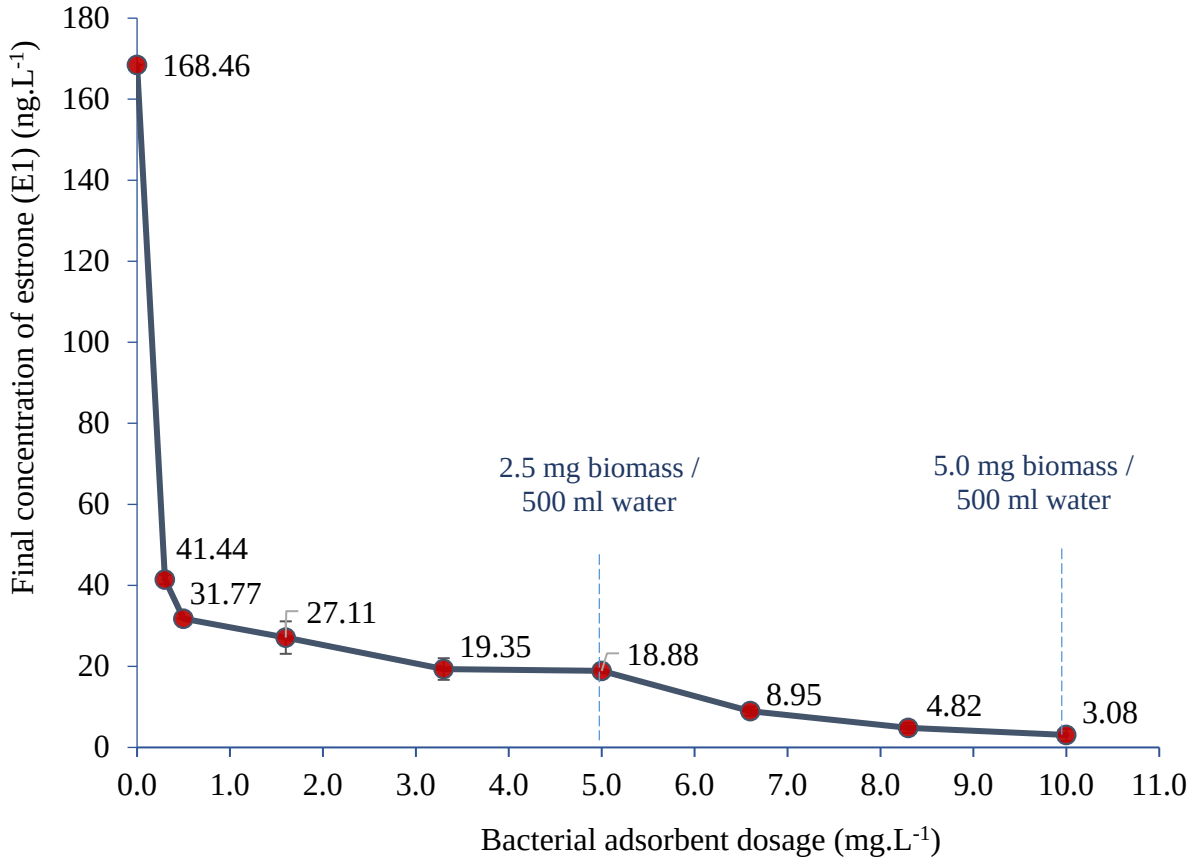


Figure 6.15. Effect of adsorbent dosage on estrone (E1) removal using SD2-1 bacterial adsorbent. Initial estrone concentration was 200 $ng.L^{-1}$, and final concentration was measured after 24 hours of adsorption.

Through statistical analysis using one-way ANOVA and Tukey pairwise comparison, it was found that adding different dosages of *Tsukamurella* sp. SD2-1 bacterial adsorbent to the reactor produced significantly different ($p < 0.05$) concentrations of estrone (Table 6.7).

The highest bacterial adsorbent dosage of 10 mg.L^{-1} (equal to 5.0 mg of biomass) added into the reactor had the highest degree of adsorption, leaving a final estrone concentration of 3.08 ± 3.08 ng.L^{-1} of E1 in the wastewater, when compared to the effect of 9 ml of adsorbent (equal to 2.5 mg of biomass) which gave a final estrone concentration of 18.88 ng.L^{-1}, which had been used in the preliminary experiment (Section 6.5). This is due to the fact that the supply of more adsorbent into the reactor will provide a higher surface area for the attachment of the estrogen, especially when a highly hydrophobic *Tsukamurella* sp. SD2-1 comes into contact with the hydrophobic estrone molecule. Therefore, adsorbent dosage has been determined to be a factor which significantly affects the performance of adsorption. Further increases in adsorbent dosage greater than 18 ml is predicted to produce even better E1 removal and lower final concentrations, but this was not tested. However, the addition of more adsorbent would add cost to the process. The use of 5.0 mg of bacterial biomass compared to 100 ng of E1 in the 500 ml reactions represents a ratio of 50,000:1 adsorbent: analyte in w/w. This achieved final estrone levels below the PNEC of E1 (3.6 ng.L^{-1}).

In comparison, the final concentration 18.88 ng.L^{-1}, achieved using 2.5 mg of the same amount of bacterial adsorbent gave less than half the final estrone concentration at 47.62 ng.L^{-1}. It is suspected that the stock solution of E1 used in this experiment had become degraded and thus lower concentrations of estrone were obtained. However, the observed pattern of the reduction in E1 concentration with bacterial dosage (the line in Figure 6.15 does not appear to have plateaued) suggests that increases in adsorbent dosage would lead to lower final concentrations of estrone, due to the higher adsorption of E1 by the additional bacterial cells.

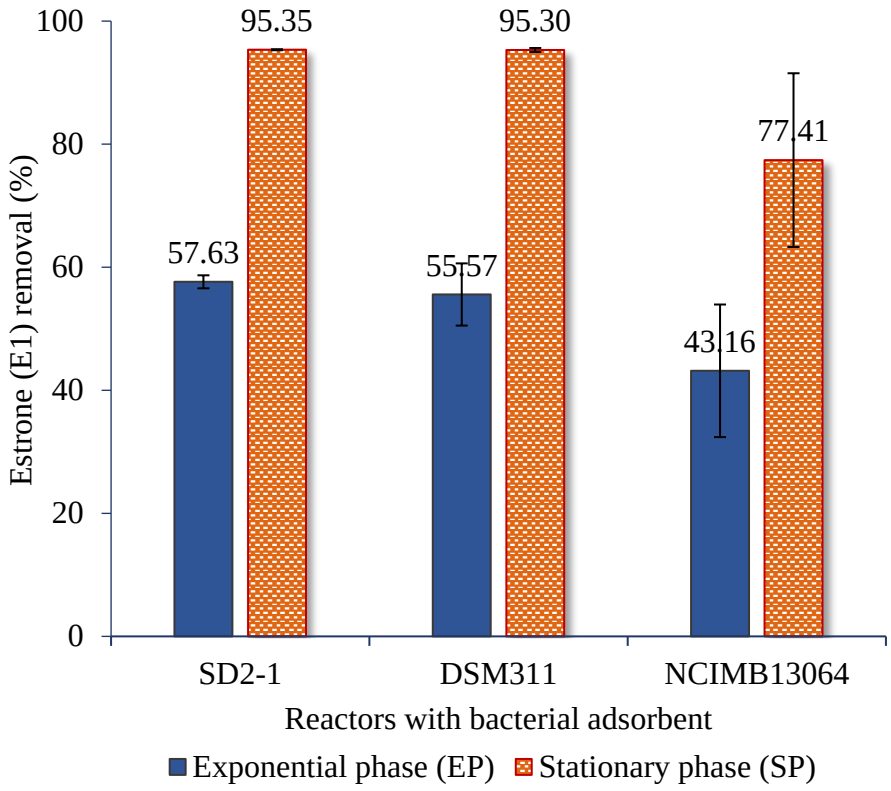
6.9.9 Estrogen removal by bacterial adsorbents prepared from different phases of growth

To investigate further enhancement of the removal of E1, the adsorption process was conducted using bacterial adsorbents prepared at different growth phases for bacterial strains showing the highest CSH values, namely *Tsukamurella* sp. SD2-1, *R. erythropolis* DSM311 and *R. erythropolis* NCIMB13064. Exponential and stationary phase bacterial cells were harvested at days 5 and 10 of the incubation period respectively, as previously identified in Chapter 4. Reaction conditions were standardised to 24 hours of contact time, 200 ng.L^{-1} E1, and 5.0 mg of bacterial adsorbent dosage. Actual volumes of adsorbent preparation that was needed for 5.0 mg of biomass at the exponential and stationary phase of each bacterial strains determined at day 5 and day 10, respectively, are presented in Table 6.8.

Table 6.8. Volumes of the biomass adsorbent preparation needed to achieve 5.0 mg of bacterial biomass, at the exponential and stationary phases of bacterial growth.

Strains	Volume of bacterial culture (ml)	
	Exponential phase (day 5)	Stationary phase (day 10)
DSM311	12.00	9.33
NCIMB	14.00	8.542
SD2-1	18.00	8.542

Figure 6.16 Degree of E1 removal by adsorption for bacterial strains with the highest CSH



(DSM311, NCIMB13064 and SD2-1), comparing biomass adsorbents prepared from the exponential and stationary growth phases. Reactions contained 5.0 mg of bacterial biomass and an initial E1 concentration of 200 ng.L^{-1}.

Figure 6.16 shows a comparison of the performance of estrogen removal by the exponential phase (EP) and stationary phase (SP) of *Tsukamurella* sp. SD2-1, *R. erythropolis* DSM311 and *R. erythropolis* NCIMB13064. High percentages of estrogen removal are identified in the batch reactors containing SD2-1 and DSM311 strains, with the SP reactor of the SD2-1 adsorbent removing 95.35 ± 0.08 % of estrogen from the water samples, compared to 57.63 ± 1.10 % in

the EP reactors. These estrogen removal levels were found to be significantly different using the 2-sample t-test ($p < 0.05$) (Table 6.9). In addition, the percentages of removal were also found to be significantly different ($p < 0.05$) in the batch reactors consisting of the stationary phase DSM311 strain (95.30 ± 0.33 %) compared to the exponential phase DSM311 (55.57 ± 5.05 %) (Figure 6.16).

Table 6.9 Analysis of the degree of estrone removal by adsorption for bacterial strains with the highest CSH (DSM311, NCIMB13064 and SD2-1), comparing biomass adsorbents prepared from the exponential and stationary growth phases. Reactions contained 5.0 mg of bacterial biomass and an initial estrone (E1) concentration of 200 ng.L^{-1}.

Bacterial strains	Estrone removal (%) *		Significance (p-value)
	Exponential phase (EP)	Stationary phase (SP)	
Tsukamurella sp. SD2-1	57.63	95.35	0.000
R. erythropolis DSM311	55.57	95.30	0.005
R. erythropolis NCIMB13064	43.16	77.41	0.044

* Estrogen removal by Live Bacteria (LB) due to adsorptiona and biodegradation, estrogen removal by thermal Inhibited Bacteria (IB) is due to adsorption only.

Interestingly, the reactors containing adsorbent from NCIMB13064 strain unexpectedly produced lower estrogen removal compared to SD2-1 and DSM311, despite having the highest cell surface hydrophobicity of these three strains. Even though the level of estrogen removal in the NCIMB13064 SP reactor was approximately 18 % lower than that in the SP reactors containing SD2-1 and DSM311 adsorbents, the NCIMB13064 SP reactor still adsorbed estrogen significantly ($p < 0.05$) better than the equivalent EP reactor with 77.41 ± 14.1 % compared to 43.2 ± 10.8 %, being observed, respectively. The higher degree of estrogen removal in SP reactors for all of the bacterial strains represents very strong evidence that different bacterial growth phases affect estrogen removal performance. Moreover, the increase in adsorption percentage in all SP reactors can be correlated with the higher CSH values of stationary bacterial cells compared to the exponential phase (Table 6.9).

Table 6.10 CSH values of bacterial strains as a function of the growth phase (data reproduced from Chapter 3)

Bacterial strains	Exponential phase CSH (%)	Stationary phase CSH (%)
Tsukamurella sp. SD2-1	86	91
R. erythropolis DSM311	87	93
R. erythropolis NCIMB13064	80	92

These results clearly support the hypothesis that a higher cell surface hydrophobicity leads to higher estrogen adsorption characteristics. However, a slightly contradictory findings is the high CSH of NCIMB13064 which gave lower estrogen removal performance than SD2-1 and DSM311, so clearly the adsorption process is strain-specific to some degree, and not simply based on the CSH values as measured by the method used in this research. Alternatively, it might have been possible that NCIMB13064 hydrophobicity had decreased during the experimental work so that the strain has lost its hydrophobic characteristics slightly before the adsorption tests were carried out. To verify this assumption, the CSH of the stationary phase for all strains (SD2-1, DSM311 and NCIMB13064) was re-measured, in which strain NCIMB13064 was actually found to be only 57 ± 9.88 %, a 35 % reduction (from the original measurement of 92 ± 1.73 %. Whereas strains of SD2-1 and DSM311 had only 1 % increase (92 ± 2.60 %) and 3 % reduction (90 ± 1.07 %) respectively.

Table 6.11 Re-measurement of CSH value for bacterial strains in stationary growth phase.

Bacterial strains	Stationary phase CSH (%)
Tsukamurella sp. SD2-1	92 ± 2.60
R. erythropolis DSM311	90 ± 1.07
R. erythropolis NCIMB13064	57 ± 9.88

This confirms that this strain had lost some of its hydrophobic characteristics, leading to a reduced estrogen removal performance. Consequently, the adapted NCIMB13064 strain is assumed to highly sensitive to environmental conditions (such as repeated culturing and storage) compared to the other bacterial strains adapted in this study. The reduction in hydrophobicity could be associated with factors such as a loss of bacterial plasmids (Lachica and Zink, 1984), mutation and changes in temperature (Ofek and Doyle, 1994). Possibly, the adapted *R.erythropolis* NCIMB13064 was more thermally sensitive than commercial *R.erythropolis.* The latter has been reported to have high tolerance to low temperatures

(Eriksson *et al.*, 2001; de Carvalho and ds Fonseca, 2005) and to the thawing process (Eriksson *et al.*, 2001). Nevertheless, the stationary phase DSM 311 and SD2-1 bacterial strains did successfully remove the estrogen from the initial concentration of 200 ng.L^{-1}, achieving final concentrations of 4.65 ng.L^{-1} and 4.70 ng.L^{-1}, which are levels very near to the predicted no-effect concentration (PNEC) of 3.6 ng.L^{-1}. As identified in the literature review, bacterial strains adapted with the hydrocarbon (n-hexadecane) have higher CSH values in their stationary phase compared to the exponential phase (Vasileva-Tonkova *et al.*, 2008).

