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UNITED KINGDOM · CHINA · MALAYSIA

The Adaptive Laboratory Evolution of an *Escherichia coli* strain with increased tolerance to styrene and ethylbenzene

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Thesis submitted to the University of Nottingham for the degree of
Doctor of Philosophy

April 2020

Abstract

Microbial bioprocesses could provide sustainable alternatives to many of the current manufacturing processes in the chemical industry. A significant amount of research and development interest is currently invested in bio-based production routes to petroleum derived aromatic monomers. However, the bio-based production of these aromatic monomers is not on a commercial scale due to product toxicity which inhibits production titres. Styrene is a mono-aromatic organic compound commonly used as an aromatic monomer in the production of many synthetic rubbers, co-polymers and polystyrene plastics. The biosynthesis of styrene has been achieved from a glucose feedstock in *Escherichia coli via* the construction of a novel pathway. The achievable bio-production titres for styrene in an *E. coli* production strain, is close to the toxicity threshold concentration, which is reported at 2.9 mmol L^{-1}. Increasing the inherent tolerance of the production strain is an ideal strategy to increase styrene production titres. However, the rational targeting of specific genes/gene product may not reveal all the factors, such as multiple mutations, that underpins the tolerance phenotype. Adaptive laboratory evolution (ALE) is a method used to generate adaptive beneficial mutations in a population, by propagating a microbial population under a selection pressure and subsequently screening to isolate the adaptive mutant. ALE was explored as a method to generate *E. coli* BW25113 strains with a tolerance to a final styrene concentration above the toxicity threshold of 2.9 mmol L^{-1}. Extensive method development was carried out to allow analytical control over the concentration of styrene, supplied as the selection pressure to a population of *E. coli* BW25113 cells. This provided useful insight into conducting ALE with other chemicals with similar physicochemical properties to styrene. The ALE experiments carried out, uncovered some new insights into the toxicity mechanisms of styrene not only being centred on its hydrophobicity and ease of entering cell membranes; but also due to the double bond located on its aromatic ring substituent. A novel two stage ALE strategy was implemented to adapt the *E. coli* BW25113 strain; firstly, to evolve resistance to the toxicity effects relating to the hydrophobicity of the aromatic ring and then to the reactive double bond of styrene.

This was done by using ethylbenzene, a structurally related compound that lacks the double bond, as a selective pressure prior to further screening using styrene. This ALE

method generated an *E. coli* BW25113 styrene tolerant mutant which could grow in 4 mmol L^{-1} styrene concentration. Subsequent genome resequencing of the mutant revealed mutations in gene commonly associated with chemical tolerance in novel locations. Findings gained from this study provide strategies to conduct ALE to evolve microbial tolerance to other hydrophobic compounds and potential gene targets which can be rationally engineered.

Acknowledgments

The completion of my PhD project and thesis would not have been possible without the support from several people and organisations.

Firstly, I would like to thank the BBSRC funding body, which has allowed this research to take place. I would like to thank my supervisors Professor Gill Stephens, Dr Anca Pordea and Dr Stephen Hall and my previous supervisor Dr Luca Rossoni, for their continued support, patience and guidance throughout this project. I have really grown as a researcher and person throughout this process and they have all been a great support in the many ups and downs I experienced during the process.

I would also like to thank my friends in the Sustainable Processing Technologies Research group for their support in the lab and always being a friendly face around office and on campus.

I would like to thank my family and friends for spurring me on to and supporting me till the very end. I would like to express my gratitude to my parents, Dr Bola Taiwo and Mrs Rev Valerie Taiwo for their instilling an important life lesson that “Learning is a continuous lifelong journey” from a very young age.

And lastly, I would like to extend my upmost gratitude to my dear husband, Segun Adebayo. I simply would not have made it across the finish line without his tremendous understanding and encouragement over the past few years.

Dedication

I dedicate this work to my father in law Oluyinka Alexander Adebayo,

I know how much you wanted to hold on to see me graduate

Rest in perfect peace

I further dedicate this work to future generations of black women looking to pursue a PhD or achieve their dreams.

If I can do it, so can you.

Just keep going take it one day at a time!

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Chapter 1

Introduction

Microbial bioprocesses could provide sustainable alternatives to many of the current manufacturing processes in the chemical industry. Micro-organisms have the capacity to produce a variety of high value chemicals from a range of renewable biomass feedstocks (Cho et al., 2015; Choi, Song, Shin, & Lee, 2015; Chung et al., 2015; Guo & Song, 2019; Jang et al., 2012; S. Y. Lee & Kim, 2015; Patermann & Aguilar, 2018; Sprenger, 2007; Yang et al., 2016). Biomass feedstocks are generated from renewable resources (CO_2, H_2O, sunlight), provide a sustainable source of organic carbon and are an attractive alternative to using petroleum based feedstocks (Isikgor & Becer, 2015). Advancements in the fields of protein/enzyme engineering, 'omics' technology, metabolic engineering, and synthetic biology have driven the development of industrial microbial strains. As a result, the bio-based industries (chemicals, plastics, pharmaceuticals biofuels and bioenergy) has experienced significant growth worldwide. In the pharmaceutical and biofuel sector, industrial bio-processes have replaced the manufacturing routes of some of the best selling drugs and the large scale production of ethanol in the USA and Brazil (Choi et al., 2015). A significant amount of research and development interest is currently invested in developing bio-based production routes to petroleum derived aromatic monomers.

The metabolic pathway engineering for the production of aromatic monomers, namely phenol, *p*-hydroxystyrene and styrene has been achieved relatively easily in a microbial host (Braga & Faria, 2020; McKenna & Nielsen, 2011; Verhoef et al.,2009; Wierckx, et al.,2005). However the bio-based production of these aromatic monomers is not on a commercial scale due to end product toxicity which inhibits production titres (Braga & Faria, 2020).

Styrene is a mono-aromatic organic compound commonly used as an aromatic monomer in the production of many synthetic rubbers, co-polymers and polystyrene plastics (Chung et al., 2015; Gilbert, 2016; James & Castor, 2011; Perry, 1954). The biosynthesis of styrene has been achieved from a glucose feedstock in *Escherichia coli*, *Saccharomyces cerevisiae* and *via* the construction of a novel pathway (McKenna & Nielsen, 2011; McKenna et al., 2014). As with other aromatic compounds, the significant barrier to bio-based styrene production is end product toxicity to the host producer cell (Gong, Nielsen, & Zhou, 2017; Ling et al., 2014; Changqing Liu et al.,

2018; McKenna et al., 2015; McKenna & Nielsen, 2011; Verhoef et al., 2007). The achievable bio-production titres for styrene in an *E. coli* production strain, is close to the toxicity threshold concentration, which was reported to be 2.9 mmol L^{-1} (McKenna & Nielsen, 2011).

Styrene is hydrophobic and tends to accumulate in hydrophobic cellular constituents, especially in the hydrophobic core of the cell membrane (McKenna & Nielsen, 2011; Sikkema et al., 1995). Accumulation in the membrane is the toxicity effect with the greatest impact, as it causes major disruption to several processes in the cell. Some toxicity effects include, increased fluidity, membrane leakage, membrane protein disruption and impacts on the overall cellular energy status (Eberlein et al., 2018; Ramos et al., 2002; Sikkema et al., 1995).

Some strategies have been employed to deal with the end product toxicity of styrene such as *in situ* styrene removal by gas stripping and solvent extraction (Lee et al., 2019; McKenna et al., 2015). These strategies resulted in increased styrene production titres but not to an industrially relevant concentration (Lee et al., 2019; McKenna et al., 2015). Increasing the inherent tolerance of the production strain appears to be the ideal strategy to increase styrene production titres. However, the rational targeting of specific genes/gene products may not reveal all the factors, such as the multiple mutations, that underpins the tolerance phenotype. Adaptive laboratory evolution (ALE) is a method used to generate adaptive beneficial mutations, by propagating a microbial population under a selection pressure and subsequently screening to isolate the adaptive mutant (Dragosits & Mattanovich, 2013; Kinnersley et al., 2019; LaCroix et al., 2015).

Chapter 2

Literature Review

2.1 Production of styrene in microbial strains

2.1.1 Applications of styrene and chemical industry production methods

Styrene is a mono-aromatic organic compound with the chemical structure C_6H_5-CH=CH_2 (James & Castor, 2011). Styrene can be found in trace amounts within several plant species; it was originally isolated from styrax tree balsam (Panten & Surburg, 2015). Styrene is commonly used as a monomer in the production of many synthetic rubbers, copolymers and polystyrene plastics (Chung et al., 2015; Gilbert, 2016; IHS, 2015; James & Castor, 2011; Perry, 1954). Styrene physically appears as a volatile clear liquid which readily polymerises to form polystyrene and co-polymers, such as acrylonitrile-butadiene- styrene, styrene acrylonitrile and styrene butadiene rubber. Of the total amount of styrene produced, about 60% goes into polystyrene production (Chung et al., 2015; Claypool et al., 2014; Gilbert, 2016; IHS, 2015; James & Castor, 2011; Perry, 1954). Polystyrene is an addition polymer made up of repeating units of styrene connected *via* carbon-carbon bonds. Polystyrene is widely used because it has physical properties that can widely differ depending on the polymerisation method. Other products of styrene include styrene copolymers, most notably acrylonitrile-butadiene- styrene, styrene acrylonitrile and styrene butadiene rubber (Claypool et al., 2014; James & Castor, 2011; K. Lee et al., 2019; Perry, 1954; Tossavainen, 1978). Aside from styrene's polymerisation products, derivatives of styrene are used in the synthesis of pharmaceuticals and other fine chemicals (James & Castor, 2011).

Styrene is most commonly produced by the dehydrogenation of petroleum derived ethylbenzene in the presence of superheated steam and an iron oxide catalyst (Gilbert, 2016; James & Castor, 2011; McKenna & Nielsen, 2011; SRI Consulting, 2009; Szala-Bilnik et al., 2017) (Figure 2.1). Following on from the dehydrogenation process are various steps to obtain and purify crude styrene. The catalytic dehydrogenation of ethylbenzene is endothermic and can be driven adiabatically or isothermally (James & Castor, 2011; Wu et al., 1981) (Figure 2.2; 2.3).

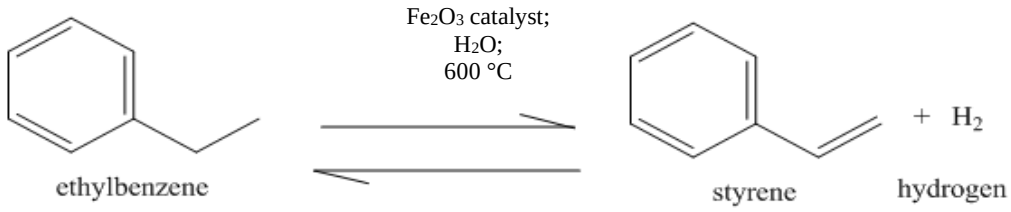


Figure 2.1 The dehydrogenation of ethylbenzene to styrene. Chemical structures drawn using ChemBioDraw Ultra 14.0 software.

Adiabatic dehydrogenation is carried out using a two-stage catalyst packed bed reactor (James & Castor, 2011; SRI Consulting, 2009; Wu et al., 1981). The feed into the reactor contains vapourised ethylbenzene mixed with superheated steam at a reaction temperature of around 640 °C which shifts the equilibrium towards styrene and provides the necessary heat for the endothermic reaction (James & Castor, 2011; SRI Consulting, 2009; Wu et al., 1981). The heat applied at entry to the first reactor is then recovered to preheat the feed into the next reactor. Ethylbenzene conversion to styrene is usually around 35% and then 65% in the first and second reactors respectively (James & Castor, 2011; SRI Consulting, 2009).

The isothermal dehydrogenation of the ethylbenzene process is carried out in a single 'shell and tube' heat exchanger reactor (James & Castor, 2011). The ethylbenzene and steam flow through the catalyst packed tubes in the reactor and heat is generated externally in the reactor shell, to drive the reaction (James & Castor, 2011). This process is not used by many styrene production plants, with around 75% still using the adiabatic process (James & Castor, 2011).