6.9.10 Comparison of estrogen removal by biodegradation and adsorption process

Given that the stationary phase bacteria have been found to have higher CSH and are also determined to be a more efficient adsorbents for estrogen, compared to the exponential phase, an evaluation of estrogen removal adopting live stationary phase bacterial cell was also conducted. A similar comparison was also carried out in the preliminary study but using stationary phase cells.

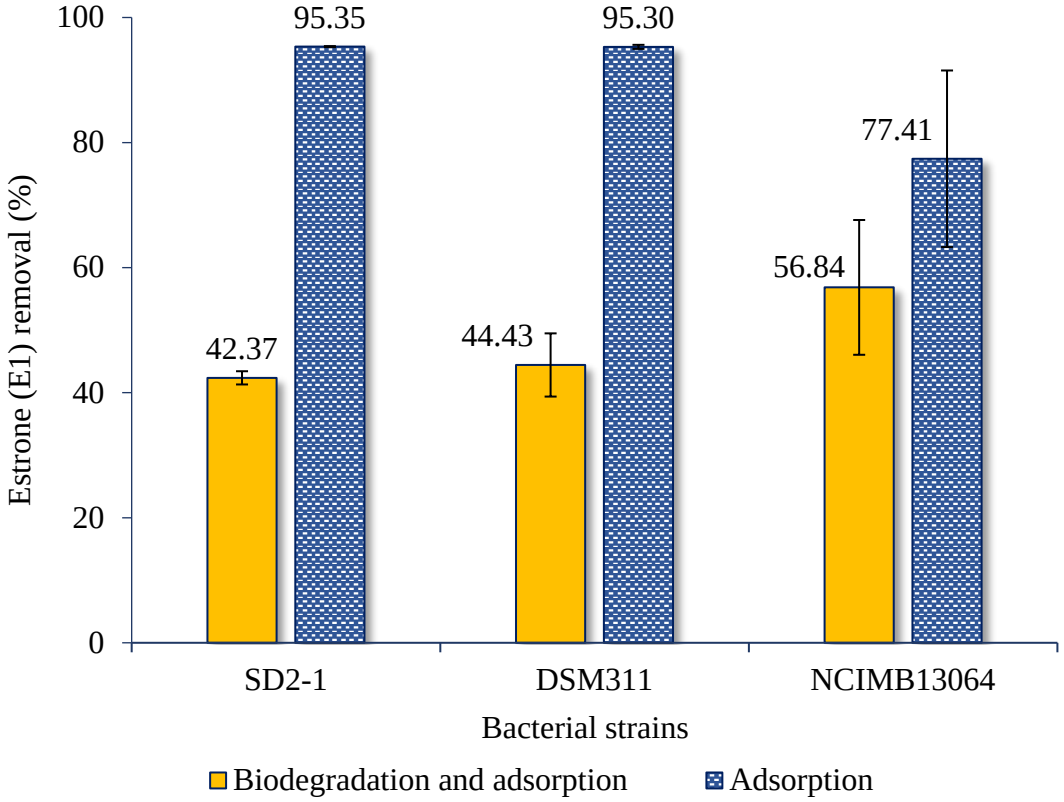


Figure 6.17 Degree of estrogen removal in reactors with adsorbent biomass from live and thermally inhibited bacterial strains prepared at the stationary growth phase. Estrogen removal by Live Bacteria (LB) due to adsorptiona and biodegradation, estrogen removal by thermal Inhibited Bacteria (IB) is due to adsorption only.

Table 6.12 Analysis of degree of estrogen removal in reactors with adsorbent biomass from live and thermally inhibited bacterial strains prepared at the stationary growth phase.

Bacterial strains (stationary phase)	Estrone removal (%)*		Significance (p-value)
	IB reactor	LB reactor	
Tsukamurella sp. SD2-1	95.35	42.37	0.000
R. erythropolis DSM311	95.30	44.43	0.003
R. erythropolis NCIMB13064	77.40	56.84	0.139

* Estrogen removal by Live Bacteria (LB) due to adsorption and biodegradation, estrogen removal by thermal Inhibited Bacteria (IB) is due to adsorption only.

Figure 6.17 shows the level of estrogen removal in the reactors containing the thermally inhibited (IB) and live (LB) hydrophobic-adapted bacterial strains. Results of a 2-sample t-test (Table 6.12), confirm that reactors fed with the LB cells (SD2-1 and DSM311 strains) had significantly ($p < 0.05$) lower estrogen removal (42.37 % and 44.43 %) compared to the equivalent IB reactors, 95.35 % and 95.30 %, respectively. This result is similar to those obtained in the preliminary study (Section 6.9.6). These results confirm that performance of estrogen removal using live bacterial cell is poor due to the interference factors, notably the presence of biosurfactant produced by the live bacterial cells. In addition, the turbid water samples and strong bad odour produced in the LB reactors also indicate the production of biosurfactants and that some biodegradation process (such as degradation of carried over hexadecane) was occurring. Besides *R.erythropolis, Tsukamurella* sp. also has been shown to produce glycolipid biosurfactants, specifically trehalose compounds, when it is cultivated in media supplied with hexadecane growth substrate (Kügler *et al.*, 2014). In contrast, the clear and odourless water samples previously observed in the IB reactors imply that biodegradation did not take place in these reactors, and the estrogen removal which occurred was solely due to adsorption. However, removal of estrogen in reactors containing NCIMB13064 bacterial strains was found to have no significant ($p > 0.05$) connection with the thermal inhibitory factor (Table 6.12). This happened due to the reduction of the CSH of the stationary phase NCIMB13064 bacterial cells, hence leading to low adsorption (77.41 %) in these IB reactors.

In a recent publication, a biodegradation study of estrogen removal was conducted using a live hydrocarbon-adapted bacterial strain (Fernández *et al.*, 2017). Complete removal of E1 was obtained using a *Bacillus* sp. adapted with the hydrocarbon phenanthrene, which is in contrast to the low estrogen removal from biodegradation observed in the current study. Assuming that

both *R. erythropolis* and *Bacillus* sp. are estrogen degraders (O'Grady *et al.*, 2009; Larcher and Yargeau, 2013), the high estrogen removal reported by Fernández *et al.* (2017) is probably because the *Bacillus* sp. had previously been isolated using hydrocarbon, and then adapted to estrogens before being employed in the estrogen removal study. Therefore, the hydrocarbon-adapted bacteria had already undergone adaptation to estrogen, and induction of estrogen degradative enzyme had occurred, allowing them to degrade the estrogen. Furthermore, biosurfactant was probably absent as no hydrocarbon involved in the latter bacterial adaptation culture, immediately before the estrogen removal test was conducted. Moreover, no CSH measurement was carried out on the bacteria in that study, and estrogen removal by adsorption process was not conducted. However, this is the only study found in the literature which employed hydrocarbon-adapted bacteria for estrogen removal. The current investigation confirms that even though the biodegradation process was found to be low, the adsorption process produced a very high level of estrogen removal (95.35 %), which is very comparable to the complete removal of estrone reported by Fernández *et al.* (2017).

6.10 Conclusions

In making use of hydrocarbon-adapted bacteria as adsorbents, different degrees of estrogen removal were found among the different bacterial strains due to their diverse CSH values. The SD2-1 bacterial strain was found to produce significantly better estrogen removal compared to the other bacterial strains considered here, due to its high CSH, when the ratio of biomass adsorbent to estrogen was fixed at 25,000:1 (w/w). However the estrogen removal was found to be moderate due to the low ratio of biomass to adsorbent used.

From the optimisation experiments, the minimum contact time was found to be 30 minutes, with the best removal observed for 60 minutes contact, with no further improvements in removal efficiency arising for longer contact time. These findings have confirmed the hypothesis that the performance in the adsorption of estrogen increases with contact time in the reactor, but this is limited to the first hour only.

The optimum adsorbent dosage was found to be at 50,000:1 (w/w) of bacterial adsorbent to estrogen analyte. Contact time and dose of adsorbent are both known to be factors that influence the adsorption process (Snyder *et al.*, 2007; Ifelebuegu *et al.*, 2015) and the current study supports the alternative hypothesis that performance of estrogen adsorption increases with the amount of bacterial biomass and contact time applied in the batch reactor.

Moreover, further increase of estrogen removal efficiency was demonstrated using stationary growth phase compared to the exponential phase bacterial cells. This verified the alternative

hypothesis, that different stages of bacterial growth affect estrogen removal by adsorption process. As the stationary phase cells of the bacterial strains have higher CSH compared to exponential phase cells, and produced higher estrogen removal, this also supports the hypothesis that the CSH of the bacterial strain correlates with efficiency in estrogen removal.

However, the adapted *R. erythropolis* strain NCIMB13064 was found to produce lower estrogen removal due to the reduction of its CSH, and it was concluded to be a more sensitive strain (to the loss of hydrophobic properties) compared to its parent strain (*R. erythropolis*), and this reduction in CSH affected its capability to adsorb the hydrophobic estrogen molecules.

Apart from adsorption, the removal of estrogen via biodegradation in live hydrocarbon-adapted bacteria was not found to be a major contributing process, particularly for live bacteria where interfering factors (biosurfactant production) were considered to reduce estrogen removal efficiency.

Overall, the bacterial strains of *Rhodococcus erythropolis* DSM311and *Tsukamurella* sp. SD2-1 have been successfully hydrocarbon-adapted to become hydrophobic strains (high CSH) , and these strains have been shown to be very effective bacterial adsorbents for estrone removal by a predominantly adsorption-based process. There is considerable potential to employ these bacterial strains, or an adsorbent material based on attenuated cells of these bacteria, for large-scale tertiary removal of estrogen from wastewater. These bacteria are readily “hydrophobic-adapted” and could be produced cheaply utilising low-cost hydrocarbon substrates, which may include diesel fuel as an extremely low-cost hydrocarbon growth substrate.

In summary, bacterial cells with high CSH were found to produce higher estrogen removal and were effective at an adsorbent dosage of 500 $\mu l.L^{-1}$. An optimum contact time of 60 minutes, and use of biomass harvested from the stationary growth phase (10 days bacterial cultivation) were identified as the most effective conditions for estrogen removal. Therefore, the objective (Section 1.3 (3)) of investigating any correlation between the cell surface hydrophobicity (CSH) of bacterial strains and their estrogen adsorption performance has been achieved.

Chapter 7 Fed-batch culture bacteria for estrone (E1) removal

7.1 Introduction

In stationary growth phase, bacterial strains of *Rhodococcus erythropolis* DSM311 and *Tsukamurella* sp. SD2-1 were found to effectively remove 95.00 - 95.35 % of estrone from the initial concentration of 200 $ng.L^{-1}$ to final concentrations between 9.3 – 9.4 $ng.L^{-1}$. To investigate the potential for complete, or at least higher, removal of estrone, the bacterial strains were hydrophobic-adapted for a longer incubation periods of more than 10 days. To ensure that the bacteria were fully adapted to the hydrocarbon without being affected by possible inhibitory effects from excess substrate, and at the same time avoiding substrate limitation, a fed-batch cultivation technique was employed. The process was conducted over a longer incubation of 20 days, thus doubling the adaptation period of the previous batch culture. Longer exposure to the hydrophobic growth substrate was expected to produce more hydrophobic bacterial biomass adsorbent for improved adsorption of estrone (E1).

In a previous study, Tzintzun-Camacho *et al.* (2012) used 13 $g.L^{-1}$ of n-hexadecane, which is equal to 16.8 $ml.L^{-1}$ of growth substrate in a hydrocarbon-adaptation study with an incubation period of 15 days. In comparison, the adaptation of bacteria used in the current study (Chapter 4) was conducted using 0.5 $ml.L^{-1}$ of n-hexadecane for a maximum of 10 days. The high amount of substrate used by these authors, 30 times higher than the current study, was not preferred as it was considered that might inhibit bacterial growth, and the presence of dead bacteria might have produced a low cell surface hydrophobicity (CSH) value. The researchers found that *Acinetobacter bouvetii,* with an initial value of 80 % CSH, started to show a decrease in CSH to 60 % after only four days of incubation, and continued to decrease to an extremely low CSH of 6 % after 15 days of incubation. This scenario presumably took place due to increases in the number of dead cells following the inhibition, therefore, the CSH could not increase higher than the 80 % value. Moreover, use of a higher volume of 25 $ml.L^{-1}$ of growth substrate for the adaptation of *Tsukamurella tyrosinosolven* (a similar bacterium to *Tsukamurella* sp. SD2-1 used in the current study) in 10 days of batch cultivation, also produced a low CSH of 29 % (Tebyanian *et al.*, 2013), compared to 91 % obtained with the same genus in the current study.

Therefore, to avoid producing low CSH, a fed-batch cultivation method was considered to be the most appropriate technique for the longer adaptation of bacteria that employed a minimal quantity of hydrocarbon substrate to avoid the inhibition of growth. The investigation sought to confirm the actual effect of longer adaptation process on the CSH of the bacteria, and how it affected the efficiency of the bacterial biomass adsorbent for the sorption of estrone.

7.1.1 Aims

To investigate whether the culturing conditions for production of the hydrophobic bacterial strains affected their CSH and estrogen adsorption characteristics.

7.1.2 Objectives

To study the optimization of the estrone removal process using the fed-batch cultivation of bacterial biomass production, and to gain a better understanding of the effect of a longer incubation period on bacterial adaptation of CSH and estrogen adsorption efficiency.

7.1.3 Hypotheses

The alternative hypotheses of this study are as follows:

1. The hydrophobic bacterial cell adsorbents are capable of removing estrone from wastewater below the relevant environmental quality standard values.
2. Fed-batch bacterial cultivation for periods exceeding 10 days has a significant effect on cell CSH level, and the performance of the bacterial biomass for estrogen adsorption.

7.2 Methodology

The experimental work plan is demonstrated in Figure 7.1, and the methodology is described in more detail below. Part per trillion concentration (200 $ng.L^{-1}$) of E1 was used for all adsorption process in this chapter.

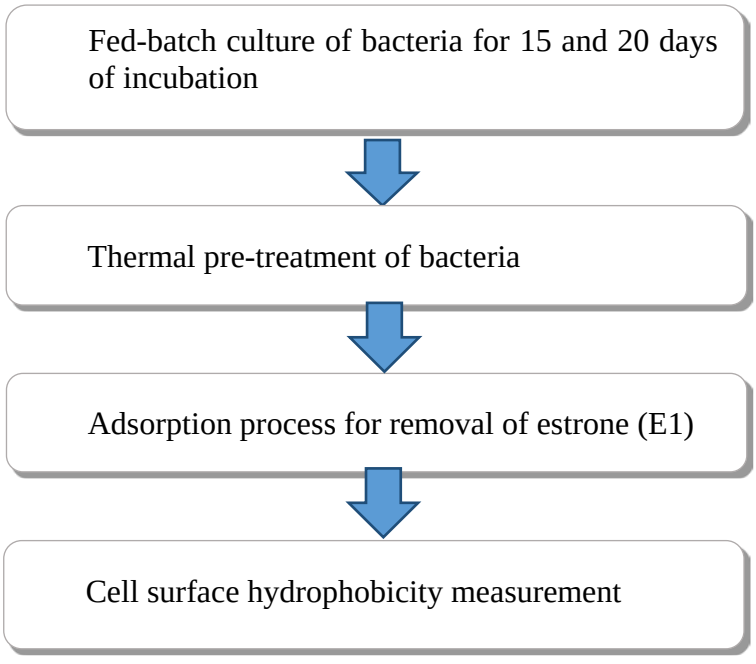


Figure 7.1 Flow of work plan for the enhancement of estrone removal by fed-batch cultivated bacteria

7.2.1 Fed-batch cultivation and biomass production

Biomass weight was measured for the bacterial strains SD2-1 and DSM311 that were cultivated in the fed-batch, to establish the volume of bacterial culture that represented the 5.0 mg of bacterial biomass required to be supplied to the reactors during assays.

Bacterial strains SD2-1 and DSM 311 were cultured on NA plates, and single colonies of the bacteria were then cultured in 10 ml of liquid MM1 medium with 500 µl.L^{-1} of n-hexadecane growth substrate in glass universal tubes for a fed-batch culture as displayed in Figure 7.2. The cultivation was carried out in 25 °C and 155 rpm incubator for 15 and 20 days. After 10 days of incubation, and standing at room temperature for 3 hours, all of the liquid phases in the bacterial cultures were discarded, leaving the settled bacteria cells at the bottom of the tubes. 10 ml of new liquid MM1 medium and 5 µl of n-hexadecane were added to the tubes under aseptic conditions in order to avoid contamination. The solution was incubated under the same conditions for another 5 days. At the end of 15 days incubation, measurement of the biomass weight for the day 15 bacterial culture was conducted according to the quantification of bacterial biomass protocol described in Section 6.2.1.

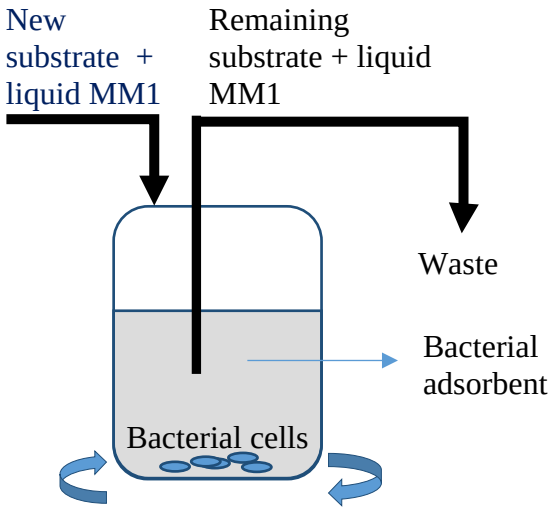


Figure 7.2 Schematic diagram of the fed-batch culture method for producing the bacterial adsorbent. Liquid MM1 growth media and n-hexadecane growth substrate were renewed after 10 days and 15 days of incubation. The bacterial culture were collected at 15 and 20 days.

The process was repeated in a separate set of universal tubes over 20 days of incubation. These batches were fed three times, the original feed on day 0, and then further medium replacement as described above on day 10 and day 15. The day 20 biomass was quantified at the end of the

incubation as described above. All of the bacterial strains were cultivated and quantified in triplicate.

7.2.2 Pre-treatment of bacterial adsorbent

The bacterial adsorbent was thermally pre-treated in an oven at 85 °C for 1 hour according to the procedure detailed in Section 6.3.2.

7.2.3 Adsorption process for treatment of estrone

The adsorption process was conducted using the bacterial adsorbents SD2-1 and DSM311 which were harvested at day 15 and 20 in the reactor in triplicate according to the adsorption process described in Section 6.6 and measurement of estrone concentration in Section 6.6.1. Data on degree of E1 removal, as well as E1 final concentration for treatment using day 5 and day 10 of bacterial age (culture period) were reproduced from the adsorption assays in (Section 6.9.9).

7.2.4 Cell surface hydrophobicity measurement

CSH was determined for the bacterial strains SD2-1 and DSM311after the longer incubation periods, 15 and 20 days, was conducted according to the microbial attachment to hydrocarbon (MATH) protocol described in Section 5.3.1.

7.3 Results

7.3.1 Biomass weight

The volumes of bacterial culture representing 5.0 mg biomass are reported in Table 7.1. The respective amounts of thermally pre-treated bacterial culture collected at days 15 and 20 of incubation were added to each reactor in duplicate.

Table 7.1 Biomass weight of the hydrophobic-adapted bacterial strains SD2-1 and DSM311 and their amounts in volumes that represent 5.0 mg of biomass as determined at Day 15 and Day 20 of incubation.

Bacterial strain	Biomass weight (mg/10 ml)		Volume of bacterial culture representing 5.0 mg biomass (ml)	
	Day 15	Day 20	Day 15	Day 20
SD2-1	8.7	10.6	5.827	4.740
DSM311	10.3	12.80	4.893	3.937

7.3.2 Estrone removal by batch reactors

Figure 7.3 displays the final concentrations of estrone (E1) in the batch reactors containing thermally pre-treated biomass from bacterial strains DSM311 and SD2-1 grown in fed-batch culture for 5, 10, 15 and 20 days.

The final concentrations of estrone shown in Figure 7.3 decline rapidly in a linear trend over with the length, which a significant ($p < 0.05$) reduction in concentration has been detected from the initial concentration to day 5, followed by day 10 (Table 7.2) as previously reported in Chapter 6. The reduction in estrone concentrations demonstrates a high degree of adsorption, and this was attributed to the increased bacterial CSH levels from their exponential to stationary growth phases (Figure 7.4 and Figure 7.5).

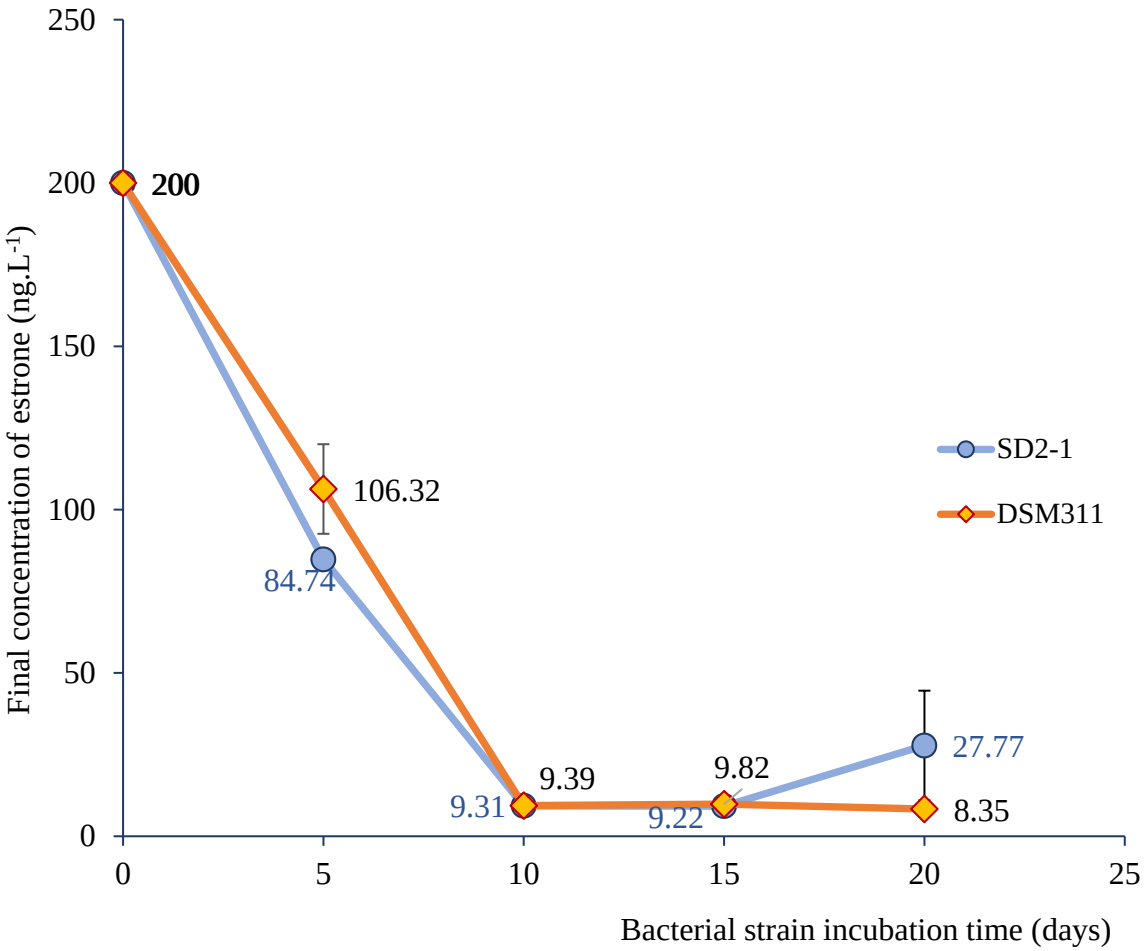


Figure 7.3 Final concentration of estrone in the batch reactors containing thermally pre-treated hydrophobic-adapted biomass from bacterial strains SD2-1 and DSM311. Initial estrone (E1) concentration was 200 $ng.L^{-1}$.

Table 7.2 Final concentration of estrone in the batch reactors containing thermally pre-treated hydrophobic-adapted biomass from bacterial strains SD2-1 and DSM311. Initial estrone (E1) concentration was 200 ng.L^{-1}.

Strains	Final concentration of estrone in rectors (ng.L^{-1}) ([1]Different incubation time bacteria)				
	D0	D5	D10	D15	D20
SD2-1	200[a]	84.74 [b]	9.31[c]	9.22 [c]	27.77 [c]
DSM311	200[a]	106.32[b]	9.39[c]	9.82 [c]	8.35 [c]

[1] Different incubation time; D5: Day 5; D10: Day 10; D15: Day 15; D20: Day 20. Data for D5 and D10 was reproduced from adsorption assays (Section 6.9.9).

[a, b] Means that do not share a letter are significantly different ($p < 0.05$). Comparison between days of incubation.

Table 7.3 Degree of estrone removal in the batch reactors containing thermally pre-treated hydrophobic-adapted biomass from bacterial strains SD2-1 and DSM311. Initial estrone (E1) concentration was 200 ng.L^{-1}.

Strains	Removal of estrone (%) ([1]Different incubation time bacteria)				
	D0	D5	D10	D15	D20
SD2-1	0.0[a]	57.63[b]	95.35 [c]	95.39 [c]	86.11 [c]
DSM311	0.0[a]	55.57 [b]	95.30 [c]	95.09 [c]	95.83 [c]

[1] Different incubation time; D5: Day 5; D10: Day 10; D15: Day 15; D20: Day 20. Data for D5 and D10 was reproduced from adsorption assays (Section 6.9.9).

[a, b, c] Means that do not share a letter are significantly different ($p < 0.05$). Comparison between days of incubation.

The estrone concentration then stabilised between days 10 to 20 for both the SD2-1 and DSM311 bacterial strains. Surprisingly, the use of a longer culture growth period of 15 days for the SD2-1 bacterial strain was observed to further decrease the E1 concentration by a small amount 1.0 % lower than the final concentration of day 10 cells. This change was not found to be significant ($p > 0.05$), (Table 7.3). In terms of the removal efficiency, 95.39 % of E1 was removed from the SD2-1 reactor compared to 95.35 % as obtained previously using the day 10 bacterial adsorbent of the same strain (Table 7.3).

In comparison, the DSM311 bacterial strain did not show improved E1 removal with biomass grown for periods in excess of 10 days, the reactor with 15 day biomass having a slightly (around 5 %) higher final concentration of E1 than the 10 day biomass, and the mean final concentration of estrogen was 9.82 $ng.L^{-1}$ and 9.39 $ng.L^{-1}$ for the 10 day and 15 day reactors, respectively. The DSM311 bacterial strain that incubated for 20 days was found to produce a higher E1 removal efficiency of 95.83 %, which represents a final concentration of 8.35 $ng.L^{-1}$.

In contrast, the concentration of E1 in the reactor with *Tsukamurella* sp. SD2-1 bacterial adsorbent from 20 day biomass was found to increase to 27.77 $ng.L^{-1}$. This is an unexpected increase in concentration and can be explained by the existence of aggregation effects in the hydrocarbon-adapted bacterial strains. This aggregation caused the bacterial cells to clump together during growth, and this persisted after heat treatment and remained in adsorbent biomass used in the reactors. This reduced the surface area of the bacteria during the adsorption reaction, and exposed fewer binding sites for the attachment of estrone molecules. The lower degree of estrone removal with the 20 day biomass of the SD2-1 bacterial strain occurred because a more severe aggregation effect was observed in this strain compared to DSM311, especially for the longest culture period.

Statistical analysis using one-way analysis of variance (ANOVA) and the Tukey pairwise test shown in Table 7.2 and 7.3 indicate that none of the final E1 concentrations in the reactors containing day 15 (D15) and day 20 (D20) biomass from both bacterial strains were significantly different from the concentrations with biomass taken from the day 10 cultures. Further investigation of the CSH values of the strains at day 15 and day 20 was conducted to investigate the reason for insignificant increases in E1 removal being observed with biomass taken from the 15 day and 20 day culture period compared to the 10 day biomass (Section 7.3.3).