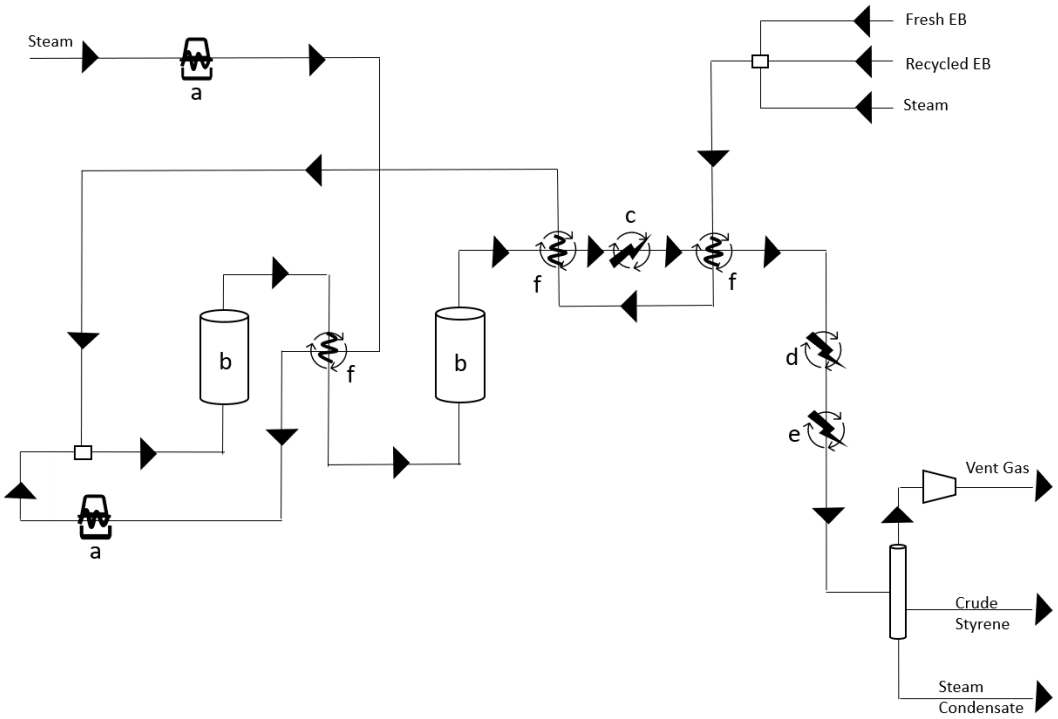


Figure 2.2 Adiabatic dehydrogenation of ethylbenzene (EB) a) Steam superheater b) Reactor c) High pressure steam d) Low-pressure steam e) Condenser f) Heat exchanger

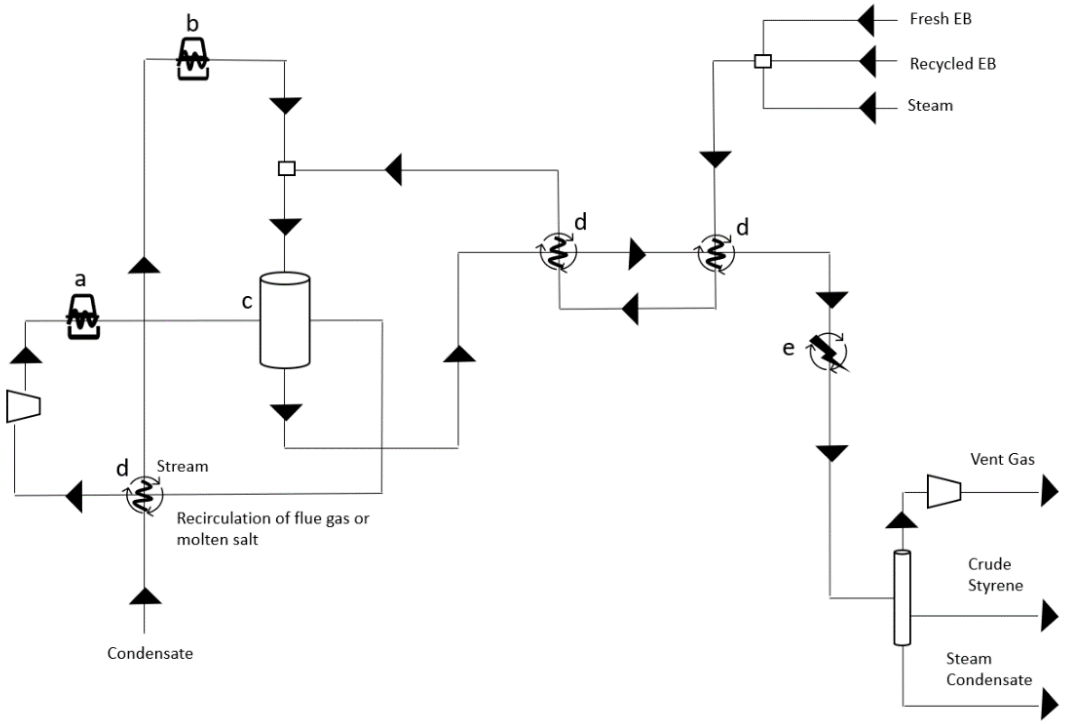


Figure 2.3 Isothermal dehydrogenation of ethylbenzene (EB) a) Heater b) Steam superheater c) Reactor d) Heat exchanger e) Condenser

Other commercial styrene production methods, aside from ethylbenzene dehydrogenation, include the co-production by oxidation and dehydration with propylene oxide from ethylbenzene via α- methyl benzyl alcohol. There are also other styrene production methods that have not been currently commercialised . Styrene can also be produced by the diels- alder dimerisation of 1, 3 butadiene to 4-vinylcyclohexene and then dehydrogenation to styrene (James & Castor, 2011). This production route has been considered low cost, providing the cost and availability of butadiene makes the overall process economically attractive. Another lower cost and available starting material for the production of styrene is toluene. There are several production routes to styrene with toluene including: the oxidation of toluene to give stilbene, which is then further reacted with ethylene to give styrene and also the alkylation of toluene over a zeolite catalyst (James & Castor, 2011). Table 2.1 summarises various styrene commerical production methods including the relevant licensor/developer.

Process	Licensor/Developer
EB Dehydrogenation	
Adiabatic, steam reheat	Dow
Adiabatic, steam reheat	Fina/Badger
Adiabatic, steam reheat (CLASSIC)	Lummus Crest/Monsanto/UOP
Adiabatic, oxidative reheat (Smart™)	Lummus Crest/Monsanto/UOP
Adiabatic, oxidative rehear (Styro-Plus™)	UOP
Isothermal, molten salt bath heating	Montedison/DWE/Lurgi
Coproduction with propylene oxide EB oxidation, propylene epoxidation with EB hydroperoxide and α-methyl benzyl alcohol dehydration.	Arco
Coproduction with propylene oxide EB oxidation, propylene epoxidation with EB hydroperoxide and α-methyl benzyl alcohol dehydration	Shell

Table 2.1 The available commercial processes for producing styrene and the licensor/developer

During the next phase, the effluent from the reactor is condensed and separated to obtain crude styrene. Crude styrene contains styrene (64%) plus a mixture of by-products formed by unwanted side reactions during the ethylbenzene dehydrogenation process (benzene 1%; toluene 2%; unreacted ethylbenzene 32%; others 1%) (James & Castor, 2011). In the final step crude styrene is distilled at elevated temperatures to separate the different components. This process is fairly simple however the residence time at high temperatures needs to be monitored to reduce styrene polymerisation (James & Castor, 2011). Polymerisation inhibitors, usually aromatic compounds with amino, nitro, or hydroxyl groups, are also used through the distillation process to further prevent polymerisation (James & Castor, 2011).

US figures from the typical styrene production process, via the catalytic dehydrogenation of ethylbenzene produced 4.68 million metric tons of styrene in 2019; generally overall production volumes has stayed within this range over the last 5 years (American Chem Council, 2020). However, the styrene production process from ethylbenzene is extremely energy intensive and reportedly consumes around 190 trillion British thermal units (US DOE, 2012).

2.1.2 Engineering the shikimate pathway

Styrene bio-production occurs *via* an extension of the shikimate pathway, through phenylalanine (K. Lee et al., 2019; Lian et al., 2016; McKenna et al., 2014; McKenna & Nielsen, 2011). The shikimate pathway is the metabolic pathway present in microorganisms and plants which produces the aromatic amino acids, phenylalanine, tyrosine and tryptophan (Braga & Faria, 2020; Cui et al., 2014; Chang Liu et al., 2015; Liu et al., 2019; Munack et al., 2016; Noda & Kondo, 2017) (Figure 2.4).

The shikimate pathway starts with a condensation reaction of the glycolysis intermediate, phosphoenolpyruvate (PEP), with the pentose phosphate pathway intermediate, erythrose-4-phosphate (E4P) (Braga & Faria, 2020). This reaction yields the first pathway intermediate 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), which is catalysed by DAHP synthase (Báez-Viveros et al., 2004) (Figure 2.4). The pathway then proceeds through six enzymatic reaction steps to produce chorismate, which is the final product of the shikimate pathway (Braga & Faria, 2020). Chorismate is the branch point molecule that starts off the separate biosynthetic pathways leading to tryptophan, tyrosine and phenylalanine (Noda & Kondo, 2017)

(Figure 2.4). The conversion of chorismate (CHO) to phenylalanine occurs via a conversion to 4-hydroxyphenylpyruvate (4-HPP) catalysed by chorismate mutase and prephenate dehydratase, and finally to phenylalanine by an aminotransferase (Figure 2.4) (Báez-Viveros et al., 2004; Braga & Faria, 2020; Noda & Kondo, 2017).

The shikimate pathway is regulated at the transcriptional and enzymatic level (Braga & Faria, 2020). Strategies to enhance phenylalanine production involved relieving bottleneck enzymes located at the first E4P and PEP condensation reaction step and the two reaction steps that convert chorismate to 4-HPP (Noda & Kondo, 2017; Tribe et al., 1976). These enzymes are feedback inhibited by phenylalanine above a certain concentration threshold (Báez-Viveros et al., 2004; Braga & Faria, 2020; Herrmann, 1995; Ikeda, 2005; Noda & Kondo, 2017). The generation of phenylalanine feedback resistant enzymes, has been achieved by random and directed mutagenesis at specific amino acid residues that regulated activity (Hartmann et al., 2003; Kikuchi et al., 1997) A further strategy involves introducing derivatives of genes that encode feedback resistant enzymes, resistant to allosteric inhibition by pathway end-products (Braga & Faria, 2020; Ger et al., 1994; Jossek et al., 2001). Other approaches include the deletion of *tyrR*, that controls the transcriptional repression of the DAHP synthase gene (Huang et al., 2013).

A strain generated from this engineering effort was the *E. coli* NST74 strain, which was used as the background host for the styrene production pathway (McKenna & Nielsen, 2011; Tribe, 1987). *E. coli* NST74 had feedback resistant gene derivatives of the genes that encoded the DAHP synthases, chorismate mutase and also prephenate dehydratase (Tribe, 1987). The DNA-binding transcriptional regulator *tyrR* and the phenylalanine operator gene *pheO* were also mutated in the strain to alleviate repression (Tribe, 1987). As a result of these key mutations, phenylalanine titres as high as 1.98 g L^{-1} were achieved in 1 L bioreactor experiments (Tribe 1987); making the strain an ideal host for styrene production.

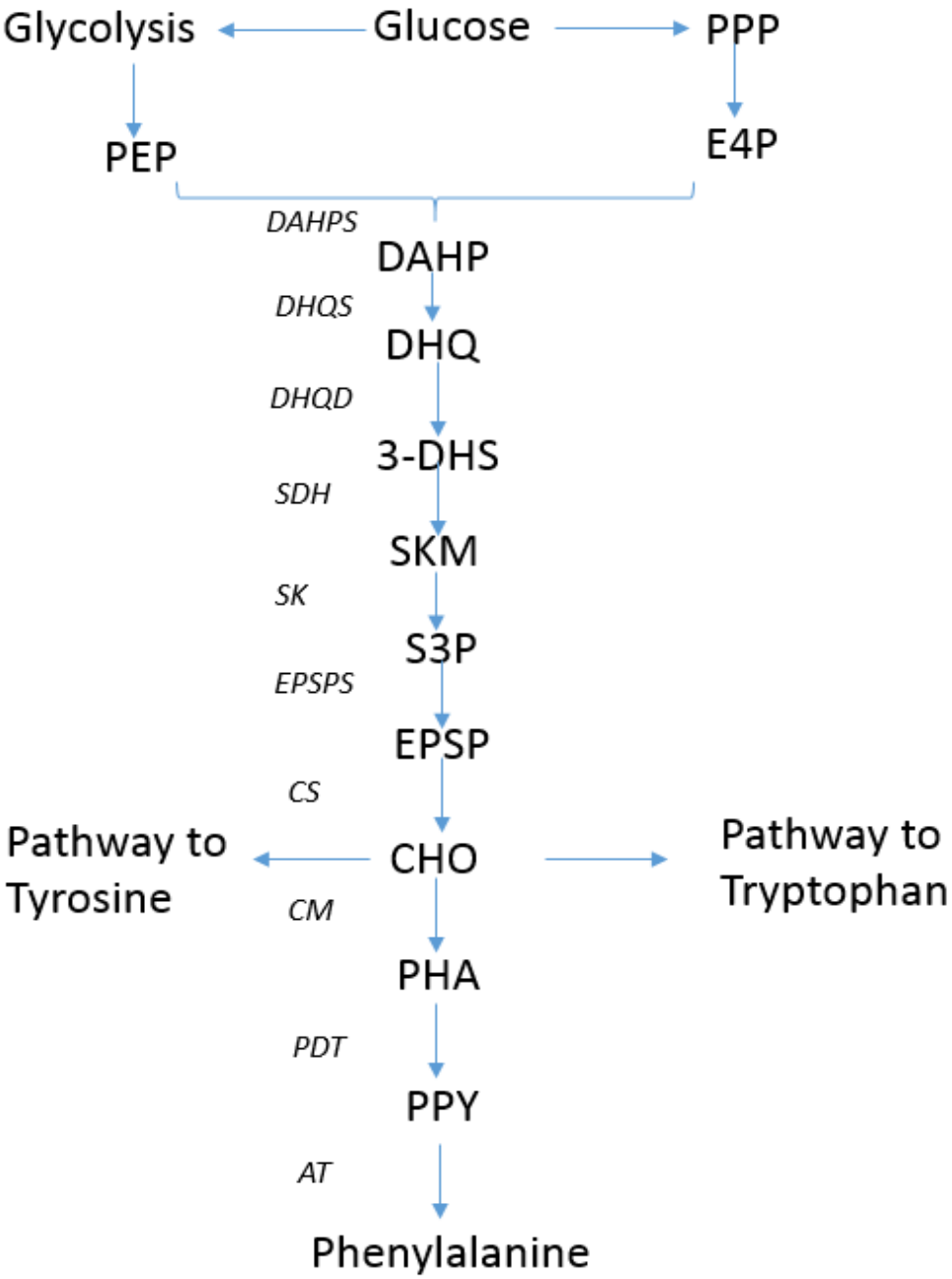


Figure 2.4 The shikimate pathway extended towards phenylalanine. **PPP**, **pentose phosphate pathway;** E4P erythrose 4-phosphate; PEP phosphoenolpyruvate; *DAHPS, DAHP synthase*; DAHP, 3-Deoxy-D-arabinoheptulosonate 7-phosphate; *DHQS, 3-dehydroquinate synthase*; DHQ, 3-dehydroquinate; *DHQD, 3-dehydroquinate dehydratase*; 3-DHS, 3-dehydroshikimate; *SDH, shikimate 5-dehydrogenase*; SKM, shikimate; *SK, shikimate kinase*; S3P, shikimate 3-phosphate; *EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase*; EPSP, 5-enolpyruvylshikimate-3-phosphate; *CS, chorismate synthase*; CHO, chorismate; *CM, chorismate mutase;* PHA, prephenate; *PDT, prephenate dehydratase*; PPY, phenylpyruvate; *AT, aminotransferase*

2.1.3 Engineering *E. coli* for styrene production

The styrene bio-production pathway was engineered in *E. coli* NST74, a strain which had been engineered to produce phenylalanine in excess (Chung et al., 2015; Jang et al., 2012; McKenna & Nielsen, 2011) (Figure 2.5). The pathway extends from the shikimate pathway product phenylalanine *via* two enzymatic reactions (Chung et al., 2015; Jang et al., 2012; McKenna & Nielsen, 2011). The reaction steps involves a phenylalanine ammonia lyase (PAL) and a phenylacrylate decarboxylase (PADC), which first deaminates phenylalanine to *trans*-cinnamic acid and subsequently decarboxylates it to styrene (Adkins et al., 2014; Lian et al., 2016; McKenna & Nielsen, 2011; McKenna et al., 2014) (Figure 2.5).