7.3.3 Effect of extended fed-batch culture periods on cell surface hydrophobicity and estrogen adsorption

The CSH of the bacterial strain SD2-1 was measured for culture periods of 3 to 20 days and results are shown in Figure 7.4.

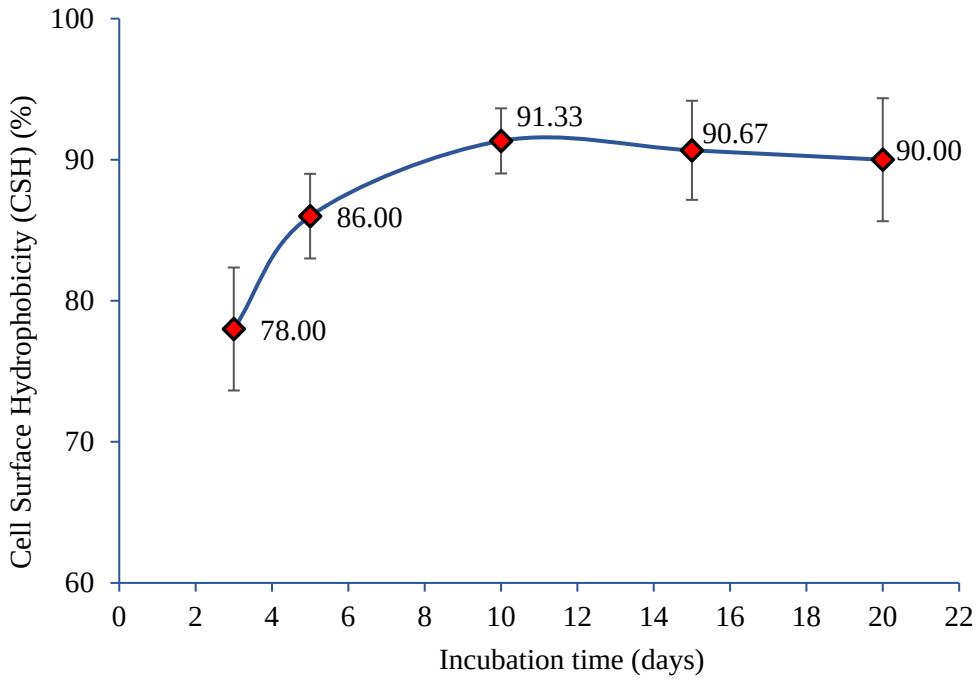


Figure 7.4 Cell surface hydrophobicity of *Tsukamurella* sp. SD2-1, measured for culture periods of 3 to 20 days.

The CSH of strain SD2-1 has increased continuously over the first 10 days of culture, starting at 78 ± 4.35 % on day 3 and reaching a maximum of 91.33 ± 2.30 % on day 10 (Figure 7.4). Then it decreases to 90.67 ± 3.51 % on day 15, and continued to decline slightly for the day 20 biomass to 90 ± 4.35 %. A total CSH reduction of 1.33 % after day 10 onwards explained the absence of significant improvement in the degree of estrone removal in the reactors containing the 15 day and 20 day SD2-1 biomass. This reduction in CSH may in part explain the higher final estrone concentration, increasing from 9.22 $ng.L^{-1}$ to 27.77 $ng.L^{-1}$ for reactors containing biomass from the 15 day and 20 day cultures, respectively (Figure 7.3). This was thought to occur due to the small reduction in CSH (0.67 %) and the severe aggregation effect on SD2-1 bacterial cells. Reductions in surface area ultimately decrease the amount of E1 that can be adsorbed. The effect of aggregation on bacteria grown for longer incubation periods has also been discussed in Chapter 4, and is a phenomenon also reported in previous studies (Flemming and Wingender, 2010).

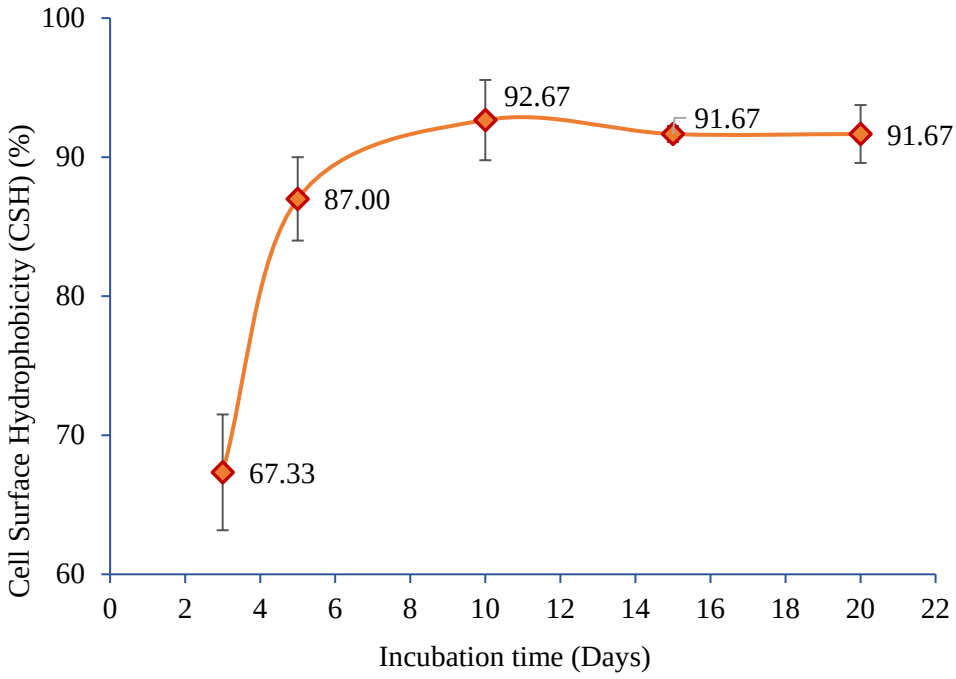


Figure 7.5 Cell surface hydrophobicity of the fed-batch culture of the hydrophobic-adapted bacterial strains of DSM311, enumerated every five days of incubation.

A similar trend of small reductions in CSH were also observed in the adapted DSM311 strain for incubation periods in excess of 10 days (Figure 7.5). Specifically, the CSH of 92.67 ± 2.89 % measured on day 10 reduced by 1 % for a 15 day incubation to 91.67 ± 0.58 %, and to 91.67 ± 2.08 % at day 20. The small reduction in CSH at day 15 yielded a slightly lower degree of E1 removal in the D15 reactor compared to D10 reactor (Table 7.3). However, higher removal took place in the D20 reactor, with final E1 8.35 ng.L^{-1}, despite the biomass having almost identical CSH to the day 15 biomass. Nevertheless, this improved E1 removal efficiency, compared to D15 reactor, was insignificant and probably due to experimental error such as the actual numbers of bacterial cells growing in separate culture tubes, i.e. having slightly different growth rates.

Overall, the insignificant difference in the removal of estrone between bacterial cultures incubated for 10 days compared 15 and 20 days was considered to reflect the almost constant CSH of the bacterial strains over this period. This is probably due to the fact that the bacterial cells are in the same growth stage between day 10 and 20, i.e. the stationary phase. The CSH was predicted to decrease gradually as the bacteria started to enter their death phase.

Even though use of fed-batch cultivation for periods longer than 10 days cultivation in the current study did not produce a higher CSH strain, the use of the lower growth substrate

concentration ensured the production of good CSH levels of 91.0 % and 92.3 % for SD2-1 and DSM311 respectively, compared to the 80 % (Tzintzun-Camacho *et al.*, 2012) and 29.0 % (Tebyanian *et al.*, 2013) obtained in the previous studies.

7.4 Conclusion

Reactors containing bacterial adsorbent grown in culture periods in excess of 10 days did not give any significant improvement in the estrogen removal efficiency because the bacterial cells were in the same stationary growth phase, and generally had similar CSH levels. It is concluded that the adsorption of estrone using the thermally pre-treated biomass of hydrocarbon-adapted bacterial strains is affected by the level of bacterial CSH, which is also related to different growth phases. The alternative hypothesis, that fed-batch bacterial cultivation in excess of 10 days (i.e: 15 and 20 days) has a significant effect on the estrone removal process is therefore rejected.

Therefore, the objective (Section 1.3 (4)) to demonstrate enhanced estrogen removal by using fed-batch cultivation with bacterial growth periods in excess of 10 days (i.e: 15 and 20 days) was not achieved. Nevertheless, 95.30 - 95.35 % of estrone being adsorbed by using the D10 bacterial adsorbent of DSM311 and SD2-1, respectively, are considered to provide good removal performance, reducing estrone from 200 $ng.L^{-1}$ down to 9.31 - 9.39 $ng.L^{-1}$, and almost achieving the PNEC standards of 3.6 $ng.L^{-1}$ (European Commission, 2016).

Chapter 8 Comparison of the estrone removal efficiency of bacterial and commercial adsorbents

8.1 Introduction

The bacterial isolate SD2-1 was found to exhibit high cell surface hydrophobicity (CSH) in its stationary growth phase and to give a high degree of estrone (E1) adsorption, and was therefore selected to evaluate its performance in comparison with commercial adsorbents, namely activated carbon and zeolite. In addition, variations in the bacterial thermal pre-treatment method were also considered in this study, where observations were made on the effect of different temperatures on the morphology of the bacteria and their estrone adsorption efficiency.

8.1.1 Aims

To compare the E1 adsorption properties of adsorbents prepared from hydrophobic adapted bacteria with that of the commercial adsorbents.

8.1.2 Objectives

In order to accomplish the aforementioned aim, the following objectives were set:

1. To compare the performance of the thermally pre-treatment hydrophobic SD2-1 bacterial strains with commercial adsorbents, activated carbon and zeolite, for their capacity to remove E1 from a synthetic wastewater.
2. To investigate the effect of the pre-treatment temperature used to activate the SD2-1 bacterial biomass on the performance of E1 adsorption from a synthetic wastewater.
3. To investigate specific aspects of E1 adsorption process, including the mechanism of interaction and the reversibility of the adsorption-desorption process.
4. To determine the adsorption isotherm parameters using the best fitted isotherm model for E1 adsorption using adsorbents from the SD2-1 bacterial strain.

8.1.3 Hypotheses

1. The removal of estrogen from wastewater using hydrophobic-adapted SD2-1 bacterial adsorbents is a viable alternative removal technology in comparison with activated carbon adsorption.
2. Temperature of thermal pre-treatment affects the E1 adsorbing performance of SD2-1 bacterial adsorbents.

3. The adsorption of E1 using the hydrophobic-adapted SD2-1 bacterial biomass is mainly attributed to its hydrophobicity.
4. The adsorption of E1 using the hydrophobic-adapted SD2-1 bacterial biomass is a reversible process.

8.1.4 Comparison of adsorption properties of SD2-1 bacterial adsorbents with activated carbon and zeolite

Activated carbon (AC) is an excellent material that has been used in various adsorption processes for the removal of a variety of emerging contaminants, including estrogen (Grassi *et al.*, 2013; Hemidouche *et al.*, 2017). This is due to its high surface area and high affinity for various types of pollutants. However, there are several issues, and drawbacks with its commercial application, particularly the high embodied energy requirement linked in its production, and the high production cost (Snyder *et al.*, 2007). Details of the characteristics of activated carbon have been discussed in the literature review in Chapter 2.

Zeolites are alternative adsorbents, being crystalline microporous aluminosilicate minerals, which have been used widely in industrial applications as efficient molecular sieves and adsorbent materials (Gleichmann *et al.*, 2016). Naturally occurring zeolites, however, are rarely pure and vary in pore size, often making them unsuitable for practical applications (Ackley *et al.*, 2003). Thus, commercial zeolites are usually synthetic materials, with uniform pore structures and composition. They have been produced industrially in large quantities using silica, alumina and sodium hydroxide via a heating process, and this is costly. In recent studies, alternative cheaper raw materials have been suggested such as clay mineral (Ugal *et al.*, 2010), silica content materials and industrial fly ash (Franus *et al.*, 2014; Tauanov *et al.*, 2017). These synthetic zeolites generally have microporous structures which can accommodate various cations for adsorption including heavy metals (Na *et al.*, 2011). They have also been recorded as good adsorbents for emerging contaminants such as perfluorinated compound (PFC) (Ochoa-Herrera and Sierra-Alvarez, 2008), which have hydrophobic characteristics similar to estrone.

Therefore, considering the potential drawbacks associated with conventional adsorbents, the hydrophobic-adapted *Tsukamurella* sp. SD2-1 could be a potential alternative adsorbent for removing estrone, and possibly other estrogens, from wastewater. Furthermore, because *Tsukamurella* sp. SD2-1 biomass comprises small bacterial cells, this provides a high collective surface area, which facilitates adsorption processes. One of the main advantages of *Tsukamurella* sp. SD2-1 biomass is that it can be produced cheaply in large quantities, particularly as relatively low temperatures are needed for its cultivation, thus requiring less

energy. In addition, the SD2-1 biomass exhibits high hydrophobicity, and has been shown to produce very high adsorption performance (Chapter 6), but this needs to be put into perspective with the performance of AC and zeolites. Therefore, determining the performance of *Tsukamurella* sp. SD2-1 in adsorbing estrone compared to activated carbon and zeolite, could give an indication of the potential commercial applicability the *Tsukamurella* sp. SD2-1 bacterial adsorbent for treating wastewaters that contain estrogens.

8.1.5 Effect of different thermal pre-treatment temperatures on bacterial morphology and biomass adsorption characteristics

An extensive study was conducted to assess the effects of different thermal pre-treatments temperatures on the performance of *Tsukamurella* sp. SD2-1 in adsorbing estrone. A higher temperature of 100 °C was applied in thermal pre-treatment process, compared to the 85 °C adopted in the previous assays. The bacterial cells pre-treated at 100 °C were assumed to be disrupted at this higher temperature, which is close to the autoclaving temperature of 121 °C (Lunsman and Lick, 2005). In theory, the broken cells of bacteria might provide a higher surface area for higher adsorption compared to the intact cells pre-treated at 85 °C. Alternatively, changes in the bacterial morphology of either broken or shrunken cells might affect their adsorption properties negatively. These factors were evaluated in this section.

8.1.6 Desorption of adsorbed estrone

A desorption experiment was also carried out to provide supporting evidence that the removal of estrone which was observed in reactors containing thermally pre-treated bacterial biomass (Section 8.1.5) was due to an adsorption mechanism, rather than a biodegradation or modification mechanism, in which case it should be recoverable by desorbing back into the liquid phase. Up to the current point, the adsorption of estrone by *Tsukamurella* sp. SD2-1 biomass has been assumed to be a physical adsorption process because the removal of hydrophobic estrogen from the aqueous phase is related to the hydrophobicity of the bacterial biomass. Therefore, the adsorption of estrone by the bacterial adsorbents was predicted to be a reversible process because physical adsorption (physisorption) is commonly a reversible process (Zhang *et al.*, 2012). However, even so, 100 % analyte recovery is not always achievable from an adsorbed state because the efficiency of the desorption process can be affected by many factors; namely, morphology of the adsorbent, temperature, activation energy and the reaction order (Amiaud *et al.*, 2015).

8.1.7 Isotherm model

The performance of adsorption can be further understood and predicted by using adsorption models, and two of the most commonly used models, the Freundlich and Langmuir adsorption models, were evaluated in the current study. General Freundlich and Langmuir models can be represented by the equations (7.1) and (7.2) respectively.

$$\text{Log } q_e = \text{Log } K_f + (1/n) \text{ Log } C_e \qquad \text{(Equation 7.1)}$$

$$q_e = \frac{K_L.q_m.C_e}{1 + K_L.C_e} \qquad \text{(Equation 7.2)}$$

Where q_e is the amount of adsorbate per mass unit of adsorbent at equilibrium, K_f is the adsorbent capacity and C_e is the final adsorbate concentration, 1/n is the Freundlich adsorption capacity, q_m as the maximum adsorption capacity, and K_L is the Langmuir adsorption constant. Selection the most suitable isotherm model for the adsorption of estrone using the hydrophobic-adapted bacteria was made by determining the best fit of the data according to its correlation coefficient (R^2).

8.2 Methodology

8.2.1 Preparation of bacterial adsorbent

Single colonies of SD2-1 bacteria were cultivated in 10 ml of liquid minimal media (MM1) with 5.0 µ of n-hexadecane added, in glass universal tubes and incubated at 20 °C and 250 rpm for 10 days (to reach stationary phase). Then the bacterial cultures were thermally pre-treated for 1 hour in ovens at temperatures of 85 °C and 100 °C. 10 µl of each pre-treated bacterial culture were taken and prepared for microscopic observation at 10,000 times magnification using a fluorescent microscope, model Nikon ECLIPSE Ci, equipped with QImaging software. The commercial adsorbents, granulated activated carbon (GAC) and zeolite, were dried in a 100 °C oven overnight and stabilised in a desiccator containing silica gel. Stable weights of 5.0 mg for the AC and zeolite were measured.

8.2.2 Adsorption process

A concentrated synthetic wastewater was prepared according to the protocol described in Section 6.3.1, and diluted one-hundred-fold with spring water. 500 ml of the diluted synthetic wastewater was added into reactors together with 1 ml of 100 $\mu g.L^{-1}$ estrone (E1) to give an initial concentration of 200 $ng.L^{-1}$. A 5.0 mg of activated carbon and zeolite were added into separate reactors. Alternatively, 8.542 ml of the SD2-1 bacterial cultures pre-treated at 85 °C and 100 °C, representing 5.0 mg of biomass dry weight, were added separately to another set

of reactors. All reactors containing each of the adsorbents were prepared in triplicate. The reactors were incubated in a shaking incubator at 20 °C and 250 rpm for 24 hours. Then samples were removed from the reactors and centrifuged at 4200 rpm for 10 minutes (Sigma 3-16P, SciQuip Ltd.) and the liquid phase was collected, leaving the settled bacterial pellet at the bottom of the centrifuge tube. All of the liquid phase samples were loaded into solid phase extraction (SPE) OASIS HLB 6cc 200 mg cartridges and extracted according to the SPE protocol described in Section 6.6.1, and the final eluate was filtered using 0.22 μm syringe filter (Stratlab Ltd.) into HPLC vials for analysis of the final concentration using the HPLC-ECD (Section 6.8). Control reactor without adsorbent has not been carried out because adsorption by the glassware was proven to be negligible, and the degree of estrogen loss is similar to the losses in cartridge (16 - 18%) (Section 6.9.3).

8.2.3 Desorption process

The remaining bacterial pellets in the centrifuge tubes were added to new reactor bottles which were filled with 500 ml of deionised water. The reactors were agitated in the incubator at 250 rpm for 24 hours at 20 °C. The water samples were then centrifuged and loaded into the SPE and the process of SPE extraction and estrone analysis was conducted as defined in Section 6.6.1. An additional control experiment was carried out using estrone dissolved in deionised water was also made, and the same parameters and protocol were adopted, except the synthetic wastewater medium was replaced with deionised water.

8.2.4 Isotherm model

Different concentrations of estrone, namely 50, 100, 200 and 500 $ng.L^{-1}$ were prepared by adding 25, 50, 100 and 250 μl of 1.0 $mg.L^{-1}$ of E1 respectively into each reactor contained 500 ml synthetic wastewater. 5.0 mg of SD2-1 bacterial adsorbent, represented in 8.542 ml of 85 °C pre-treated biomass (Section 6.3.2) was also added to each reactor. The adsorption reaction, separation of adsorbent from aqueous phase, SPE concentration and final analysis of the estrone concentration by HPLC-ECD system was carried out according Section 6.6.1 . Data of the adsorption parameter, including final concentration analysed were fitted into the isotherm models of Freundlich and Langmuir (Section 8.1.7).

8.3 Results and discussion

8.3.1 Adsorption process

Table 8.1 shows the results of one-way ANOVA and Tukey pairwise analysis of the percentages of E1 removal and adsorption capacities in reactors containing different type of adsorbents.

Table 8.1 Performance of estrone removal and estrone adsorption capacities in the aqueous phase of reactors containing different adsorbents. Type of adsorbents are: GAC, granulated activated carbon; SD2-1(85), SD2-1 strain pre-treated at 85 °C; and SD2-1(100), SD2-1 strain pre-treated at 100 °C. Initial estrone concentrations were 200 $ng.L^{-1}$.

Analysis	Type of adsorbents			
	GAC	SD2-1(85)	SD2-1 (100)	Zeolite
Final concentration of E1 ($ng.L^{-1}$)	0.00	1.78	62.14	66.55
Removal performance (%)	100[a]	99.11 [a]	68.93 [b]	66.73 [b]
[1]Adsorption capacity ($ng.mg^{-1}$)	20[a]	19.82[a]	13.79[b]	13.35 [b]

[a, b] Values that do not share a letter are significantly different. Comparison valid between types of adsorbents only.

[1] Mass of estrone (ng) adsorbed per mass of adsorbent (mg)

The estrone in the activated carbon reactors was found to have been completely removed from its initial concentration of 200 $ng.L^{-1}$, due to its high adsorption efficiency. Meanwhile, the reactor of SD2-1(85) had an E1 mean final concentration of 1.78 $ng.L^{-1}$, which represent 99.11 % of removal (Figure 8.1), achieving a final concentration below the predicted no-effect concentration (PNEC) of 3.6 $ng.L^{-1}$. In contrast, only 68.93 % of E1 has been removed from the reactor with SD2-1(100), and the final concentration of estrone in the aqueous phase was found to be 62.14 $ng.L^{-1}$, which exceeds PNEC level. A similarly low removal performance was found in the zeolite reactors, where only 66.73 % of the estrone was removed, producing a final concentration of 66.55 $ng.L^{-1}$.

According to Table 8.1, there is no significant difference between the estrone removal performance in the SD2-1(85) reactor and the activated carbon reactor which gave complete removal. The excellent performance can also be found by comparing the adsorbents' adsorption capacities (Table 8.1 and Figure 8.1), results showing the SD2-1(85) adsorption capacity (19.82 $ng.mg^{-1}$) was not significantly different from GAC (20.00 $ng.mg^{-1}$). However, SD2-1(100) and zeolite had significantly lower adsorption capacities compared to GAC.

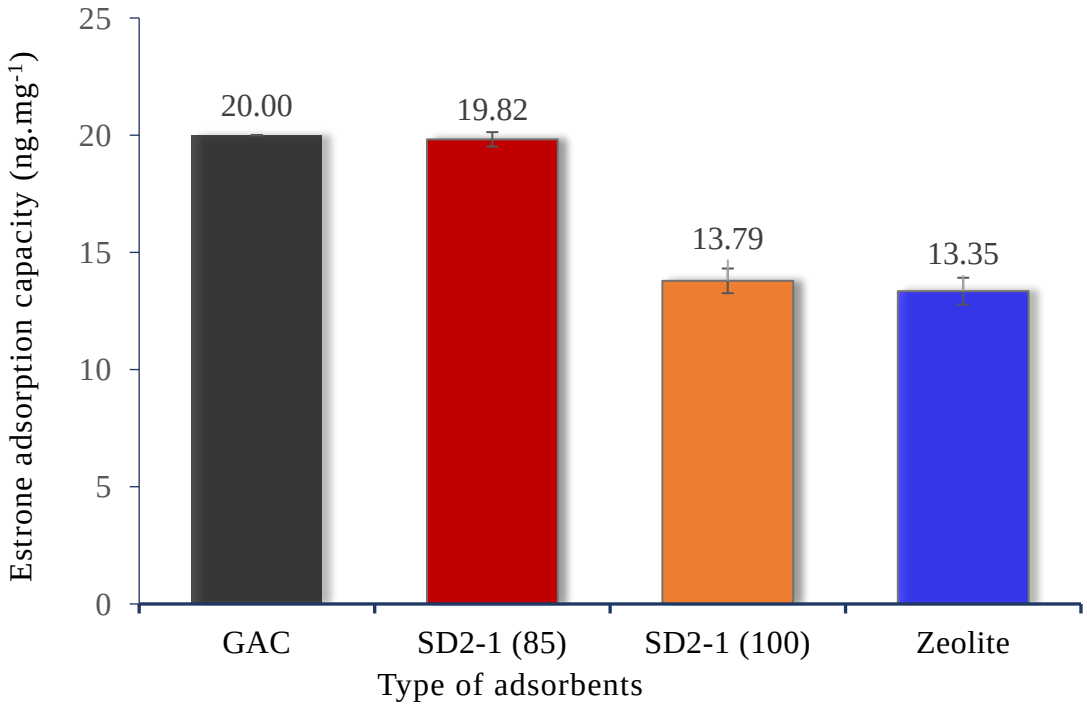


Figure 8.1 Estrone (E1) adsorption capacity of adsorbents in the aqueous phase of reactors containing different adsorbents. Type of adsorbents are: GAC, granulated activated carbon; SD2-1(85), SD2-1 strain pre-treated at 85 °C; and SD2-1(100), SD2-1 strain pre-treated at 100 °C. Initial estrone concentrations were 200 ng.L^{-1}.

This indicates that the hydrophobic SD2-1 strain is an efficient adsorbent material with a performance that is statistically indistinguishable from that of GAC. The high degree of adsorption by the SD2-1 bacteria cells and GAC are both closely related to the hydrophobic interaction between the estrone and bacterial cells, or GAC. The adsorption of negatively and neutrally charged micropollutants onto AC is also suggested to be mainly due to hydrophobic interactions, whereas the adsorption of positively charged compounds by GAC has been determined to be mainly influenced by electrostatic or ionic interactions (Margot *et al.*, 2013b). Because estrone is a neutral micropollutant it is assumed to have a highly hydrophobic affinity for activated carbon, and hydrophobicity is considered to be the dominant adsorption interaction of this compound with GAC (Margot *et al.*, 2013b; Hartmann *et al.*, 2014), apart from other mechanisms such as π-π interaction which can occur between estrone and activated carbon surface (Jiang *et al.*, 2017). Due to the equal estrone adsorption performance of GAC and bacterial adsorbent SD2-1(85), and considering the neutral charge of bacteria and hydrophobic characteristics of estrone, the adsorption mechanism most likely involves hydrophobic interaction between the bacteria and estrogen compound. During the adsorption process, the hydrophobic surface of SD2-1(85) bacterial adsorbent attract the hydrophobic estrone

molecule. Then the estrone molecules attached to the bacterial cell adsorbent (solid phase) are then removed from the aqueous phase along with the bacterial cells during the separation phase (centrifugation), and reduced levels of estrone remain in the aqueous phase, with almost complete removal being achieved after the adsorption process.

However, significantly lower degrees of estrone removal were found in the reactors with SD2-1(100), as well as for the zeolite reactor, compared to the GAC reactor (Table 8.1). The degree of removal was also found to be significantly lower than that of SD2-1(85), and thus the biomass pre-treatment temperature was found to be an influencing factor on bacterial adsorbent efficiency. As has been previously predicted (Section 8.1.5), the bacterial cells were physically disrupted by the 100 °C inhibition temperature, with cells being observed to be broken (Figure 8.3) compared to the intact cells displayed for the 85 °C pre-treatment temperature (Figure 8.2). It can be deduced that the broken cells were less suitable for the attachment of estrone molecules, and this was closely associated with the loss of their hydrophobicity characteristics (Figure 8.3), despite the assumption that broken cells have a higher surface area than intact cells.

Reactors containing zeolite adsorbent showed this adsorbent gave relatively low adsorption of estrone, presumably because this pure synthetic zeolite has a preference for cation molecules through an ionic bonding mechanism, rather than having hydrophobic adsorption properties. The zeolite that has previously been reported to be an excellent adsorbent for anionic perfluorochemical (PFC) surfactants is actually a specifically hydrophobic-modified zeolite known as NaY80 (Ochoa-Herrera and Sierra-Alvarez, 2008), and this material could not be tested in the current study, explaining the poor results for estrone removal by the pure zeolite that was used. The poor adsorption of estrone by pure zeolite also supports the theory that the adsorption of estrone requires hydrophobic interactions to be established between the adsorbent and the estrogen molecule, and the hydrophobic bacterial cell surface provides this type of surface. In addition, natural zeolite has been proven to be an inefficient adsorbent for 17β-estradiol (E2), and only functions as an excellent adsorbent with 98 % of E2 removal after it has undergone a surface-modification with the hexadecyltrimethylammonium (HDTMA) surfactant (Dávila-Estrada *et al.*, 2016) when the surface of the zeolite would be converted from being hydrophilic to hydrophobic due to the presence of the organic-rich surfactant layer on the zeolite surface following modification. These results indicate that the major mechanism in the adsorption of hydrophobic micropollutants such as estrogen and PFC is dominated by hydrophobic interactions, and this can also be achieved using hydrophobic-adapted bacterial

biomass such as that from *Tsukamurella* sp. SD2-1, an activated carbon material such as GAC, or a hydrophobic-modified zeolite.