Before the pathway was constructed, a number of PAL and PADC isoenzymes were screened in the *E. coli* NST74 strain to measure specific activity on a phenylalanine substrate (McKenna & Nielsen, 2011). PAL2 from the plant species *Arabidopsis thaliana* and FDC1 from *S. cerevisiae* were identified as the best pathway enzymes to express in *E. coli* (Cochrane et al., 2004; McKenna & Nielsen, 2011). The co-expression of these two enzymes in a phenylalanine over-producing *E. coli* strain NST74 resulted in styrene titres of 2.6 mmol L^{-1} (McKenna & Nielsen, 2011). This yield was only around 60% of what was calculated to be the theoretical styrene yield (McKenna & Nielsen, 2011). It was deduced that this was due to the end product toxicity of accumulated styrene, as the concentration of the achieved styrene yield was coincidentally similar to the toxicity threshold of the strain (McKenna & Nielsen, 2011). The styrene toxicity threshold concentration was reported to be 2.9 mmol L^{-1} (Lian et al., 2016; McKenna & Nielsen, 2011).

In a similar fashion to the approach carried out in *E. coli*, the *Pseudomonas putida* S12 strain was engineered to produce hydroxystyrene from glucose (Verhoef et al., 2009). The enzymes incorporated were PAL and a *p*-coumaric acid decarboxylase (PDC) which converted tyrosine to hydroxystyrene via *p*- coumarate (Verhoef et al., 2009) (Figure 2.6). Additionally the degradation pathways of *p*-coumaric acid were also disrupted to maximise hydroxystyrene production (Verhoef et al., 2009). The engineered strain was able to produce 4.5 mmol L^{-1} hydroxystyrene before productivity was ultimately halted due to the toxicity of the product compound (Verhoef et al., 2009). The PAL/PADC styrene production pathway has also been expressed in *S. cerevisiae*, but was only able to produce negligible styrene titres in comparison to *E. coli* (McKenna et al., 2014).

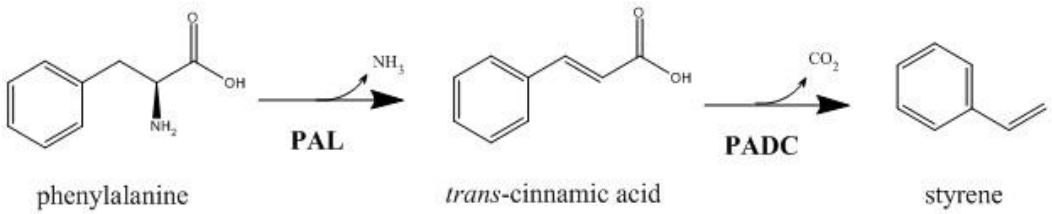


Figure 2.5 Styrene bio-production pathway to convert the precursor phenylalanine to the product styrene via the *trans*-cinnamic acid. The two-step biotransformation is achieved by the co expression of phenylalanine ammonia lyase (PAL) and a phenylacrylate decarboxylase (PADC) enzymes in an *E. coli* NST74 host. Chemical structures drawn using ChemBioDraw Ultra 14.0 software.

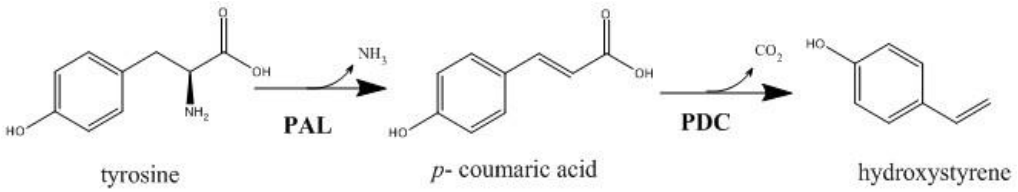


Figure 2.6 Hydroxystyrene bio-production pathway to convert the precursor tyrosine to the product hydroxystyrene via the *p*-coumaric acid. The two-step biotransformation is achieved by the co expression of phenylalanine ammonia lyase (PAL) and a *p*-coumaric acid decarboxylase (PDC) enzymes in an *P. putida* host. Chemical structures drawn using ChemBioDraw Ultra 14.0 software.

2.1.4 Toxicity of styrene and related chemicals in *E. coli*

The specific mechanism of styrene toxicity in *E. coli* is not well studied. In absence of extensive study, the toxicity mechanisms of styrene can be predicted from the way other solvents affects *E. coli* and other Gram-negative bacteria.

The cell membrane envelope of a Gram- negative bacterium consists of a thin peptidoglycan layer that is sandwiched between an outer and inner membrane (Eberlein et al., 2018; Nicolaou et al., 2010; J.-L. Ramos et al., 2015; J. L. Ramos et al., 2002; Sardessai & Bhosle, 2002; Sohlenkamp & Geiger, 2015; Weber & de Bont, 1996) (Figure 2.7). The outer membrane is not permeable and consists mainly of lipopolysaccharides (LPS) and non-specialised porins (Chng et al., 2010; Ebbensgaard et al., 2018; Eberlein et al., 2018). The inner membrane encloses the cytoplasm and consists of phospholipids and membrane proteins which is typically arranged in a planar bilayer (Eberlein et al., 2018; Jouhet, 2013). The inner membrane modulates energy transduction, lipid biosynthesis, transporting molecules in/out the cell, and regulation of intracellular environment, turgor pressure, and signal transduction (Sikkema et al., 1995). The periplasmic space in between contains the layer of peptidoglycan and forms the oxidative environment that is conducive for protein folding. The features of the cell envelope supports the physical properties, integrity, and fluidity of the membrane (Eberlein et al., 2018; Sikkema et al., 1995).

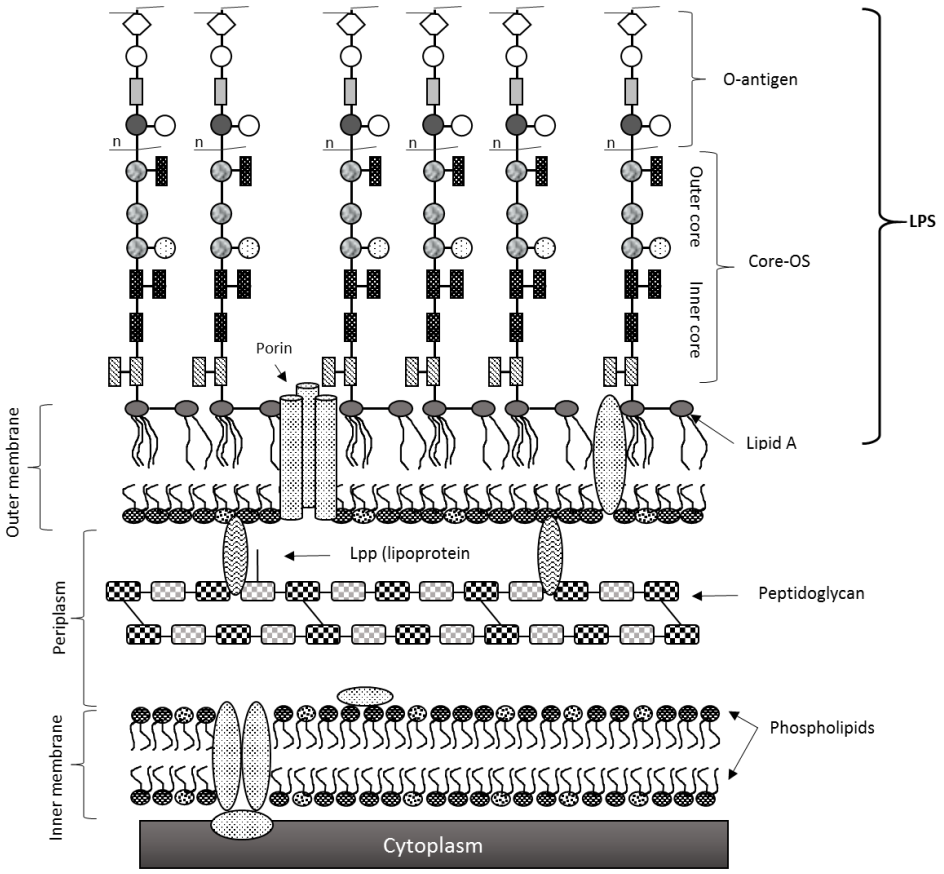


Figure 2.7 The organisation of the cell membrane envelope in Gram negative bacteria. Taken from Volgers et al., 2018

Hydrophobic chemicals can easily partition into the membrane and have a high affinity to bind to the fatty acid hydrophobic core (Eberlein et al., 2018; Nicolaou et al., 2010; J.-L. Ramos et al., 2015). The accumulation of hydrophobic agents will ultimately disrupt the organisation of the cell membrane, which is important for the maintenance of membrane fluidity and integrity (Eberlein et al., 2018; J. L. Ramos et al., 2002; Jan Sikkema et al., 1995; Frans J Weber & de Bont, 1996). A measure of toxicity often used, is the partition coefficient of a solvent in a defined octanol-water mixture (log P_{ow}) which directly correlates to how easily it can partition into the membrane (J. L. Ramos et al., 2002; Sikkema et al., 1995). Hydrocarbons are very toxic because they have a log P_{ow} of 3.0 or below, meaning they can easily partition and accumulate in the cell membrane. (J. L. Ramos et al., 2002; Sikkema et al., 1995). Predictions of the toxic action of styrene can therefore be made from its log P_{ow} value and its structure. Styrene has a Log P_{ow} = 2.95 and its structure consists of a benzene ring with a substituted vinyl group (James & Castor, 2011).

Toxicity studied in Gram- negative bacteria have been conducted with other benzene substituted compounds with similar Log P_{ow} values to styrene (Figure 2.8). Toluene, which is a methyl benzene-substituted compound (log P_{ow} = 2.68), was observed to increase the permeability of the *E. coli* inner membrane, remove inner membrane components and inactivate membrane associated systems (de Smet et al., 1978).



toluene

Log P_{ow} = 2.68

styrene

Log P_{ow} = 2.95

Figure 2.8 The chemical structure and log P_{ow} values of toluene and styrene. Chemical structures drawn using ChemBioDraw Ultra 14.0 software.

Electron microscopy photos confirmed that the toluene treated *E. coli* cells had not been lysed, and there was evidence that the outer membrane was still intact whereas the inner membrane had considerable damage (de Smet et al., 1978; Sikkema et al., 1995). This finding can be linked to styrene by recent studies on an *E. coli* styrene producer strain, which reported that only intracellular concentrations of styrene affected membrane permeability (Lian et al., 2016). It could therefore be inferred that styrene has a similar mode of toxic action in *E. coli* as was reported from toluene. The inner membrane has an important role by allowing the membrane to establish proton and ion gradients that drives proton motive force (Ramos et al., 2015; Segura et al., 1999; Jan Sikkema et al., 1995). Proton motive force ($\Delta\rho$) is an energy resource for cell used to drive processes such as solute transport and ATP synthesis (Jan Sikkema et al., 1995). Perturbations to the inner membrane causes leakage of protons and other ions and cytosolic macromolecules. If an effective proton gradient cannot be set up across the cell membrane, then there will not be the required proton motive force present to drive the phosphorylation reaction by ADP synthase. Therefore, it can be deduced that the effect of toluene, styrene and other related compounds will ultimately affect the energy status of the cell (Sikkema et al., 1995).

A further structure related toxicity action can be associated with the styrene's double bond present on the substituent of the benzene ring. The presence of this double bond makes styrene a very reactive electrophilic compound. The double bond is

electron deficient and readily able to react with thiol groups in proteins and peptides (Richter & Escher, 2005). In particular, the thiol group of the glutathione has been identified as an electrophilic reactive target. A study investigating the effect of different compounds of varying electrophilic strength on *E. coli* toxicity observed glutathione depletion as one of the toxicity effects (Richter & Escher, 2005). Glutathione has an important role in the detoxification of toxic compounds, cell signalling and maintaining intracellular redox homeostasis (Chae et al., 2011; Fahey, 2013; Loi et al., 2015; Suzuki et al., 2005; Z. Wang et al., 2018).

Although the focus of this review is the toxicity and tolerance mechanisms of *E. coli* and other Gram-negative bacteria; many of the toxicity mechanisms also apply to Gram-positive bacteria. Disruption to membrane fluidity and integrity has been observed in *Bacillus sp.* and *Rhodococcus erythropolis* upon exposure to toluene and short-chain alcohol respectively (de Carvalho et al., 2005; Pepi et al., 2008). Similar effects have also been witnessed in studies directly comparing the effects of solvents on the membrane of *E. coli* with Gram- positive *B. subtilis* (Dyrda et al., 2019). It is possible, that Gram-negative and positive bacteria possess conserved mechanisms of tolerance due to the similar effects of solvents on the membrane (Dyrda et al., 2019; Sardessai & Bhosle, 2002).

2.2 Chemical tolerance mechanisms of microbial strains

Microorganisms have intrinsic adaptive mechanisms to survive and grow in the presence of hydrophobic chemicals. Some of the important adaptive mechanisms of Gram-negative bacteria towards hydrophobic solvents are reviewed below.