Figure 8.2 Intact structure of *Tsukamurella* sp. SD2-1 bacterial cells pre-treated at 85 °C for 1 hour. Cell morphology observed at 10,000 times magnification using a fluorescence microscope.

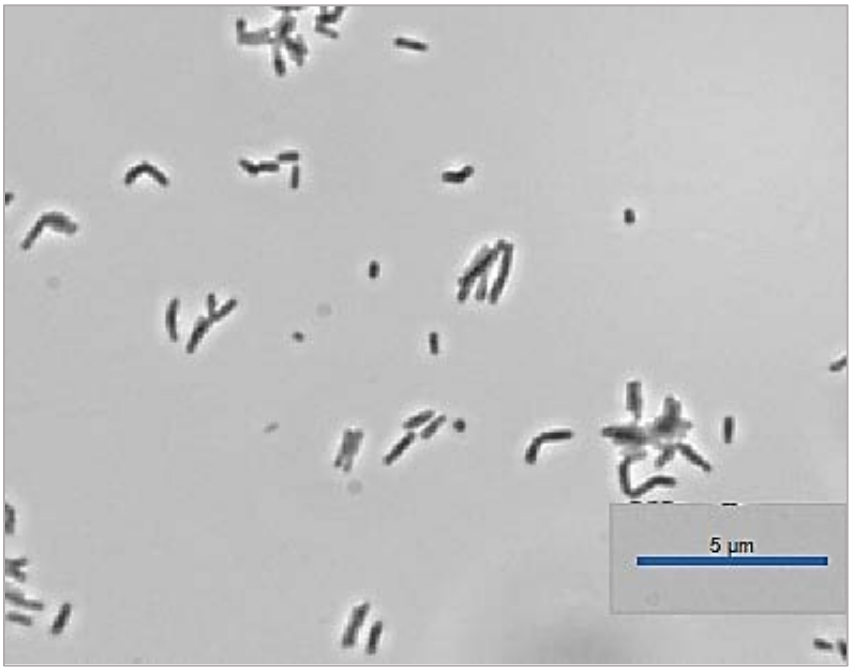
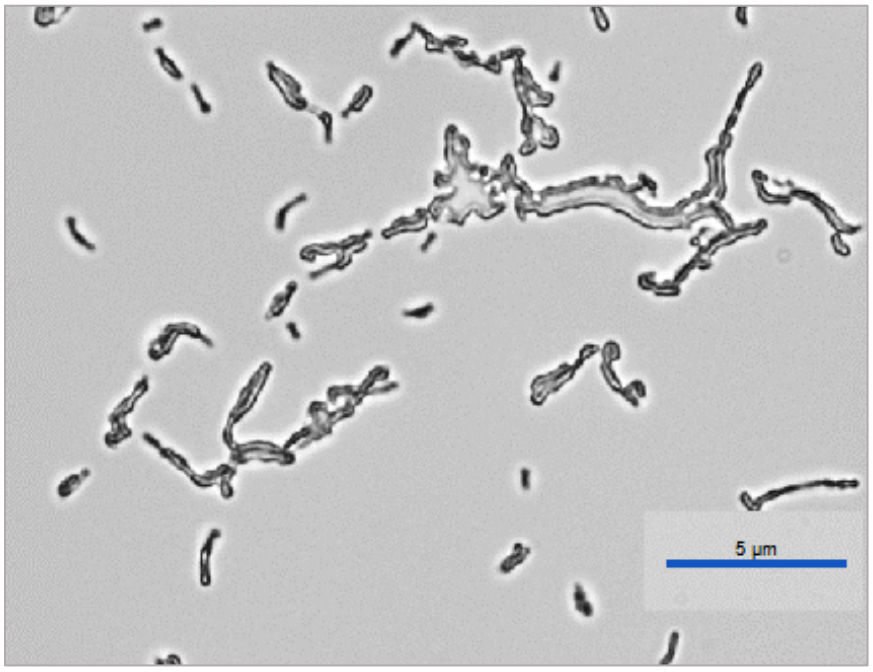


Figure 8.3 Disrupted structure of *Tsukamurella* sp. SD2-1 bacterial cells that inhibited at 100 °C for 1 hour. Cell morphology observed at 10,000 times magnification using a fluorescence microscope.



8.3.2 Desorption process

The amounts of estrone adsorbed onto the surface of the bacterial adsorbent and then desorbed back to the aqueous phase were measured to determine the reversibility of the adsorption process and the degree of estrone recovery that was possible from the adsorbent. Figure 8.4 shows the amounts of estrone that could be adsorbed to and subsequently desorbed from the bacterial biomass adsorbent.

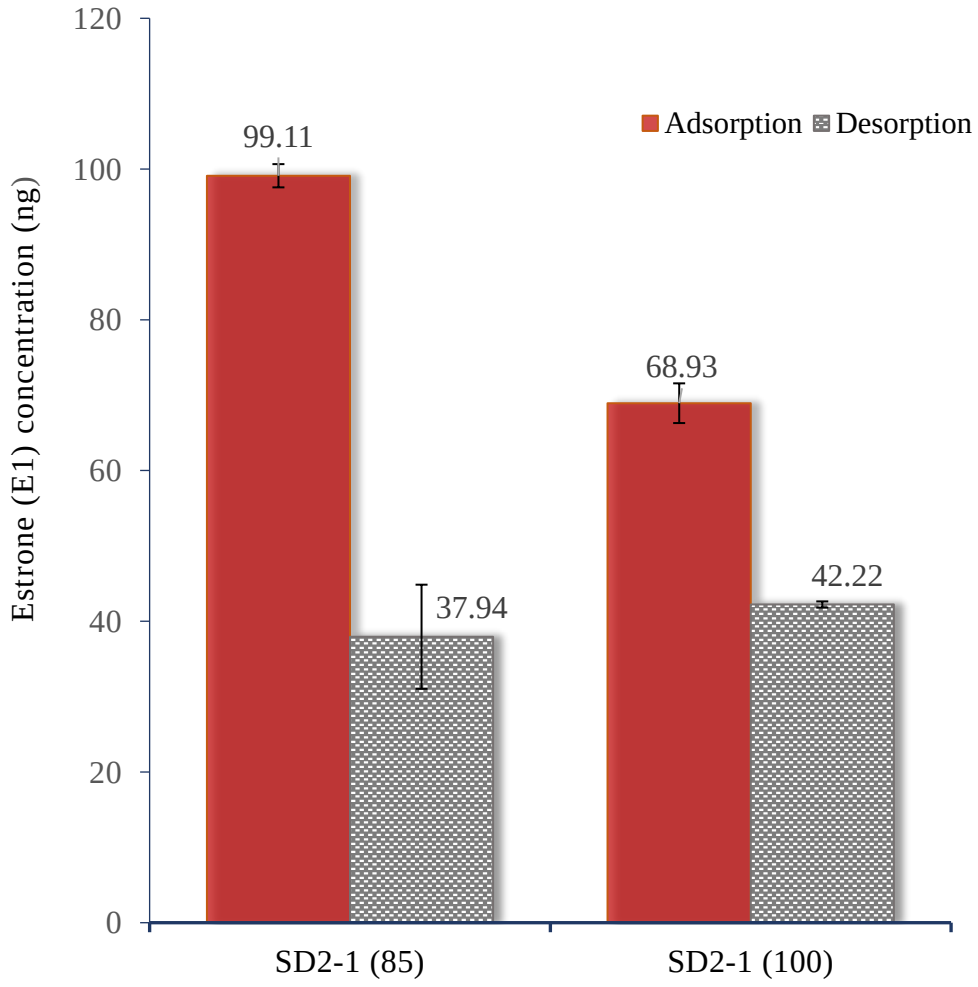


Figure 8.4 Amount of estrone found to be adsorbed from synthetic wastewater by the SD2-1 bacterial adsorbent pre-treated at temperatures of 85 °C and 100 °C and subsequently desorbed back to the aqueous phase. Reactors each contained 500 ml of synthetic wastewater and 200 ng.L^{-1} of estrone, carrying a total amount of 100 ng of estrone.

Table 8.2 shows the degree of estrone recovery, and any difference between the pre-treatment temperatures according to the 2-sample t-test and Tukey pairwise analysis using Minitab 17 statistical software.

Table 8.2 Recovery efficiency of estrone from the desorption process compared to the amount of estrone previously adsorbed onto the bacterial adsorbent. Bacterial adsorbents were pre-treated at temperatures of 85 °C and 100 °C. Reactors each contained 500 ml of synthetic wastewater and 200 ng.L^{-1} of estrone, carrying a total amount of 100 ng f estrone.

Strains	Percentage of recovery (relative to adsorption amount) Mean (%)*	St Dev
SD2-1(85)	38.28[a]	6.97
SD2-1(100)	61.25[b]	0.60

* Means that do not share a letter are significantly different.

Consequently, for the SD2-1(85) adsorbent, 38.28 ± 6.97 % of the total available estrone was found to be desorbed back into the deionised water, representing an aqueous concentration of 37.94 ng.L^{-1} being formed from the 99.11 ng of estrone bound to the intact cells of SD2-1(85) after 24 hours contact time. In comparison, 61.25 ± 0.60 % of estrone was recovered from the total amount of 68.93 ng adsorbed on the disrupted SD2-1(100) cells. Firstly, these results reveal that a very effective adsorption process was obtained using intact bacterial cells pre-treated at 85 °C. Secondly, the amount of estrone successfully recovered from the adsorbed state on bacterial cells pre-treated at 85 °C is relatively low compared to the total amount adsorbed. In contrast, a higher desorption performance was recorded for the bacterial cells pre-treated at 100 °C. Consequently, it can be deduced that the poor desorption performance observed with SD2-1(85) in comparison to SD2-1(100) was because the attachment of the estrone to the intact cell structure of SD2-1 bacterial cells was relatively stronger than to the broken SD2-1 (100) cells. In other words, higher desorption performance occurred with SD2-1(100) cells due to the ability of the broken cells to release the estrone molecules more readily.

Nevertheless, the recovery of estrone in both reactors was considered to be only moderate, at 38 – 61 %. These findings are similar to those of previous studies, where 100 % desorption and analyte recovery has not usually been obtained. However, no complete study of the adsorption and desorption processes of estrogen compounds using pure or mixed bacterial species has been conducted before, except for activated sludge and wastewater sludge which were assumed to contain microbes. Different degrees of desorption have been cited in past studies, including 60 % desorption of EE2 recovered from wastewater sludge adsorbent (Tenenbaum *et al.*, 2014), while very low levels of desorption of estrogen compounds, less than 1.1 % , were found following the adsorption of E1, E2, E3 and EE2 by activated sludge (Ren *et al.*, 2007a). These

poor recovery rates were considered to be related to the slow rate of desorption compared with the adsorption process, and that the adsorption of estrogen onto bacterial adsorbents or sludge is not completely reversible (Ren *et al.*, 2007a; Rimmer, 2010; Marti and Batista, 2014). In addition, the use of solvents in the aqueous phase of the desorption process has been shown to affect the degree of desorption of estrogen compounds (Zhang *et al.*, 2012). Specifically, the addition of 50 % acetonitrile was found to increase the percentage of desorption of EE2 to 97.2 %, double the amount of 41.2 % obtained using distilled water only. Use of co-solvent in the desorption process was also found to increase the EE2 solubility and decreased the interaction between the EE2 and the sludge (Zhang *et al.*, 2012).

In parallel work, the level of desorption of pesticides from soil particles has also been found to be lower than their adsorption, and is affected by various factors such as the controlled ambient temperature during the desorption process (Rani and Sud, 2015). The degree of recovery of the pesticide (triazophos) was found to be higher during desorption at 40 °C compared to 20 °C or 10 °C (Rani and Sud, 2015), which confirms that the desorption process requires an energy in order for the analyte molecules to be released from the adsorbent.

To investigate the desorption process further, an additional control experiment was carried out using estrone dissolved in deionised water as the adsorption medium. This showed the adsorption of estrone from deionised water by the SD2-1 bacterial strain (Figure 8.5) was much less efficient than its adsorption from synthetic wastewater (Figure 8.4), with only 38.38 ng of estrone being adsorbed in the reactor containing estrone dissolved in deionised water (Figure 8.5). This result can be explained by the fact that the deionised water would have disrupted the bacterial cells due to osmotic stress (Vadillo-Rodríguez and Dutcher, 2011), causing the bacteria to lose their previously intact hydrophobic surface and consequently decreasing their estrone adsorption characteristics. This effect was absent in the reactor containing synthetic wastewater as the background medium for the SD2-1(85) bacterial adsorbent (Figure 8.5), because the hydrophobic properties of the intact bacterial cells were well preserved in this medium. This allowed them to exert their full adsorption potential for estrone present in the wastewater sample. In addition, a very low amount of estrone was found to be subsequently desorbed back in the aqueous phase during the desorption process in deionised water. The cells that break open from osmotic stress appear visibly very different from thermally pre-treated cells, and do not possess the same estrogen adsorption capacity, probably due to the different mechanism of the processes that caused the disruption of the cells.