2.2.1 Osmotolerance molecules

Hydrophobic chemicals can easily permeate through the membrane and elicit the osmotic stress response. This happens due to their effects on membrane fluidity which increases osmotic pressure and membrane leakage (Goodarzi et al., 2010; Rowley et al., 2006). Glycine and betaine are synthesised by micro-organisms during osmotic stress and has also been shown to contribute towards chemical tolerance effects (Gonzalez et al., 2003). This has been demonstrated in ethanol tolerance in *E. coli*, whereby the tolerance was enhanced by the presence of intracellular osmoprotectant molecules (Gonzalez et al., 2003). The genes encoding glycine-betaine synthetic genes were expressed at significantly higher levels in ethanol resistant *E. coli* strains

(Gonzalez et al., 2003). Metabolomic analysis has also supported the role of osmoprotectants in chemical tolerance. Several amino acids and osmoprotectants, such as isoleucine, valine, glycine, glutamate and trehalose are key metabolites that are elevated in response to ethanol, n-butanol, and isobutanol stress in *E. coli* (Horinouchi et al., 2018; J. Wang et al., 2013).

2.2.2 *Cis – trans* isomerisation of membrane unsaturated fatty acids

The phospholipids of the inner membrane in Gram- negative bacteria can be attached to either saturated or mono-unsaturated fatty acid chains (Sohlenkamp & Geiger, 2015; Strahl & Errington, 2017). The steric arrangement of the chains in unsaturated fatty acids exist in either a *cis* or *trans* configuration (Heipieper et al., 2003; Keweloh & Heipieper, 1996; Strahl & Errington, 2017). The fatty acid chain in the *cis* configuration is bent at angle of 30°; whereas the *trans* configuration has a straighter chain only bent at an angle of 6 ° (Eberlein et al., 2018; Macdonald et al., 1985). In the *trans* configuration the fatty acid chains can be closer together which allows denser packing of the membrane phospholipids (Eberlein et al., 2018; Heipieper et al., 2003; Keweloh & Heipieper, 1996; Strahl & Errington, 2017). Hydrophobic chemical stressors can penetrate and accumulate in the inner membrane affecting membrane stability by increasing the fluidity of the membrane (Eberlein et al., 2018; Nicolaou et al., 2010; J.-L. Ramos et al., 2015; J. L. Ramos et al., 2002; Sardessai & Bhosle, 2002; Sohlenkamp & Geiger, 2015; Weber & de Bont, 1996). The conversion of fatty acid chains from the *cis* to *trans* configuration, has been observed as an adaptive response to chemical stress in *Pseudomonas sp.* and Vibrio *sp.* (Bernal et al., 2007; Eberlein et al., 2018; Heipieper et al., 2003; Molina-Santiago et al., 2017). The *cis-trans* conversion is catalysed by the *cis-trans* isomerase Cti enzyme located in the periplasm (Eberlein et al., 2018). A proposed model of action for Cti enzyme under chemical stress involves an increase in membrane fluidity allowing the enzyme access in between the fatty acid chains to catalyse *cis – trans* isomerisation (Heipieper et al., 2003). The restorative effect of Cti activity *cis – trans* isomerisation on membrane fluidity then restricts access and it can return to the periplasm (Heipieper et al., 2003).

2.2.3 Changes to headgroup composition

The phospholipids of the inner membrane can have several different headgroups, however the most prevalent in bacteria are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Perczyk et al., 2020; Pichler &

Emmerstorfer-Augustin, 2018; J. L. Ramos et al., 2002; Sohlenkamp & Geiger, 2015; Frans J Weber & de Bont, 1996) (Figure 2.9). The phospholipid headgroups all differ in structure and can ordered by size as PE < PG < CL. Several strains of bacteria have been reported to change the abundance of certain phospholipid headgroups in response to environmental stress (Perczyk et al., 2020; J.L. Ramos et al., 2015; Ramos et al., 2002). In particular, an increase in the amount of PG and CL in the membrane influences the packing geometry as an adaptive response to chemical stress (Frans J Weber & de Bont, 1996). The fluidising effect on lipid membranes can disrupt planar arrangement of the bilayer and induce a membrane micellar phase (Figure 2.10). PE is the most abundant phospholipid in Gram-negative bacteria and has a propensity to switch the membrane arrangement into the inverted micelle phase. This is because PE has a small headgroup and has an overall 'cone' shape which induces a curvature in the lipid bilayer (Jouhet, 2013; Frans J Weber & de Bont, 1996). Increasing the amount of larger headgroup phospholipids like PG, which have an overall 'cylindrical' shape restores the bilayer structure (Jouhet, 2013; Lindblom, Orädd, Rilfors, & Morein, 2002; J.-L. Ramos et al., 2015; J. L. Ramos et al., 2002; Weber & de Bont, 1996). The phospholipid headgroups can be ordered by melting temperature PG < PE < CL, therefore the increase in the ratio of CL:PE in the membrane has a rigidifying effect (J.-L. Ramos et al., 2015; J. L. Ramos et al., 2002; Frans J Weber & de Bont, 1996).

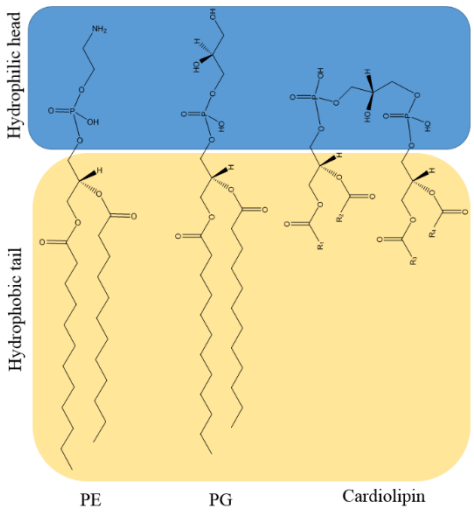


Figure 2.9 Structure of the three main phospholipids present in Gram-negative bacteria. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL)

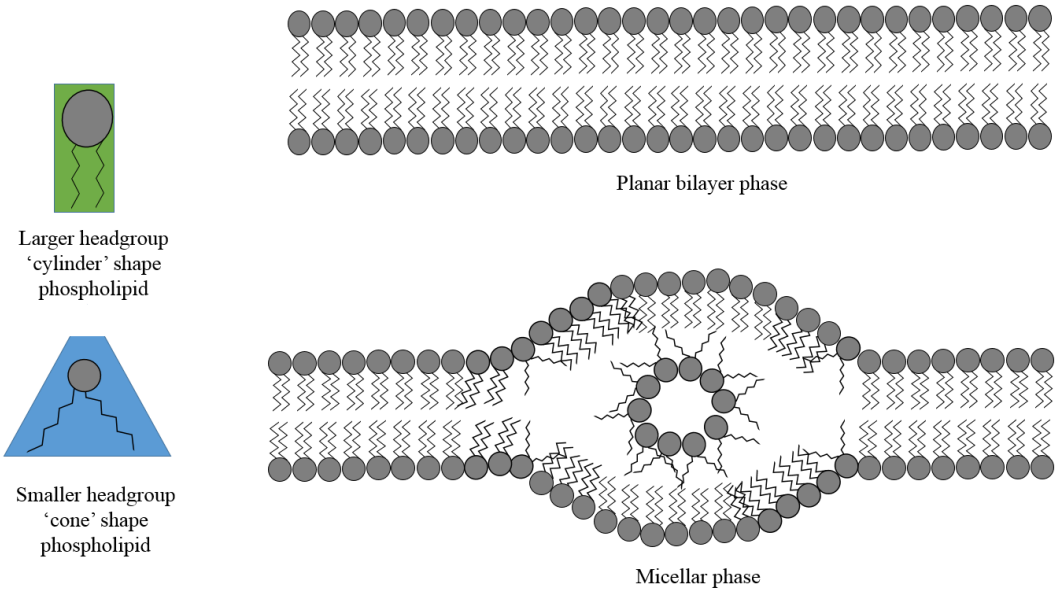


Figure 2.10 Diagram showing the concept of the lipid membrane in planar bilayer phase and micellar phase. Phospholipids with smaller headgroups have an overall 'cone' shape and their overall abundance can induce the micellar bilayer phase upon an increase in membrane fluidity. Figure adapted from (Jouhet, 2013).

Alterations in headgroup composition has demonstrated increased tolerance in mutant and engineered *E. coli* strains (Clark & Beard, 1979); and in solvent tolerant *Pseudomonas* strains (Bernal et al., 2007; J. L. Ramos et al., 2002; Frans J Weber & de Bont, 1996)

2.2.4 Modification of lipopolysaccharides

The outer membrane of Gram- negative bacteria is composed mainly of lipopolysaccharides (LPS) (Clifton et al., 2016; Ebbensgaard et al., 2018; Malojčić et al., 2014; Nikaido, 2003; Sikkema et al., 1995). LPS are made up of three components: Lipid A, a lipid moiety that anchors the LPS to the membrane; a core phosphorylated oligosaccharide (core-OS); and an O- specific polysaccharide hydrophilic chain (O-antigen) that extends out into the extracellular space (Chng et al., 2010; Clifton et al., 2016; Ebbensgaard et al., 2018; Eberlein et al., 2018; Emiola, George, & Andrews, 2015; Malojčić et al., 2014; Nikaido, 2003; Sikkema et al., 1995). The LPS molecules are thought to protect the organism against toxic hydrophobic compounds. The anionic phosphate groups of the core-OS bind to divalent cations (e.g. Mg^{2+},Ca^{2+}) and coordinate the binding of neighbouring LPS molecules *via* salt bridges (Clifton et al., 2016; Ebbensgaard et al., 2018; Ma et al., 1996). The linkage forms a dense hydrophilic layer on the cell surface consisting of the core-OS and the O antigen which

impermeable to hydrophobic compounds (Aono & Kobayashi, 1997; Clifton et al., 2016; Nikaido, 2003). The cell surface of solvent tolerant *E. coli* mutants was found to have low hydrophobicity due to increased ratio of LPS in the outer membrane (Aono & Kobayashi, 1997).

2.2.5 Release of outer membrane vesicles (OMV)

The release of OMV in response to chemical stress is most notable in Gram-negative *Pseudomonas sp.* strains (Eberlein et al., 2018; J.-L. Ramos et al., 2015; J. L. Ramos et al., 2002; Volgers et al., 2018). OMVs are spherical particles of the outer membrane, ~20 – 250 nm in diameter, filled with periplasmic content which bud and detach from the cell (Deatherage et al., 2009; Kulp & Kuehn, 2010; Volgers et al., 2018). Various proposals in literature, have been put forward to how OMVs are formed as adaptation to chemical stress (Eberlein et al., 2018; Schwechheimer & Kuehn, 2015; Volgers et al., 2018). One mechanism proposed that modifications to the specific interactions between the outer membrane lipids and the peptidoglycan layer, causes the release of OMVs (Deatherage et al., 2009; Volgers et al., 2018). Another proposal is from disruptions to the linkage between adjacent LPS molecules, which makes the charged portions to align closely and repel each other, causing the membrane to curve and promoting OMV formation (Schwechheimer & Kuehn, 2015). Increased levels of OMVs were reported in the solvent tolerant *Pseudomonas putida* IH-2000 strain (Kobayashi et al., 2000). There was a higher concentration of toluene in the OMV than was measured in the cellular membrane (Kobayashi et al., 2000)

2.2.6 Increased expression of efflux pumps

Bacterial efflux pumps (BEP) are inner membrane protein transporters which can non-discriminately facilitate the extrusion of chemical compounds out of the cell (Amaral *et al.*, 2014; Anes *et al*., 2014; Machas *et al*., 2019; Nicolaou *et al*., 2010; J.-L. Ramos *et al*., 2015) There are five major families of BEP: ATP-binding cassette (ABC) superfamily, resistance-nodule-division (RND) family, small multidrug resistance (SMR) family, major facilitator superfamily (MFS), and multidrug and toxic compound extrusion (MATE) family (Machas et al., 2019). The BEP families utilise two different types of energy source to transport compounds against the concentration gradient. ABC transporters utilise ATP hydrolysis and RND, SMR, MFS and MATE

transporters obtain their energy from proton motive force (Anes et al., 2015; Machas et al., 2019).

AcrB is a RND efflux pump present in *E. coli* that is commonly linked to chemical resistance (Anes et al., 2015; Machas et al., 2019; Mingardon et al., 2015; J. L. Ramos et al., 2002; Rojas et al., 2001) (Figure 2.12). AcrB works cooperatively with the TolC outer membrane channel and AcrA the periplasmic connector protein (Anes et al., 2015; Machas et al., 2019). The expression of AcrAB is controlled locally by a number of transcription factors (Duval & Lister, 2013; Ma et al., 1996; Machas et al., 2019; J. L. Ramos et al., 2002). The *acrR* gene is located upstream of the *acrAB* operon, and is divergently transcribed in the opposite direction (Li et al., 2007; Weston et al., 2018) (Figure 2.11). The gene product, AcrR, is a complete α-helical protein with a (HTH) motif N-terminal domain (Li et al., 2007). AcrR can bind to a number of different ligands with varying structures, causing a conformational change that then allows the transcription of *acrAB* (Li et al., 2007; Weston et al., 2018). MarA is a global transcriptional activator that increases the expression of *acrAB -tolC* (Duval & Lister, 2013; Li et al., 2007; Ma et al., 1996; White et al., 1997). Homologues of MarA are SoxS and Rob which bind to the same sequences that activate the transcription of *acrAB* (Duval & Lister, 2013). The expression of *marA* and SoxS is controlled by MarR and SoxR respectively as MarA and SoxS do not have a ligand binding domain (Duval & Lister, 2013; Li et al., 2007; Weston et al., 2018). Mutations in AcrAB regulatory genes *marR*, *soxR*, and *acrR* have been shown to increase the expression level of the AcrAB-TolC pump (Jellen-Ritter & Kern, 2001; H. Wang et al., 2001; Watanabe & Doukyu, 2012). In study looking at the genomic mutations of cyclohexane tolerant *E. coli* mutants, each mutant had a mutation either in acrR or marR (Watanabe & Doukyu, 2012). The location of some of these mutations disrupted the repressive activity and translation of the proteins (Watanabe & Doukyu, 2012). The importance of AcrAB-TolC efflux system in chemical *E. coli* tolerance has been shown in studies where the overexpression of AcrAB improved growth in the presence of 1-hexene compared to the wild type strain with native AcrAB expression levels (Mingardon et al., 2015). Lesser studied regulators of *acrAB* expression include EnvR/AcrS, PhoP and MprA. EnvR/AcrS is also a regulator of AcrEF, a multidrug efflux pump that is expressed at a lower level to AcrAB, due to it being under the control of global repressor N-HS (Hirakawa et al., 2008). EnvR/AcrS is thought to function as a switch between AcrAB and AcrEF expression (Hirakawa et al., 2008).

PhoP and MprA have also been identified as a repressor of *acrAB* due to the close proximity to the *acrA* promoter region (Monsieurs et al., 2005; Rodionov et al., 2001).

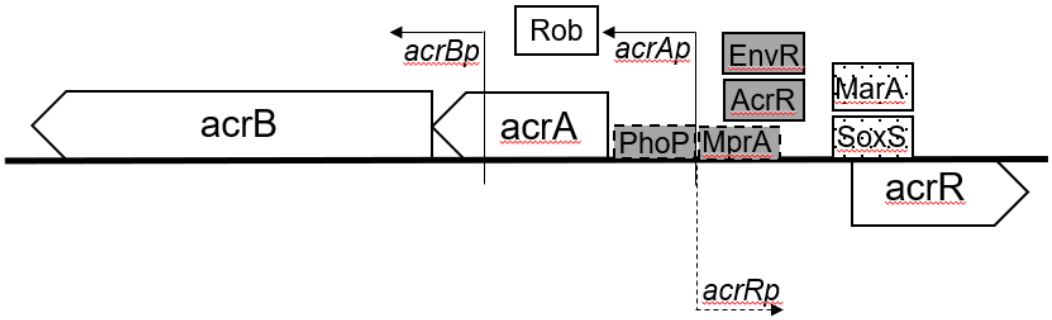


Figure 2.11 Transcription unit of the *acrAB* operon. The *acrR* gene, which is located upstream of and in the opposite direction of *acrAB*, encodes AcrR, a negative DNA-binding transcriptional regulator of *acrAB*. EnvR, PhoP and MprA have also been identified as a repressor of acrAB due to the close proximity to the acrA promoter region. MarA, SoxS and Rob bind to the same sequences and activate the transcription of *acrAB*

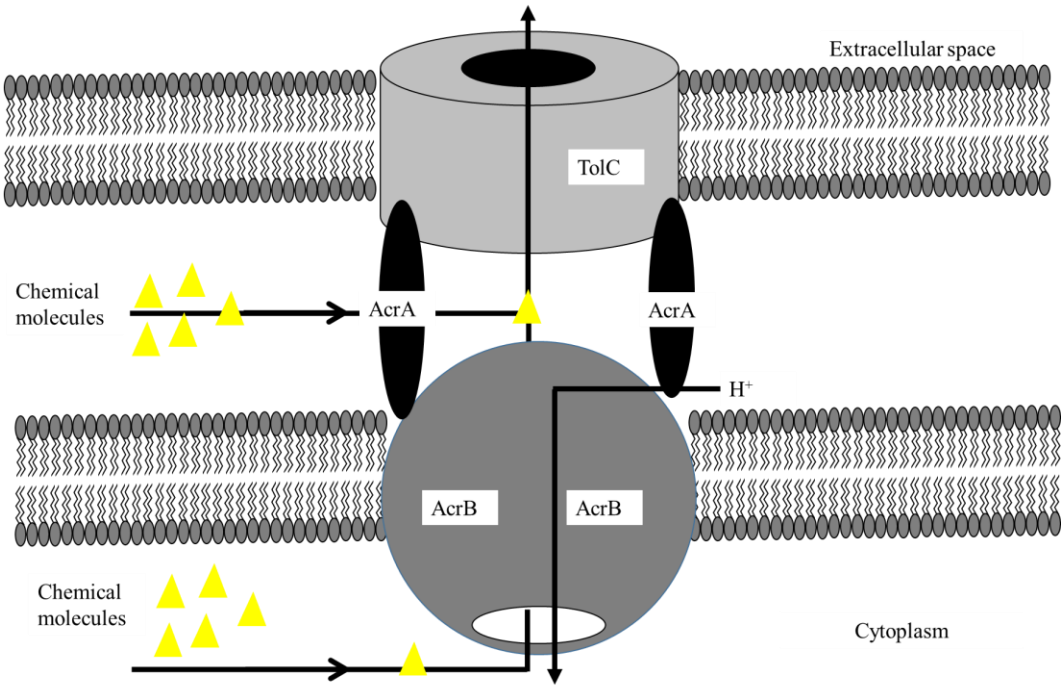


Figure 2.12 The organisation of the AcrB, AcrA and TolC proteins that make AcrAB-TolC efflux pump. AcrB (the efflux pump) is in the inner membrane and responsible for the substrate recognition/selection and energy transduction. Chemical products are extruded across the cytoplasmic membrane in coupled exchange with protons. TolC forms a channel connecting the periplasmic space with the extracellular space. Two AcrA proteins act as linkers between AcrB and TolC. Figure taken from Murakami et al., 2006.

2.3 Adaptive laboratory evolution (ALE) as a tool to enhance chemical tolerance

Microbial chemical tolerance is a complex phenotype that is determined by several different cellular mechanisms and proteins, some of which are uncharacterised. The genome basis of a tolerance phenotype in chemical resistant micro-organisms is often encoded by adaptive DNA mutations that have accumulated in the genome. This complexity means that using a rational approach to engineer microbial cell tolerance, whereby specific genes are targeted can be both challenging and ineffective (Conrad et al., 2011; LaCroix et al., 2015; Sandberg et al., 2019). A random approach for adaptive tolerance, whereby microbial strains are subjected to successive rounds of random DNA mutagenesis and then screened for the desired phenotype, is often much more effective. This way of introducing genome wide mutations can be executed by Adaptive laboratory evolution (ALE). ALE has its advantages over rational engineering methods due to its broad applicability, ease of method and the novel, non-intuitive adaptive mutations that are generated (Sandberg et al., 2019).

The fundamental design of ALE experiments is linked to the natural selection principles of genetic variation and selection; whereby micro-organisms are cultured for several generations in a selective environment to generate adaptive mutants (Conrad et al., 2011; LaCroix et al., 2015; Long & Antoniewicz, 2018; Peabody et al., 2014; Remigi et al., 2019; Sandberg et al., 2019; Van den Bergh, et al., 2018; J. Winkler et al., 2013). ALE experiments facilitate the random accumulation of mutations because of natural DNA replication errors. Fitness enhanced mutants arise in population due to adaptation to a selective environment, eventually taking over the rest of the population. Microbial populations provide the ideal adaptive landscape due to their large size ($10^8 – 10^{10}$); short generation time and high supply of mutations (Gresham & Dunham, 2014; Sandberg et al., 2019). In the context of microbial chemical toxicity, ALE can generate microbial mutant strains that possess adaptive mutations which enhance chemical tolerance.

The use of ALE to improve microbial traits has been practiced for a long time as evidenced by its use in early studies (Atwood et al., 1951). However, it was the famous long-term evolution experiment led by Richard Lenski that demonstrated the dynamic landscape by which adaptations occur. Since 1988, Lenski and colleagues have ran an ALE study by serial batch culturing 12 identical *E. coli* cultures (Lenski, 2017). Over this period of time, Lenski and colleagues reported many changes in all 12 populations

including changes in the bacteria's mutation rate, new abilities to utilise citrate as carbon source for aerobic growth; and changes in relative fitness in comparison to the ancestral strain (Lenski, 2017). It can be argued this work alongside the advent of genomic technologies, has elevated the simple methods and concepts of ALE to become a powerful tool in microbial strain optimisation (Fernández-Cabezón et al., 2019; Sandberg et al., 2019; J. Winkler et al., 2013).

ALE experiments have been used successfully to increase the tolerance of *E. coli* wild-type and engineered micro-organisms to the alcoholic compounds as listed in Table 2.2.

Evolved Strain(s)	Parent Strain	Chemical selection pressure	ALE method	Mutant Isolation method	Reference
HG228	MG1655	Ethanol	Serial transfer in batch culture for 160 generations.	Solid medium	(Goodarzi et al., 2010)
LY01	BL21-DE3	Ethanol	Serial transfer in batch culture for 3 months.	Solid medium	(Gonzalez et al., 2003)
Ethanol tolerant ethanologenic strains	FBR5	Ethanol	Colony transfer (x3) on solid medium.	Solid medium	(Lupino et al., 2018)
IPA tolerant strains A-F	MSD42	Isopropanol	Serial transfer in microtitre plates for 24 days.	Solid medium	(Horinouchi, Sakai, et al., 2017)
B500	MG1655 MG1655 Δ*lacZ*	*n*-butanol	Serial transfer in batch culture for 500 generations.	Solid medium	(Dragosits et al., 2013)
n-butanol tolerant strains MG1-6 MY1-4	BW25113	*n*-butanol	Chemostat	Solid medium	(Reyes et al., 2012)

Table 2.2 A compiled table of relevant studies that have used ALE to generate an E. coli strain that is tolerant to various alcoholic compounds

2.3.1 Principles of ALE

ALE is an evolutionary engineering approach, that identifies genome-wide mutations which contribute towards the overall fitness or desired phenotype (Long & Antoniewicz, 2018; Peabody et al., 2014; Sandberg et al., 2019). The main goal is to select a mutant clone isolate from an evolved population, that displays the target phenotype, such as enhanced tolerance, substrate utilisation, growth rate or metabolite

production (Fernández-Cabezón et al., 2019; Sandberg et al., 2019; J. Winkler et al., 2013).

Population structure is an important principle in ALE as it governs the attainment of a single adaptive mutant. The most commonly assumed population structure in ALE is clonal replacement, where an adaptive mutant sweeps through the population replacing the parent population (Conrad et al., 2011; Raynes & Sniegowski, 2014; Van den Bergh et al., 2018). At any one time the population is assumed homogenous except when the selective sweep is taking place (de Visser & Rozen, 2006; Raynes & Sniegowski, 2014). However, multiple mutant lineages can exist within a population whereby they compete under selection to take over the population, this is called clonal interference (Cvijović et al., 2018; de Visser & Rozen, 2006; Kinnersley et al., 2019; Van den Bergh et al., 2018). With this population structure, the assumption cannot be made that the population is homogenous until isolates from the population are characterised to confirm a selective sweep.

Another important principle in ALE is mutation fixation. When a mutation is fixed in a population the frequency of the mutation is present in all individuals of the population (Van den Bergh et al., 2018) . Genetic drift is the process which increases the frequency of non-adaptive mutations in a population to achieve majority fixation. In ALE, the adaptive mutant fixation is in direct competition with genetic drift, where mutations occur under non-selective conditions (Masel, 2011; Van den Bergh et al., 2018). Genetic drift can affect the success of ALE as it changes the frequency of an adaptive mutation in a population and determines fixation (Masel, 2011; Van den Bergh et al., 2018). In ALE experiments, random sampling from heterogeneous populations result in genetic bottlenecks, where the population cannot pass adaptive mutations on to progeny because the genetic drift process counteracts the fixation of adaptive mutations (Masel, 2011; Van den Bergh et al., 2018). Lastly, the selective environment is an important principle in ALE experiments (LaCroix et al., 2017). ALE experiments use a selective growth environment as a parameter to generate mutant strains (Sandberg et al., 2019). The experimental design of the selective environment can couple the desired phenotype to growth and/or survival (Sandberg et al., 2019). In a growth-coupled tolerance strategy, the selective environment is designed to select an adaptive mutant that displayed enhanced growth/survival in the presence of an inhibitory concentration of the stressor (Sandberg et al., 2019).

2.3.1 ALE methods

2.3.1.1 Batch sub-culturing

A batch culture is a closed culturing system, whereby a nutrient source is added to cell culture which proceeds to grow until the substrate concentration is fully consumed (Pirt, 1975). The provision of nutrients in a batch culture is reflected in four distinct growth phases; lag, log, stationary and death phase (Figure 2.13) (Pirt, 1975). During this time the culture experiences periods of 'feast' and 'famine' where there is an excess of nutrients at the start (lag) the nutrients are consumed at an exponential rate (log) until there is no more nutrients (stationary phase) (LaCroix et al., 2017; Pirt, 1975). The onset of death phase could be due to lack of nutrient or an accumulation of toxic metabolites (Pirt, 1975).

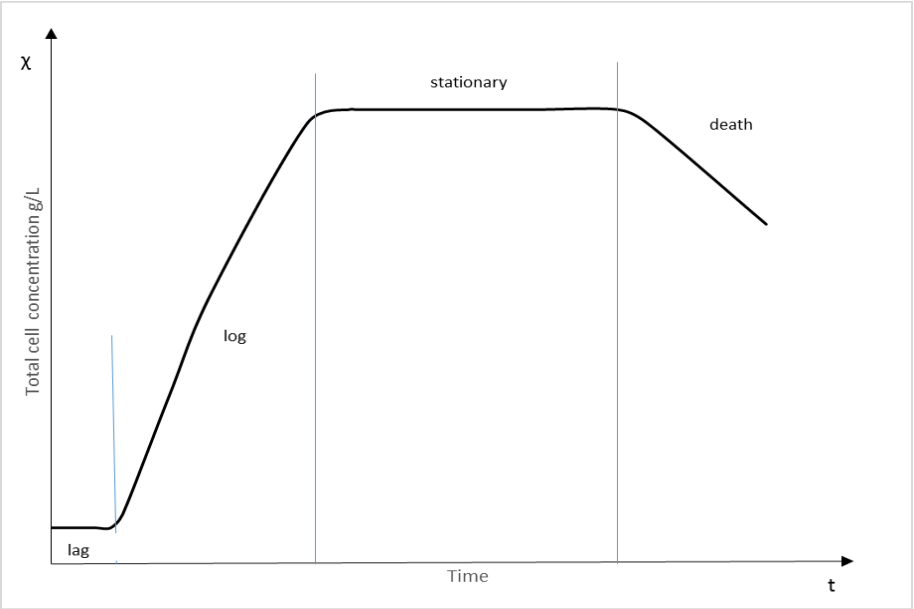


Figure 2.13 Batch growth curve showing the four phases; lag phase, log phase (exponential), stationary phase and death phase. Figure adapted from (Pirt, 1975)

Batch culturing is most commonly carried out in shake flasks, but the method is not limited to a particular culture vessel, and can be performed in bioreactor or micro-titre plates (Dragosits & Mattanovich, 2013; Horinouchi et al., 2014). Important considerations apply to the culture and headspace volumes when performing batch culturing to allow sufficient O_2 exchange (J. Winkler et al., 2013).