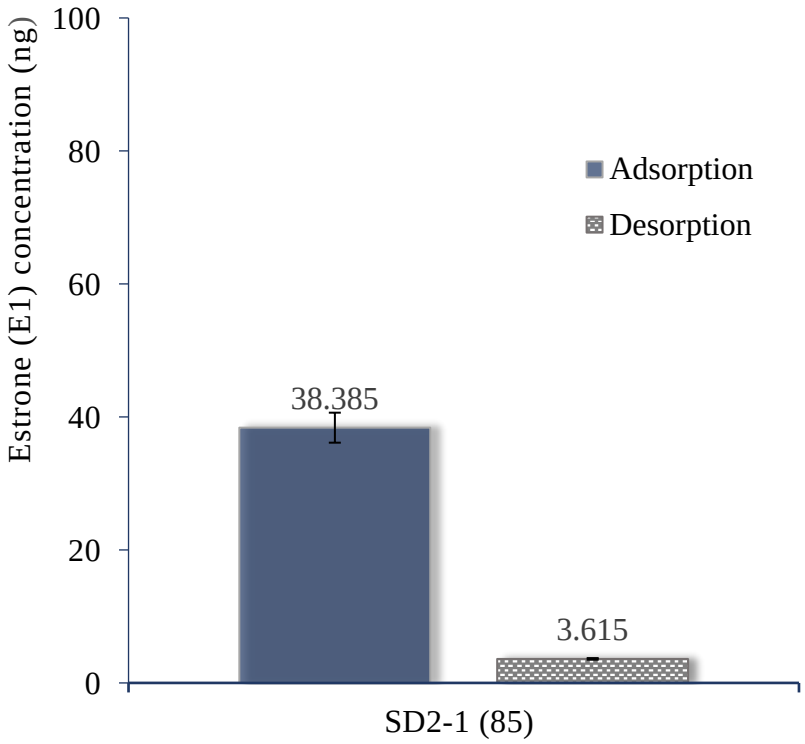


Figure 8.5 Amount of estrone found to be adsorbed from deionised water by the SD2-1 bacterial adsorbent pre-treated at temperatures of 85 °C and subsequently desorbed back to the aqueous phase. Reactors each contained 500 ml of synthetic wastewater and 200 ng.L^{-1} of estrone, carrying a total amount of 100 ng of estrone.

8.3.3 Isotherm model

Data of the final concentrations of estrone following adsorption, using different initial concentration, were plotted according to the Freundlich and Langmuir model (Section 8.1.7) and their correlation coefficients evaluated. The modeling results show that the estrone adsorption data is not perfectly linear, however it is best fitted in the Freundlich model with its correlation coefficient (R^2) of 0.9103 (Figure 8.7), compared to lower R^2 value of 0.741 for the linear Langmuir model (Figure 8.6). Therefore only the Freundlich model was used in calculation of isotherm parameters. The sorption coefficient (K_f) is described as an estimation of the adsorption capacity, and the sorption constant (1/n) is seen as a measure of the strength of adsorption (Gomes *et al.*, 2011; Ifelebuegu, 2012).

Table 8.3 Transformed data on the adsorption of estrone (E1) from different initial concentrations as fitted to the isotherm models.

Initial concentration, C_o (ngL-1)	Final concentration, C_e (ngL^{-1})	Amount of estrone adsorbed/ adsorbent weight, q_e ($ng.mg^{-1}$)	C_e/q_e (mg)	Log C_e	Log q_e
50.00	0.36	10.00	0.04	-0.44	1.00
200.00	4.60	39.08	0.12	0.66	1.59
350.00	92.18	51.56	1.79	1.96	1.71
500.00	118.83	76.23	1.56	2.07	1.88
700.00	180.00	103.90	1.74	2.26	2.02
1000.00	259.00	148.20	1.75	2.41	2.17

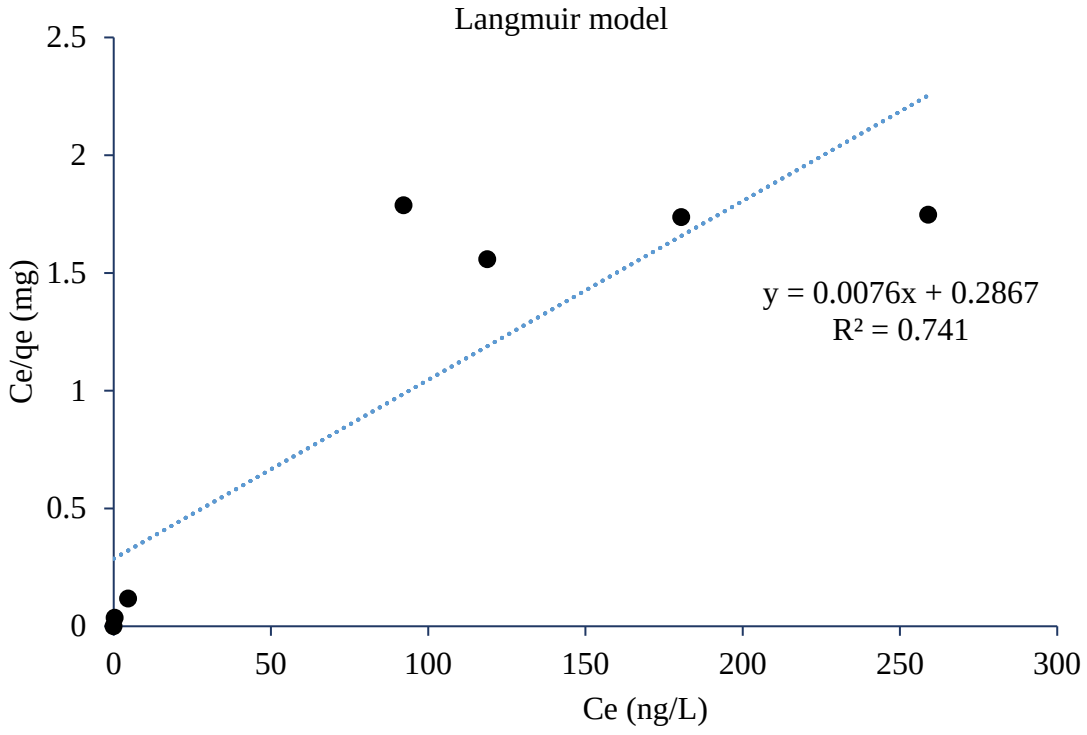


Figure 8.6 Data on estrone adsorption fitted into the Langmuir model

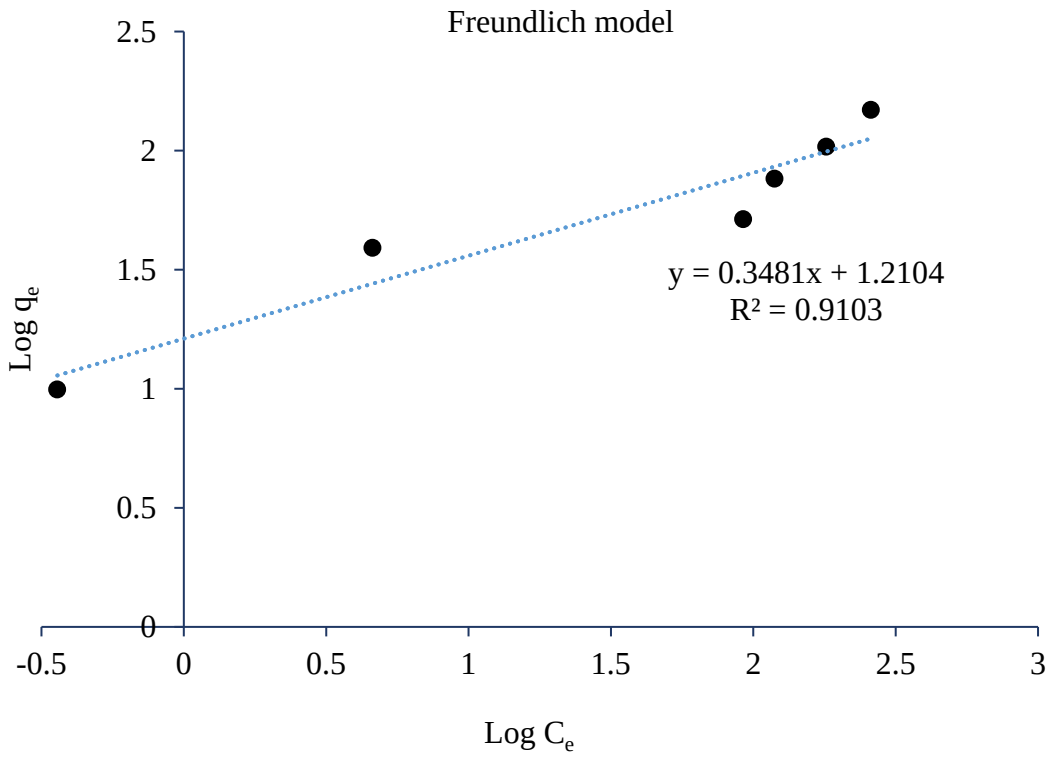


Figure 8.7 Data on estrone adsorption fitted into the Freundlich model

Using experimentally determined sorption data from Table 8.4, the empirical relationship of the Freundlich isotherm was established and value of the adsorption capacity (K_f) and sorption constant (1/n) of the SD2-1 bacterial adsorbent for E1 were found to be 16.23 ng.mg^{-1} and 0.3481 respectively (Table 8.4). Consequently, the adsorption capacity of the SD2-1 bacterial biomass was found to be half that of the 29.84 ng.mg^{-1} found for GAC in previous work (Li *et al.*, 2012). However, the GAC adsorption capacity was measured using a drinking water sample, which would have contained much lower background concentrations of organic matter compared to the synthetic wastewater used in the present study. Therefore the organic matter would have reduced the performance of adsorbent for the specific estrone analyte. The presence of organic matter in real wastewater has been shown to reduce the adsorption capacity of GAC for estrogens by approximately 34 – 37 % when compared to adsorption from a pure water sample, e.g. ultra-pure water, as well as drinking water (Hartmann *et al.*, 2014; Hemidouche *et al.*, 2017). Consequently, the adsorption capacity of the hydrophobic-adapted bacterial adsorbent (SD2-1) is to be very comparable capacity to the GAC, and the lower K_f values seen in this study was mainly due to the interference of dissolved background organic matter present in the synthetic wastewater medium.

Table 8.4 Freundlich sorption coefficient for the estrone in the adsorption process using the adapted-bacterial adsorbent from strain SD2-1.

Isotherm model	Log K_f	K_f ($ng.mg^{-1}$)	1/n
Freundlich	1.21	16.23	0.3481

8.4 Conclusions

The SD2-1 adsorbent pre-treated at an ideal temperature of 85 °C was found to be a very efficient material for the adsorption of estrogen from synthetic wastewater, with a performance level comparable to that of activated carbon. It has successfully been show to reduce estrone levels from an initial concentration of 200 $ng.L^{-1}$ to a final concentration of 1.78 $ng.L^{-1}$, below the PNEC for E1. Therefore the alternative hypothesis is accepted, which is that removal of estrogen using a hydrophobic bacterial cell adsorbent is a potentially viable alternative removal technology compared to activated carbon and the objective (Section 1.3 (5)) of comparing the performance of the thermally pre-treated SD2-1 bacterial adsorbents to commercial adsorbents was achieved.

The use of higher temperatures for the pre-treated of bacterial cells, 100 °C pre-treatment, was found to disrupt the structure of the bacterial cells and to alter their hydrophobicity, thereby producing lower levels of estrone removal. This supports the hypothesis that bacterial pre-treated temperature has a critical effect on the performance of the bacterial biomass adsorbing estrogens. In addition, the adsorption of estrone to the bacterial adsorbent and activated carbon were concluded to operate by the same mechanism, namely hydrophobic interaction with the estrogen adsorbate, rather than by ionic bonding. This explained the high efficiency seen with the hydrophobic-adapted bacterial adsorbent in removing estrone from the synthetic wastewater.

Furthermore, recovery of estrone during the desorption process was linked to the degree of adsorption achieved during the adsorption step. Poor desorption and low degree of estrone recovery was affected by the factor of cell morphology of the bacterial cells, with higher degrees of estrone desorption and recovery being achieved from cells that were disrupted. Use of deionised water as the aqueous phase for the desorption process without solvent also reduced degree of the recovery.

Finally, the adsorption of estrone using the bacterial adsorbent of SD2-1 strain fitted the linear Freundlich model, with 16.23 $ng.mg^{-1}$ adsorption capacity, and after taking the effects of interfering background organic matter into account, the SD2-1 adsorbent was considered to

have comparable performance to activated carbon. The adsorption of estrone was identified to be associated with a physisorption process that was only partially reversible.

Chapter 9 General conclusions and recommendations

The aim of this study was to explore the potential of using hydrophobic bacteria as sustainable adsorbents for the removal of estrogens from wastewater and to evaluate their capability of removing selected estrogens to below their Environmental Quality Standard. The research involved a preliminary study of the removal of estradiol (E2) from water, followed by a more comprehensive investigation of the removal of estrone (E1) from synthetic wastewater, and how the results compared to the requirements of the the Water Framework Directive Watch List. The annual average concentration in the Environmental Quality Standard (AA-EQS) for E2 is recommended to be below 0.4 $ng.L^{-1}$. However, since an AA-EQS has not yet been established for E1, the predicted no-effect concentration (PNEC) of 3.6 $ng.L^{-1}$ is recommended by the WFD to be a level that is considered to be safe when present in water (European Commission, 2016). In the long-term, the complete removal of the estrogen compounds from water is an ultimate goal, which is a concern for all environmentalists. Several findings that have been identified throughout this study support the aim and also contribute new knowledge relevant to this issue. The main outcomes from this study, as well as recommendations that can be considered as a consequence of the work, are summarised below.