Batch culturing as an ALE method sets up repeated cycles of log growth and stationary phase by sub-culturing in the presence of a selection pressure (Adams &

Rosenzweig, 2014; Dragosits & Mattanovich, 2013; Kram et al., 2017; LaCroix et al., 2017; Sandberg et al., 2019; Van den Bergh et al., 2018). The concentration of the selection pressure is then increased over successive sub-cultures until the desired growth characteristic is achieved (Dragosits & Mattanovich, 2013; LaCroix et al., 2017; Van den Bergh et al., 2018; J. Winkler et al., 2013). Increased fitness in batch can be observed by decreased lag phase duration and enhanced cell viability in the presence of the selection pressure (Gresham & Dunham, 2014; Sandberg et al., 2019)

Under most circumstances, the batch sub-culturing experimental method is simple, and just involves repeated transferrals of an inoculation sample of one culture to fresh medium (Sandberg et al., 2019; Van den Bergh et al., 2018). Batch sub-culturing is a popular ALE technique due the method being easy to perform, cheap and parallel experiments can be performed at once (Dragosits & Mattanovich, 2013).

Batch sub-culturing has succesfully been used to enhance tolerance in *E. coli* to various stressors (Table 2.2). Improvements in *E. coli* tolerance to four different stressor conditions (osmotic, acidic, oxidative, *n*-butanol) was achieved after evolving *E. coli* for over 500 generations by batch sub-culturing (Dragosits et al., 2013). The relative fitness of the evolved strains under 0.6% v/v butanol concentration was much higher than the non- evolved control (Dragosits et al., 2013). Tolerance to ethanol was enhanced in ethanologenic *E. coli* strain LY01 by batch subculturing resulting in improvement to ethanol production by 51% from a glucose feedstock (Gonzalez et al., 2003). Batch subculturing of *E. coli* also resulted in strains capable of growing in a 14% higher ethanol concentration than the wild type (Goodarzi et al., 2010). A final example of comes from improvement of *E .coli* strain to isopropanol after batch sub culturing, gradually increasing the isopropanol concentration from 0 mM to 500 mM isopropanol concentration in 50 mM steps (Horinouchi, Sakai, et al., 2017). One of the final generated mutant strains that could grow at 350mM; whereas the wild type failed to grow at this concentration (Horinouchi, Sakai, et al., 2017).

An overall limitation of the batch culturing is that experimental parameters, such as growth rate, population density, pH, dissolved O_2 and nutrient supply can not be easily controlled (Dragosits & Mattanovich, 2013). However there are limitations that apply to batch sub-culturing specifically as an ALE method. Sub-culturing a batch at the stationary phase time point, when the cultures has depleted resources for growth, could select for mutants with tolerance towards starvation or O_2 limited conditions;

rather than with the intended selection pressure (Fernández-Cabezón et al., 2019; Sandberg et al., 2019). A solution could be to maintain the batch culture in log phase when repeatedly sub-culturing (Fernández-Cabezón et al., 2019) but this can prove to be labourious process for the analyst (Sandberg et al., 2019). Horinouchi et al., 2014 reported on an automated batch sub-culture system for ALE which could maintain 'hundreds of independent culture series in exponential growth'. Many successful ALE studies using this system have come from this research group (Horinouchi et al., 2010, 2015, 2018; Horinouchi, Suzuki, et al., 2017) but they reported a 'major drawback' was the cost of operation (Horinouchi et al., 2014). This could mean many research groups may not have the resources (time, cost, labour) to carry out ALE batch culturing at its full potential.

A further limitation is that ALE batch sub-culturing is susceptible to populations bottlenecks due to the effects of genetic drift (LaCroix et al., 2017; Masel, 2011; Van den Bergh et al., 2018; J. Winkler et al., 2013). An inoculation sample, taken from a previous culture to initiate growth in the subsequent culture, can contain random individuals that may not all possess the adaptive mutations (Van den Bergh et al., 2018). The subsequent culture may succumb to the effects of genetic drift, whereby the adaptive mutations is outcompeted and becomes extinct in the population (Van den Bergh et al., 2018).

2.3.1.2 Chemostat culturing

A chemostat is a type of continuous culture that operates as an open culturing system, whereby there is an constant feed of a nutrient source and output of cell biomass (Pirt, 1975). This system sustains cells in constant exponential growth by introducing medium at a rate that is equal to the specific growth rate of the micro-organism. This is known as steady state, where specific growth rate occurs at a constant rate and cell biomass concentration is constant

At steady state cell specific growth rate is equal to the dilution rate (Pirt, 1975).

$$\mu = D \qquad \text{(Equation 2.1)}$$

Where: μ = specific growth rate; D = dilution rate

(Equation 2.1) is therefore used to calculate the dilution rate to use in a chemostat system based on the specific growth rate of the micro-organism. The maximum growth rate is achieved when the substrate concentration in the feed is equal to the substrate concentration in the culture volume.

$$\mu = D_c = \frac{\mu_{max}[S]_f}{S_f + K_s}$$

(Equation 2.2)

Where: μ = specific growth rate; Dc = Critical (maximum) dilution rate; μ_{max} = maximum growth rate; $[S]_f$ = substrate concentration in feed; K_s = affinity of cells towards *S*.

A plot of chemostat steady state growth showing cell and substrate concentration against dilution can be seen in Figure 2.14.

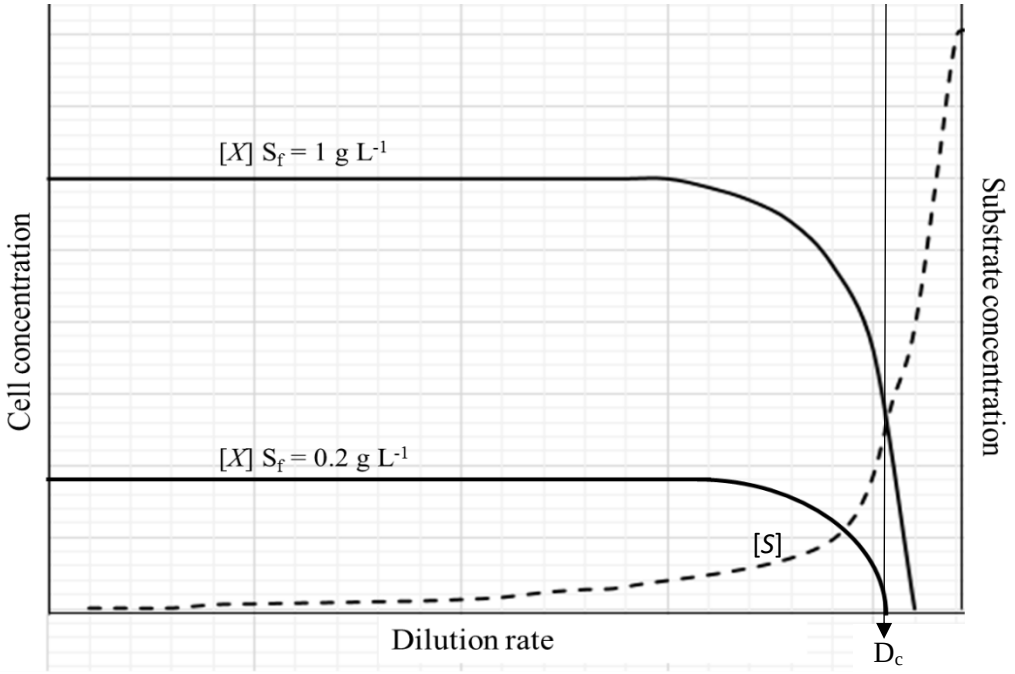


Figure 2.14 Chemostat steady state of cells [*X*] (black solid line) and growth limiting substrate [*S*] (dashed line) Figure adapted from (Pirt, 1975)

Chemostat cultures consist of a culture in steady state by feeding in a defined medium where one nutrient is present at a growth limiting concentration (Dragosits & Mattanovich, 2013; LaCroix et al., 2017; Sandberg et al., 2019; Van den Bergh et al., 2018; Ziv et al., 2013). In comparison with batch culture, the maximum cell output at the same volume is exceeded by chemostat culture. This is because chemostat culture operates at constant exponential growth rate whereas the average of bacterial growth rate over the four phases of batch culture is a much lower (Pirt, 1975).

Chemostat cultures are usually set up in continuously stirred bioreactors, with feed lines delivering O_2, pH maintenance solutions and an effluent line for culture to flow out (Van den Bergh et al., 2018). Chemostat culturing can be adapted to become an ALE method by delivering a selection pressure at a growth inhibiting concentration and selecting for mutants with and increased μ_{max} (J. Winkler et al., 2013). As chemostat cultures run at steady state, growth inhibition can be observed by a deviation from steady state which indicates $\mu \neq$ dilution rate (Gresham & Dunham, 2014; Gresham & Hong, 2015). On the other hand, an adaptive event in chemostat can be observed by the return of cell concentration back to steady state (Gresham & Dunham, 2014; Gresham & Hong, 2015).

The main advantage chemostat culturing offers is there are smaller genetic bottlenecks due to the continual maintenance of a large population (Gresham & Dunham, 2014; Van den Bergh et al., 2018). In chemostat cultures adaptive mutations are not opposed by genetic drift, and have the opportunity to increase to a frequency in the population where it is fixed (Gresham & Dunham, 2014). Chemostat culturing also allows the operator to have tight control over the experimental parameters, including the selective environment, which is very important in ALE (Gresham & Dunham, 2014).

Reyes et al., 2012 performed ALE using a chemostat system whereby the concentration of *n*-butanol was increased in a step-wise fashion with an initial concentration of 0.5% (v/v) and raised to 1.3% (v/v) every 20-30 generations. This experiment was unique because they were able to visualise the landscape of adaptation by viewing the proportions of different fluorescently labelled cells (Reyes et al., 2012). In the end they were able to isolate mutant *E. coli* with enhanced fitness in comparison to the wild type at 0.8% v/v *n*-butanol (Reyes et al., 2012).

2.4 Random mutagenesis methods for the generation of chemical tolerant microbial strains

Random mutagenesis is an overall approach that underpins several methods that uses a mutagen to induce genetic diversity in a microbial population. It differs from ALE as its methods directly cause mutations and are not reliant on them occurring spontaneously. When combined with an appropriate screening technique, random mutagenesis can be very effective in isolating adaptive mutants (Harper et al., 2011;

Sandberg et al., 2019; Selifonova et al., 2001; Y. Zhang et al., 2009). This section provides an overview of some commonly used random mutagenesis methods.

2.3.4.1 Chemical mutagenesis

Chemical mutagenesis exposes a bacterial population to a mutagen which chemically alters the DNA structure (Parekh et al., 2000). The mutagenic chemicals that are widely used in chemical mutagenesis include; base analogues, alkylating agents and intercalating agents (Parekh et al., 2000). Chemical mutagens usually causes damage through deletion, addition, substitution of bases or breakage of DNA strands (Parekh et al., 2000; Singer & Kusmierek, 1982). Chemical mutagenesis with alkylating agents (*N*-methyl-*N*′-nitro-*N*- nitrosoguanidine (NTG) and ethyl methanesulfonate is the most common methods. NTG mutagenesis was used to generate tolerant *E. coli* (Yoon et al., 2007) and *C. acetobutylicum* strains (Syed et al., 2008). However, because of the aggressive nature of the method, chemical mutagenesis is known to cause deleterious mutations or even multiple mutations at one genomic site, which can counter the positive effects of any adaptive mutations attained (Bonomo et al., 2006).

2.3.4.2 UV mutagenesis

The UVB portion (<280-400 nm) of UV light can be used to induce mutations in microorganisms (Ikehata & Ono, 2011; Todd & Glickman, 1982). Exposure of microorganisms to UV irradiation induces dipyrimidine photoproducts such as cyclobutane pyrimidine dimers (CPDs) (Ikehata & Ono, 2011). Dipyrimidine photoproducts cause transition-type (pyrimidine-to-pyrimidine) mutations, namely, cytosine (C)-to-thymine (T) or CC-to-TT mutations at dipyrimidine sites 4, 5 (Todd & Glickman, 1982). Three rounds of UV mutagenesis generated a *Pichia stipitis* strain that could tolerate and produce ethanol from hardwood spent sulphite liquor (Bajwa et al., 2009).

2.3.4.3 Transposon mutagenesis

Transposons are segments of DNA that can insert into various locations in the genome mediated by the transposase enzyme (Rau et al., 2016; van Opijnen & Camilli, 2013). The use of transposons is applied in insertional mutagenesis, whereby library of mutant bacterial strains are constructed with transposons inserted at random locations (van Opijnen & Camilli, 2013). The mutant library can then be screened against various

stressors to isolate tolerant strains or identify new gene functions/interactions that contribute towards tolerance (Kadoya et al., 2015; Rau et al., 2016; van Opijnen & Camilli, 2013). A transposon mutant library of *E. coli* was screened against chemicals with different functional groups, to elucidate a link between chemical structure and toxicity (Rau et al., 2016).

2.3.4.4 Mutator strains

Mutator bacterial strains possess mutations in DNA repair or replication genes, which increases the over spontaneous mutation rate (Fowler & Schaaper, 1997; Raynes & Sniegowski, 2014; Wielgoss et al., 2013). An example of a mutator strain is the *E. coli* strain XL1-Red® (Stratagene) which has mutations in *mutS* that encode a component of the methyl-directed mismatch repair system (Kang et al., 2019; Muteeb & Sen, 2010). Genes expressed in mutator strains are subjected to a higher rate of mutation, likewise mutator genes expressed in a strain of interest has the same effect(Kang et al., 2019; Le Chat et al., 2006; Muteeb & Sen, 2010; Selifonova et al., 2001; Zhu et al., 2015). Under screening conditions, recombinant mutator strains can be selected which contain novel mutations that display a desirable phenotype (Kang et al., 2019). However, there is a critical threshold of mutation rate, which if surpassed, can decrease fitness (Kinnersley et al., 2019; Sprouffske et al., 2018; Swings et al., 2017). Long term hypermutation is detrimental due to the accumulation of deleterious mutations (Sprouffske et al., 2018). An intermediate mutation rate has been linked with fitter microbial lineages (Sprouffske et al., 2018). This can be facilitated by cloning mutator genes in vector based systems under the control of an inducible promoter (Badran & Liu, 2015; Zhu et al., 2015). Six genes involved in the *E. coli* stress response, DNA replication were expressed in an *E. coli* mutator strain and adaptively involved under n-butanol stress (Zhu et al., 2015). The mutator population was then adaptively evolved under *n*-butanol selection, which generated a mutant strain which could tolerate n-butanol at a 70% higher concentration that the parental strain (Zhu et al., 2015).

2.3.4.5 Genome shuffling

Genome shuffling recombines the entire genome to generate novel combinations of mutations (Luan et al., 2013; Nicolaou et al., 2010; Patnaik, 2008; Shi et al., 2009).

The method is usually performed on a population that had previously been diversified by random mutagenic methods (Nicolaou et al., 2010). Protoplast fusion is a commonly used genome shuffling method, where two mutant populations are recombined to evaluate the sum of their combined mutations under selection (Patnaik, 2008; Shi et al., 2009; J. Winkler et al., 2010). Genome shuffling was used in combination with UV mutagenesis to generate an ethanol tolerant *S. cerevisiae* strain that had enhanced production capability (Shi et al., 2009).

NTG mutagenised populations of the *C. beijerinckii* strain were recombined which generated an isopropanol tolerant strain (Gérando et al., 2016). Protoplast fusion has also been used to recombine genomes from different bacterial organisms (J. Winkler et al., 2010). *Lactobacillus brevis* is naturally tolerant to *n*-butanol and when recombined with *E. coli* generated a novel hybrid strain with enhanced butanol resistance (J. Winkler et al., 2010).

2.3.4.6 Global transcription machinery engineering (gTME)

gTME introduces mutations into transcription and sigma factors to alter the promoter preference of RNA polymerase (H Alper & Stephanopoulos, 2007). This modifies the transcription and expression of multiple genes which can be screened under a selection pressure (H Alper & Stephanopoulos, 2007; Horinouchi et al., 2010) The standard method used to create the transcription/sigma factor mutant libraries is error prone PCR(H Alper & Stephanopoulos, 2007; Hal Alper et al., 2006). This approach has successfully generated E. coli strains with enhanced ethanol tolerance (H Alper & Stephanopoulos, 2007; Tan et al., 2016)**,** and lycopene production (H Alper & Stephanopoulos, 2007).

2.5 Screening

Screening is an essential process for the selection of an adaptive mutant from an ALE evolved microbial population that exhibit desirable characteristics. An effective screen needs to be both high throughput to generate an effective output. Screening for chemical tolerant strains in a microbial population must be accurate and sensitive, to prevent the false discovery of mutants. Functional screening parameters for the assessment of tolerance are growth rate, lag, efficiency/yield and also cell viability

(Blomberg, 2011; Warringer et al., 2003, 2008). Batch culture or solid medium can be set up as tolerance competition assays which include functional screening parameters; to isolate mutant strains from the wildtype. Prior to conducting a tolerance assay a predetermined concentration of a stressor will need to be set (Zingaro et al., 2013).

Growth rate screening in batch culture requires bacterial cells to be kept in exponential phase by repeated dilution in fresh medium (Blomberg, 2011). A batch culturing platform can also be used for the comparison of growth lag duration and overall cell yield OD_{600} (Sandberg et al., 2019; Van den Bergh et al., 2018). However, the introduction of automation to batch culturing is becoming increasingly important to facilitate a high throughput process. Typically this could involve a set up whereby automated screens are conducted in parallel to overall improve chances of mutant isolation, (Blomberg, 2011; Horinouchi et al., 2014; Sandberg et al., 2019). *E. coli* strains isolated from numerous studies by Horinouchi and colleagues were screened using an automated culture system, which maintained hundreds of independent culture series in exponential growth phase in parallel.

Screening for cell viability and survival is best conducted on solid agar medium in the presence of a chemical stressor either overlaid on top or in a supersaturated vapour concentration. This method allows mutant colony isolates to be spatially distributed and selected for further analysis.

2.6 Conclusions and Outlook

ALE is a highly impactful method to facilitate the isolation of many industrially relevant phenotypes, especially enhanced chemical tolerance. ALE methodologies when coupled with adequate screening and selection assays can select for complex phenotypes like tolerance, which often involves numerous underlying genetic factors. ALE is unique from other random mutagenesis methods in that it imposes a restrictive selection regime on a population that is relevant to the overall desired characteristic. This then generates a mutagenic profile that is tailored to the selection pressure, which can then be isolated and subjected to further analysis to elucidate the basis of the phenotype. An additional benefit of using ALE comes from the ability to design experiments, where a specific culturing condition and parameter can be set up which thereby functions as a selection pressure.

Chapter 3

Aims and Objectives

3.1 Aims and Objectives

The aim of this study was to overcome the issues caused by styrene toxicity by generating a styrene tolerant *E. coli* BW25113 strain using ALE. To fulfil this aim the following objectives were set:

1. **Develop an ALE method which can be used to enhance the tolerance of *E. coli* BW25113 cells to hydrophobic compounds.**

   This objective was to use styrene as a case study to develop an ALE method which can enhance the tolerance of *E. coli* BW25113 to hydrophobic compounds.

   E. coli BW25113 is the parent strain of the Keio collection, and was selected because it is widely used as a host strain for genetic study and manipulation (Grenier et al., 2014). The styrene toxicity threshold concentration has been determined in *E. coli* BW25113 as 2.9 mmol L^{-1} (McKenna & Nielsen, 2011). Therefore, a styrene aqueous concentration ≥ 2.9 mmol L^{-1} is a styrene selective environment, which can select and isolate styrene tolerant *E. coli* BW25113 mutants. However, maintaining a constant aqueous styrene concentration is challenging due to its physicochemical properties. Styrene, like other hydrophobic solvents is poorly soluble in water and is very volatile (James & Castor, 2011). As maintaining a constant selective environment is important in ALE, careful method development and validation is required. The objective was therefore to determine the best methods to conduct ALE with styrene. Batch and chemostat culture with liquid phase and vapour phase styrene were evaluated by monitoring aqueous phase concentration over time and the effect on *E. coli* BW25113 cells.

2. **Generate and isolate styrene tolerant *E. coli* BW25113 mutants using developed ALE batch and continuous culture methods.**

   The objective was to apply the ALE methods developed in (1) to generate a styrene tolerant *E. coli* BW25113 strain with adaptive resistance to styrene toxicity threshold concentration = 2.9 mmol L^{-1} .

3. **Generate and isolate styrene and ethylbenzene tolerant *E. coli* BW25113 mutants using the developed ALE and screening methods with ethylbenzene as a selection pressure.**

   Due to some difficulty in isolating styrene tolerant mutants, ALE was conducted using ethylbenzene at it is structurally related and has similar physicochemical properties to styrene but is less toxic to *E. coli* BW25113 cells. The objective was to apply a two-step approach by first generating a population with tolerance to ethylbenzene and then isolating styrene tolerant strains within the population.

4. **Characterise the gene mutations of styrene tolerant *E. coli* BW25113 mutants.**

   Comparing the genome resequencing data of the mutant with a genome of a non- adapted wild- type genome which was used as a reference, to highlight gene mutations which could underpin the tolerance phenotype.

Chapter 4

Materials and methods

4.1 Chemicals and reagents

All chemicals, reagents and glassware were purchased from either Sigma Aldrich (Gillingham, UK) or Fisher Scientific (Loughborough, UK). Granulated LB broth was purchased from Melford Biolaboratories Ltd (Ipswich UK), whilst the bacteriological agar was from Oxoid Ltd (Altrincham, UK).

4.2 Batch culture vessels

The culture vessels for all batch culture experiments with styrene liquid were performed in 250 ml Quickfit® glass flasks. Batch culture experiments with chemical vapour were performed in 250 mL Quickfit® modified shake flasks. The modifications to the flask were made by the glassblowing workshop based in the faculty of Chemistry, University of Nottingham. The modifications included a glass tube (ø – 1 cm and 6 cm length) glass welded to the bottom of the flask, forming a central vial unit.



Figure 4.1 Photograph of the modified Quickfit® shake flask with a central glass vial welded to the bottom. The addition of the glass vial allowed for there to be a containment vessel where different volumes of styrene could be added. The modification allowed or styrene vapour to be tested as a selection pressure in batch culture.

4.3 Strains and media

The parental bacterial strain used in this study was *Escherichia coli* BW25113 which was purchased from the American Type Culture Collection (ATCC). *E. coli* BW25113 is a common laboratory strain and a parent strain for approximately 4,000 single-gene deletion mutants (Datsenko & Wanner, 2000).

Strain	Features	Source/ Reference
E. coli BW25113	From *E. coli* MG1655 [Δ(*araDaraB*) 567 Δ(*rhaD-rhaB*)568 Δ*lacZ4787* (*::rrnB-3*) *hsdR514 rph*-1]	ATCC (Datsenko & Wanner, 2000)
mEtBCH 1	From *E. coli* BW25113: *bamA*(Ala41Val) *gsiA*(indels) *yghQ*(Tyr207Phe) *alsC*(Gly295Ala) *lptE*(Glu139Lys) *fhuA*(Truncated at Tyr554) *acrB*(Thr489Ile) *acrB*(Phe178Leu) *ptsG*(Gly13Ala) *phoQ*(Met466Ile) *opgG*(indels) *lsrD*(Val199Met) *rsxD*(indels) *lpxM*(indels) *mtgA*(indels) *ycdT*(indels) *pncC*(Ile65Leu) *orn*(Pro103Leu) *acrR*(indels) *nac*(Asp194Asn) *galS*(indels) *rlha*(Ala230Thr) *rplM*(Asp141Gly) *rho*(Met245Ile) *ydbC*(Ala266Asp) *aspS*(Ala60Thr) *rhlB*(indels) *hflX*(Ala224Val) *gdhA*(Truncated at Trp311) *gltB*(Gly1345Ser) *ahpC*(Gly142Ala) *katG*(Phe642Val) *rob*(Arg156His) *ydaU*(indels) *yfeS*(Gly115Ser) *ybgQ*(indels) *yhjJ*(Tyr417His) *yadE*(Leu96His) *yicI*(Val710Ala) *ycbF*(indels)	This study

Table 4.1 Bacterial strains used in this study

LB was routinely used for *E. coli* BW25113 growth for stock cultures whilst MSX or MS was used as a minimal growth medium whenever styrene was added. MS medium (pH 7) was prepared as per (Stephens & Dalton, 1987) containing 2 g L^{-1} KH_2PO_4, 3 g L^{-1} NH_4Cl, 0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 2 mL L^{-1} Vishniac trace elements solution [130 mmol L^{-1} EDTA, 7.64 mmol L^{-1} $ZnSO_4$, 25 mmol L^{-1} $MnCl_2$, 18.5 mmol L^{-1} $FeSO_4$, 0.89 mmol L^{-1} $(NH_4)_6Mo_7O_{24}$, 6.4 mmol L^{-1} $CuSO_4$, 6.72 mmol L^{-1} $CoCl_2$,

37.4 mmol L^{-1} $CaCl_2$]. MSX was adapted from a recipe of MS medium taken from (Stephens & Dalton, 1987) but with 6 g L^{-1} KH_2PO_4.

4.4 Tolerance assay batch culture with styrene/ethylbenzene liquid

50 mL of MSX medium contained inside a 250 mL Quickfit® glass flask was inoculated with *E. coli* BW25113 cells from an overnight seed culture at a starting $OD_{600} \approx 0.1$. The culture was then grown to $OD_{600} \approx 0.2$ and then styrene/ethylbenzene was added to the culture volume at a final concentration dependant on the experiment conditions. The Quickfit® glass flask was sealed with a glass stopper and incubated in an orbital shaker at 250 rpm and 37°C. To analyse growth, culture samples were taken at regular time-points and analysed by UV-vis spectrophotometry. To analyse survival, culture samples were taken at regular time and grown on 25 mL MSX medium plates. The total volume of the medium was always 25% or less of the cultivation vessel to ensure a sufficient oxygen supply.

4.5 Tolerance assay batch culture with styrene/ethylbenzene vapour

Styrene/ethylbenzene was added to the vial inside the modified Quickfit® glass flask at a volume dependent on the experimental conditions. 50 mL MSX medium was then added to the flask space surrounding the vial. The Quickfit® glass flask was sealed with a glass stopper and incubated in an orbital shaker at 250 rpm and 37°C for ≥12 h to allow the styrene/ethylbenzene to reach equilibrium concentration in the medium. After 12 h, the 20 mL MSX medium was inoculated with *E. coli* BW25113 cells from an overnight seed culture at a starting OD600 = 0.1. The cultures were incubated in an orbital shaker at 250 rpm and 37°C. To analyse growth, culture samples were taken at regular time-points and analysed by UV-vis spectrophotometry. To analyse survival, culture samples were taken at regular time and grown on solid medium plates. The total volume of the media was always 25% or less of the cultivation vessel to ensure a sufficient oxygen supply.