9.1 General conclusions

9.1.1 Adaptation of bacterial strains into hydrophobic adsorbents

To ensure the efficient removal of estrogens from water, this study focuses on the interaction between the estrogen and hydrophobic microorganisms, particularly the study of hydrophobic-adapted bacterial strains that might be developed further as potentially viable adsorbents for use in drinking water treatment. Initial findings revealed that the adaptation of commercial strains of *Rhodococcus erythropolis* strains DSM 311 and NCIMB13064 after culturing on a hydrocarbon growth substrate, were found to show increased bacterial cell surface hydrophobicity (CSH) by up to 6 %. The type of hydrocarbon used as growth substrate was found to be a major factor in the adaptation process, and the medium chain-length hydrocarbon n-hexadecane was identified as an appropriate growth substrate for the adaptation process. Moreover, short-chain alkanes such as n-hexane were found to be too toxic to the bacteria, and inhibited their growth, due to the lower octanol-water partition coefficients, which made this substrate much more water-soluble and able to disrupt the bacterial cells. Initial 7-day enrichment cultures were conducted to isolate bacteria from diesel contaminated-soil, and this provided several new hydrophobic strains. Of these, strain SD2-1, later identified as a *Tsukamurella* sp., was found to be the most hydrophobic strain under test. A longer soil-

enrichment incubation period of 21 days provided more hydrophobic isolates in addition to those from the 7-day enrichment. The *R. erythropolis* NCIMB13064, *R. erythropolis* DSM311 and *Tsukamurella* sp. SD2-1 strains have been determined to be the most hydrophobic bacterial strains to arise from the adaptation process during the growth cycle. Unfortunately, the adapted *R. erythropolis* NCIMB13064 strain was found to experience a loss of hydrophobicity when the glycerol stock was stored over 2 months with a 35 % reduction in CSH compared to the strain that was preserved. This could have been due to loss of genetic material (e.g. plasmids) causing physiological changes. Another factor that was found to affect the successful adaptation of hydrophobic characteristics is the dosage of the growth substrate. 50 µl of n-hexadecane in a 100 ml of culture (500 $\mu l.L^{-1}$) was determined to be the optimum substrate dosage for bacterial growth. However, higher concentrations of n-hexadecane should be avoided because its toxicity could inhibit bacterial growth and, furthermore, would add additional cost to adsorbent production. Interestingly, the CSH of the adapted bacteria were identified to increase rapidly at the beginning of the batch growth cycle. The CSH increased during the exponential growth phase, reaching high CSH values that persisted into the stationary phase for most strains. In theory, the hydrophobicity increased when the membrane lipid composition of the bacteria cells altered so that they became saturated when exposed to the hydrocarbon. As the incubation period extended into the exponential phase, the bacteria became more fully adapted, and generated greater hydrophobic characteristics in the cells, meeting the objective of investigating the CSH characteristics during different phases of bacterial growth. Nevertheless, the levels of hydrophobicity gradually decreased when the bacteria entered, or approached the death phase. Consequently, further increases in hydrophobicity did not occur with extended growth periods, rather, there was often a reduction in CSH due to cell death. In conclusion, the *R. erythropolis* NCIMB13064, *R. erythropolis* DSM311 and *Tsukamurella* sp. SD2-1 were found to have their highest CSH levels of 90 – 93 % in their stationary phase, and were adopted as the best potential adsorbents for the removal of hydrophobic micropollutants such as estrone in subsequent experiments. The high CSH values obtained meant that the objective of adapting bacterial species into a potentially useful hydrophobic adsorbent by using a hydrophobic growth substrate has been achieved.

9.1.2 Removal of estrone

Different degrees of estrone (E1) removal from synthetic wastewater were obtained using the adapted bacterial strains, and this was considered to involve both adsorption and biodegradation processes. However, the main outcome of these experiments was to show that the *R. erythropolis* DSM311 and *Tsukamurella* sp. SD2-1 with the highest CSH values also provided

the highest removal rates of E1. However, the E1 removal efficiency of the adapted *R. erythropolis* NCIMB13064, was very low because it had lost its hydrophobicity. This indicates a very strong connection between the bacterial CSH and the estrone adsorption efficiency of the biomass, and meets Objective 3 (Section 1.3).

Apart from the CSH of the bacterial adsorbent, contact time and bacterial adsorbent dosage were also found to affect the efficiency of the E1 removal process. In addition, the temperature used for bacterial pre-treatment was also found to indirectly influence the adsorption efficiency, with poor E1 adsorption resulting from the loss of hydrophobic attachment sites of the disrupted cells following 100 °C pre-treatment, compared to the 85 °C pre-treatment that preserved the intact cells.

Further investigation of E1 removal using bacteria in different growth phases indicated that cells in the stationary growth phase give higher E1 adsorption performance than those in the exponential phase, and this correlated with higher CSH in the stationary phase. Providing longer growth incubation periods (more than 10 days) during extended fed-batch cultures of the bacterial cells that were then used as adsorbents, as identified in the objectives, however, did not produce a significant increase in the adsorption efficiency. This was due to the almost constant bacterial CSH values obtained, regardless of the growth incubation period, and only very small insignificant differences were found for the degree of E1 removal by these cultures, compared to the single batch cultures.

In contrast, an unexpected lower E1 removal rate was observed using live bacterial cells, even though the conditions allowed for both biodegradation and adsorption processes to occur. The lower degree of E1 removal was finally determined to be due to the presence of the interfering effect of biosurfactants, which solubilised the E1 within the aqueous phase. Because *R. erythropolis* has been reported as an estrogen-degrading bacteria (O'Grady *et al.*, 2009; Larcher and Yargeau, 2013) it should have degraded E1 in these experiments. However, the presence of the biosurfactants appeared to reduce the efficiency of biodegradation as well as adsorption. The biosurfactant of trehalose lipid, a possible component of the biosurfactants, has been shown to increase E1 solubility (Kügler *et al.*, 2014), which possibly gave poor attachment to the hydrophobic bacterial cells, and reduced uptake and biodegradation . This was thought to arise due to the presence of residues of hydrocarbon growth substrates being carried over with the bacterial cells when added to the reactor, allowing the cells to grow in the reactor and produced biosurfactant. Thus, in future studies, the live bacterial cells should be separated from the aqueous phase (containing the n-hexadecane residue) prior to being added to the reactor for the biodegradation and adsorption of E1.

In experiments comparing the bacterial adsorbents from stationary phase *Tsukamurella* sp. SD2-1 with commercial adsorbents, granular activated carbon and zeolite, it was shown that very high E1 removal efficiency (99 %) could be achieved with the bacteria adsorbent, which was close to the complete removal shown by the activated carbon. After adsorption by stationary phase *Tsukamurella* sp. SD2-1 cells, the final aqueous E1 concentration was recorded as 1.78 $ng.L^{-1}$, which is below the PNEC level for this hormone, proving that the SD2-1 strain isolated from soil could potentially provide an effective drinking water treatment process for use at full-scale. In contrast, synthetic zeolites gave lower removal performance compared to SD2-1 and granular activated carbon, due the different (ionic) bonding mechanism provided by these adsorbents. This indicates that major route for the adsorption of E1 in this study was through hydrophobic interaction with the adsorbent, and explains similarity in the efficiency of the hydrophobic adsorbent SD2-1 with that of granular activated carbon. Therefore, as identified in the objectives, the capacity of removal of E1 by the SD2-1 bacterial adsorbent was found to be comparable to the commercial adsorbent activated carbon, but higher than zeolite.

In experiments that investigated the E1 adsorption-desorption reversibility, only 37 % of the E1 could be recovered from the bacterial adsorbent due to the slow desorption rate, the heterogeneous physicochemical properties of the bacteria and aqueous medium. This low degree of recovery from the bacterial adsorbent is supported by previous research, in which only 60 % (Tenenbaum *et al.,* 2014) and less than 10% (Ren *et al.,* 2007a) of estrogen could be recovered from activated sludge, due to desorption of estrogens being a slower process than adsorption (Rimmer, 2010; Marti and Batista, 2014). These studies used activated sludge which contains inorganic, organic and microbiological components instead of pure strains of bacteria, and to date there is no study reporting the desorption of estrogen pollutants from pure strain bacterial adsorbents.

In conclusion, adaptation of bacteria in minimal media and n-hexadecane has successfully improved the hydrophobicity of a commercial strain of *R. erythropolis* (DSM311), and produced a very hydrophobic isolate of *Tsukamurella* SD2-1. These hydrophobic strains shown to be efficient adsorbents for the removal of estrone below its PNEC level, and to have almost comparable performance to that of granular activated carbon.

9.2 Implication of the current research

From this investigation, bacterial adsorbents appear to be excellent alternatives to commercial adsorbents for reducing estrogen concentrations in municipal wastewater before discharge to the environment, or during potable water treatment, ensuring the reduction of E1 concentrations to levels below those causing adverse environmental effects or human health implications.

Potentially, the use of hydrophobic bacterial adsorbents could reduce the cost of estrogens removal without compromising the high performance already provided by the use of activated carbon. Consequently, the bacterial adsorbents identified in this bench-scale study could readily be upscaled to a pilot scale study, to identify whether a cheaper hydrocarbon substrate material might be adopted instead of the laboratory grade of n-hexadecane used in this research, in order to demonstrate further the low cost treatment objectives of this study. In addition, hydrophobic-adapted bacterial adsorbents could also be applied to the adsorption of other estrogen compounds, notably the synthetic estrogen 17α-ethinylestradiol (EE2). Even though it is frequently found in lower concentrations than estrone (E1) in real wastewater, EE2 has been determined to be a more potent estrogen compound than estrone. Moreover, the hydrophobic-adapted *R. erythropolis* DSM311 and *Tsukamurella* spp. SD2-1 may also have potential in the removal of other hydrophobic emerging contaminants, where the effectiveness of removal can be expected to be related to the level of hydrophobicity of the bacterial cell which determines the level of adsorption. Moreover, hydrophobic adsorption is a non-specific process, unlike biodegradation which requires specific catabolic enzymes for different contaminants.

9.3 Recommendation for future work

There are areas for improvement in the current studies which could potentially be investigated to gain a better understanding of the underlying adsorption mechanisms, and to produce more efficient techniques in the elimination of estrogens from water. The performance of the adapted bacterial strains in removing E1 in terms of both adsorption and biodegradation process was assessed, and two of the highest CSH strains were found to provide high adsorption performance. Further studies on the estrogen removal process using mixtures of pure hydrophobic bacterial species could potentially give a better understanding of how interactions in the microbial community affect performance in the biodegradation and adsorption of estrogen. In addition, their variability in enzyme content and physicochemical properties might lead to compensatory effects, which improve the estrogen catabolic activity, and enable higher rates of attachment of estrogen molecules in the adsorption process. Mixtures of strains with the highest CSH values of SD2-1 and DSM311 would be the most prominent combination to be investigated to provide answers as to how this combination removes estrogen differently from the pure strains. Furthermore, an extensive study related to biodegradation could also be conducted using the adapted strains. It is proposed that the addition of live hydrocarbon-adapted bacterial strains into adsorption reactors should be made without carrying over culture media, because the hydrocarbon residues carried over promote the production of biosurfactants that inhibit the efficiency of biodegradation. To implement the addition of only bacterial cells, their

separation from the liquid phase can be achieved adopting a centrifugation step. As an estrogen-degrading species, live *R. erythropolis* is expected to be able to degrade and adsorb estrogen well as long as biosurfactants are absent. However, the biodegradation performance of *Tsukamurella* sp. SD2-1 strain is uncertain since it has not been reported to be an estrogen degrading-species in any past studies, and therefore its biodegradation properties should be determined in future work.

Apart from the design of experiments, the use of different but closely related growth substrates, notably with longer molecular chains of aliphatic alkane, should be investigated. Aliphatic alkanes with high carbon content of more than n-C12, such as n-C14 and n-C16, are recommended (Serebrennikova *et al.*, 2014) due to their higher octanol-water partition coefficients and hydrophobicity. In addition, a cheap alternative growth substrate, such as diesel, should be evaluated for the cost-effective adaptation of bacteria on a large scale, because the lower price of such chemicals could reduce the cost of estrogen treatment significantly, and this would be potentially more appropriate in a pilot study.

Finally, a continuous growth reactor should be evaluated which can provide an optimised environment for the production of bacterial biomass adsorbent, allowing the cells to develop their maximum levels of hydrophobicity, and ultimately to enhance their performance in estrogen removal. Further growth studies should be explored in which bacteria are grown as biofilms on porous or segmented surfaces that can provide high surface area for contact with the process water. These biofilm support matrices could be made from cheap hydrophobic materials, such as polypropylene or polystyrene, which should facilitate the attachment of the growing hydrophobic bacterial biofilm. This approach would also generate an immobilised form of the biomass that could be used in a conventional packed bed filter in much the same way as GAC filters contain particles of carbon adsorbent. Such “bioadsorbent” filters could be operated in parallel, one for hydrophobic biomass formation, the other for estrogen treatment, with the roles of treatment flow and biomass growth being alternated when the adsorption capacity of the adsorbent had been reached.

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Appendix 1

Clean DNA sequences for the soil-isolates bacteria and significant species detected in BLAST.

Strains	Clean sequences of samples (based on the F8FpL (forward) and P806R (reverse) primers)	Significant species (%)
SD1-1	CTGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCG GGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAA GCCTGGGAAACTGGGTCTAATACCGGATATGACTTCAGGTTGCATGACTTGGGGTGGAAAGA TTTATCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAG GCGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCA GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGA CGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAG TGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG GGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTT GTGAAAACCAGCAGCTCAACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGG GGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGC GAAGGCCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGG ATTAGAT	99 % *Rhodococcus erythropolis*
SD2-1	CCTGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTAAGGCCCTTC GGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGGGTGACCTGCCCTGTACTTCGGGATA AGCCTGGGAAACTGGGTCTAATACCGGATATGACCTTCCCCTGCATGGGGGTTGGTGGAAAG CTTTTGCGGTACAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAA GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCG ACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGACGAAGCGCAA GTGACGGTACCTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTA GGGTGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCACGTCGT CTGTGAAAACCCGAGGCTTAACCTCGGGCCTGCAGGCGATACGGGCAGACTTGAGTACTGTA GGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTG GCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGAGTAGCGAACAG GATTAGATA	99 % *Tsukamurella tyrosinosolven* 100 % *Tsukamurella pulmonis* 100 % *Tsukamurella pseudospumae*

Strains	Clean sequences of samples (based on the F8FpL (forward) and P806R (reverse) primers)	Significant species (%)
PD2-1	CTACATGCAGTCGAGCGGTTAGGCCTTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACAC GTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGA CTTCAGGTTGCATGACTTGGGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCCTATC AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGA CCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGT AAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTA AAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCTTGC AGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAA ATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGC TGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGAT	99 % *Rhodococcus erythropolis*
PD2-2	CGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCGG GGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAG CCTGGGAAACTGGGTCTAATACCGGATATGACCTCAGGTTGCATGACTTGGGGTGGAAAGAT TTATCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGG CGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGAC GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGT GACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG GTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTG TGAAAACCAGCAGCTCAACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGG GAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCG AAGGCGGGTCTCTGGGCAGTAACTAGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGA TTAG	99 % *Rhodococcus erythropolis*

Strains	Clean sequences of samples (based on the F8FpL (forward) and P806R (reverse) primers)	Significant species (%)
PD2-3	CTTAACACATGCAAGTCGAGCGGTAGGCCTTTCGGGGTACACGAGCGGCGAACGGGTGAGTA ACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGAT ATGACNTCAGGTTGCATGACTTGGGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCC TATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGG TGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA TATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT TGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCT AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCG TAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCTTG CAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGA AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACG CTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAG	99 % *Rhodococcus erythropolis*

Appendix 2

Phylogenetic tree for the soil-isolates strains