4.6 Styrene/ethylbenzene ALE chemostat

Styrene/ethylbenzene ALE chemostat conditions were set up in a custom-built bioreactor (Figure 4.2). The bioreactor consisted of a 100 mL glass bioreactor vessel with a PTFE lined butyl rubber head plate closed with a clamp. The vessel had an

inbuilt water jacket which was maintained at 37 °C by the continuous passing of temperature controlled from a water bath into the water jacket. The media feed was introduced with peristaltic pumps through the silicone tubing and into a metal rod (ø – 1mm). A connection between the headspace of flask and O_2 gas from an air compressor was provided by another metal rod connected to PTFE tubing with a 0.20 μm filter for sterilisation. The working volume was determined as 57 mL by measurement of volume loss after mixing and aeration for 1h. The reaction output was drained down to an attached waste pot through a waste arm. Mixing was achieved by magnetic stirring. MS medium was fed into the vessel via the media feed line and the reactor vessel was inoculated with a sterile syringe containing cells from an overnight culture via an inoculation port. The OD_{600} of the culture was obtained by taking samples via a sampling port and measuring on a UV-vis spectrophotometer. pH was measured by an autoclavable pH probe inserted through the head plate into the culture medium. Deviations from the 6.8-7.1 range were corrected with solutions of a base (0.25 M NH_4OH) which were pumped in via peristaltic pumps and tubing. The growth parameters were set at: dilution rate 0.1 h^{-1}; agitation control 500 rpm; air supply 0.2 L min− 1; temperature 37 °C; and pH 7. The glucose concentration in the MSX media was 1 g L-1. A styrene syringe pump was connected to the vessel via PTFE tubing and was calibrated to administer styrene/ethylbenzene a flow rate at a range between 0 – 3.2 mL h^{-1}, depending on the experimental conditions. The syringe pump was switched on when the cell concentration was at steady state. Steady state was defined as two or more OD_{600} readings ± 5% obtained for subsequent samples taken in a time period which accounts for 5 volume changes of the reactor = 2.4 days. Glucose limitation was confirmed by increasing the glucose concentration of the inflow MS medium from 1 g L^{-1} to 2 g L^{-1} and measurement of *E. coli* BW25113 OD_{600} response (Appendix F)

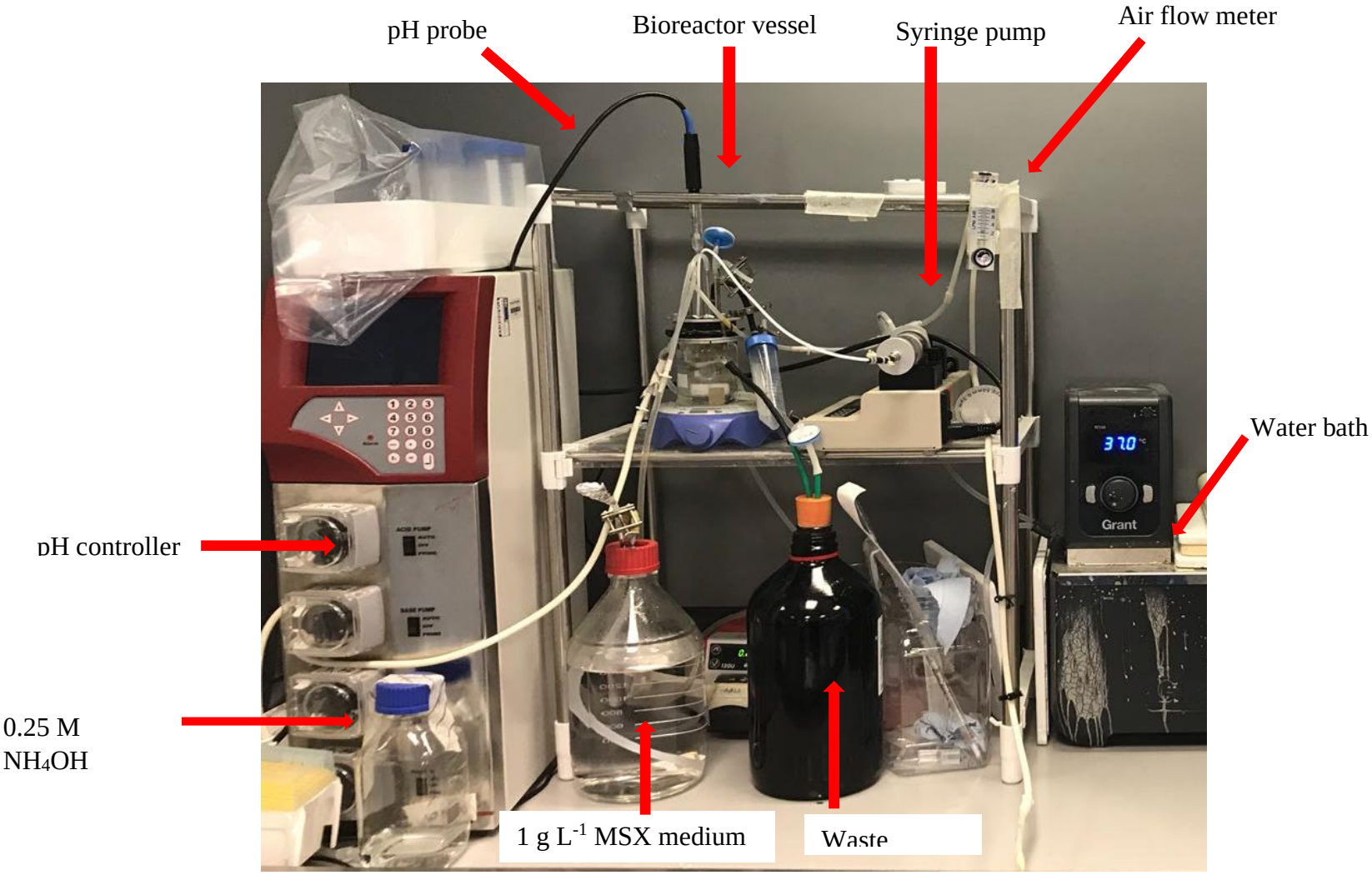


Figure 4.2 Photograph of the custom built chemostat used in this study to conduct ALE.

4.7 Styrene/ethylbenzene tolerant mutant isolation

To generate styrene/ethylbenzene tolerant clonal isolates, an ALE *E. coli* BW25113 population samples were spread on MSX plates that had an aseptically created hole in the agar. For the styrene experiments 100 µL of styrene were added to the hole. For the ethylbenzene experiments 500 µL of ethylbenzene were added to the hole. The plates were then placed inside a 6 L desiccator which was sealed before incubation at 37 °C for 48 h. The resulting colonies were then picked for further analysis.

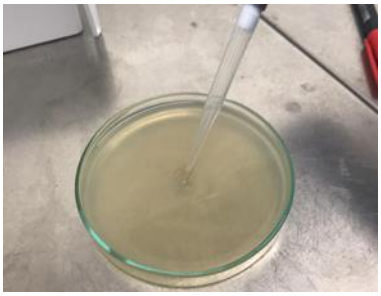


Figure 4.3 Photograph showing how styrene/ethylbenzene was added to an aseptically created hole in the MSX agar plate.

4.8 Sample preparation for GC-MS

Styrene/ethylbenzene extractions were performed on a 15 mL sample of either the supernatant of an inoculated culture sample or an inoculated sample. The 15 mL extraction sample was added to 5 mL hexane in a 40 mL glass vial and vortexed for 5 mins. The 5 mL organic phase was retained and the aqueous phase was combined again with a fresh 5 mL of hexane. This process was repeated a total of 3 times to attain a total hexane extraction phase of 15 mL+ *t*-butyl benzene (10 mM) which was used as the internal standard. A 1 mL sample was taken for GC-MS analysis.

4.9 Analytical determination of styrene/ethylbenzene concentration

Styrene and ethylbenzene were detected and quantified using an Agilent 7890 A GC-MS System equipped with an Agilent capillary HP5-MS column (0.32mm x 30 m x 0.5 µm), an Electron Impact ionisation source and a 5975C inert MSD with quadrupole mass analyser. The same detection method was used for both styrene and ethylbenzene. Helium was used as carrier gas at 1.2 mL/min. Data analysis was performed using MSD ChemStation software and by comparison with the mass spectra and retention times of authentic standards. For detection of styrene and ethylbenzene in samples aqueous phase (1 µL) was injected using an autosampler a split ratio of

200:1. The inlet temperature was 250℃ the oven temperature was initially kept at 45℃ for 5 min and increased to 300℃ at 20℃/min. The final temperature was held for 10 min. Concentrations were estimated based on a calibration curve constructed of styrene and ethylbenzene extractions from calibration samples containing styrene (within a range of 1 – 5 mmol L^{-1}) or ethylbenzene (within a range 1 – 10 mmol L^{-1} ethylbenzene). Reference samples of each chemical were made up in the same concentration range in hexane + *t*-butyl benzene (10 mM) which was used as the internal standard.

Recovery % was calculated as: $\frac{[Calibration\ sample\ peak\ area]}{[Reference\ sample\ peak\ area]} \times 100$

The styrene in the hexane extract was identified by comparing its relative retention time with that of styrene standard.

4.10 DNA sequencing

Overnight cultures of the styrene tolerant *E. coli* BW25113 mutant mEtBCH 1 were used to inoculate a 50 mL LB culture and grown to exponential phase. The cells were collected by centrifugation at 16,000 × g for 2 min and 25 °C and genomic DNA was extracted using the DNEasy spin column kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction.

The genome resequencing and bioinformatics was conducted at Deep Seq, an external sequencing facility based at Queen's Medical Centre, Nottingham. A barcoded resequencing library was prepared using Nextera v2 technology from the genomic mutant sample that was provided after extraction. The resulting library was then sequenced to provide enough reads for 50x coverage with 250 paired end reads by the MiSeq platform (Illumina). Bioinformatic analysis was conducted using the GATK v 3.3.0 (DePristo et al., 2011) to look for germline short variants by mapping the mutant genomic DNA to the *E. coli* BW25113 parental strain/reference genome (Escherichia coli strain BW25113, assembly ASM75055v1, annotation version 34 from Ensembl) (Datsenko & Wanner, 2000). For each potential point mutation, the coverage reads of more than 10 and a ratio of variant read to wild type read more than 0.6 were extracted.

4.11 Structural analysis

The locations of the mutations were studied on published structures of AcrB (PDB ID: 2DHH), AcrR (PDB ID: 3BCG), PhoQ histidine kinase phosphotransfer domain in

complex with ATP (PDB ID: 1ID0). Rendered images of the protein structures were generated using Jmol: an open source Java viewer for chemical structures in 3D.

Chapter 5

Method development for adaptive laboratory evolution with styrene

5.1 Introduction

This chapter reports on the development of methods which can be used for ALE, to generate an *E. coli* BW25113 styrene tolerant mutant under a constant styrene selective environment. The ALE method needed to allow analytical control over the concentration of styrene, supplied as the selection pressure to a population of *E. coli* BW25113 cells. The ALE method also needed to be representative of how the styrene could accumulate in liquid phase to a toxic concentration in an industrial fermentation. Batch sub-culturing and chemostat culturing, where styrene is added in the liquid phase, were considered as potential ALE methods to be used in this study.

Previous ALE studies that used the alcoholic compounds in liquid phase as a selection pressure, were successful in generating alcohol tolerant *E. coli* BW25113 mutants strains (Dragosits et al., 2013; Gonzalez et al., 2003; Goodarzi et al., 2010; Horinouchi et al., 2017; Lupino et al., 2018; Reyes et al., 2012). However, in comparison to the alcohols, styrene is more hydrophobic and has a water solubility that is an order of magnitude lower. This presents a challenge when using styrene and other chemicals with similar physicochemical properties in microbial cultures.

In addition to any ALE method, there is a need for a robust mutant isolation method, as well as a screening method to identify their tolerance to styrene. Previous studies have used styrene as a selection pressure, in vapour or gaseous phase, to screen and isolate styrene degrading micro-organisms from a mixed population (Hartmans et al., 1990; Oelschlagel et al., 2014; Omori et al., 1975; Portune, Pérez et al., 2015; Przybulewska et al., 2006). Although the techniques in these studies demonstrate how effectively styrene in vapour phase can be used to screen a microbial population; the context in which styrene vapour is used differs. In those studies, styrene was acting as a carbon source for growth and they were screening for styrene degradation capability, not styrene tolerance.

To date there have been no studies that have used styrene as a selection pressure, in ALE or to isolate styrene tolerant mutants. Therefore, there is a need to develop robust methods for this purpose.

5.2 Results and Discussion

5.2.1 Analytical determination of styrene in the aqueous phase.

The aim of this first experiment was to validate the quantitative determination of styrene in batch and chemostat cultures. Hexane was chosen as the extraction solvent because styrene is soluble in hexane and hexane is both low-density and insoluble in water. The method extracted styrene from the aqueous cultures after mixing with hexane solvent. The organic hexane phase could then be analysed by GC-MS. Styrene working standard solutions were made up in 50 mL MSX medium in 250 mL Quickfit® glass flasks, with styrene added to a final concentration between 1 - 5 mmol L^{-1}. Concentrations above styrene's solubility limit in water (3.46 mmol L^{-1} calculated in Appendix G) were reported in mmol L^{-1}, for comparison to the toxicity threshold concentration. Styrene recovery was calculated from the GC-MS relative peak area of the styrene working standard solutions, expressed as a percentage of the reference sample. The quantitative determination of styrene was validated, by assessment of how reproducible the styrene recovery percentage was across the samples.

Styrene concentration mmol L^{-1}	Recovery (%)
1	29.39 ± 0.056
2	29.90 ± 0.021
4	35.02 ± 0.023
5	36.95 ± 0.067

Table 5.1 Recovery (%) of styrene extracted with hexane solvent styrene working standard solutions. 3 x 5 mL hexane extractions were performed on 15 mL sample taken from the styrene working standard solutions containing 1- 5 mmol L^{-1} styrene. Percentage recovery = $\frac{[styrene\ standard\ ]}{[styrene\ reference]} \times 100$. The results and standard deviations are the average of triplicate experiments.

The recovery percentage of styrene extracted from the aqueous phase was relatively low, but consistent across the range of styrene working standard solutions (Table 5.1).

A standard curve was constructed from the data obtained to quantitatively determine styrene concentration in the aqueous phase of the batch cultures (Appendix A).

Next, a separate calibration curve was constructed to quantitatively determine the styrene concentration in the aqueous phase of the chemostat culture. A separate calibration was required because the concentration of the styrene in the chemostat aqueous phase was expected to be lower due to gas stripping. The chemostat was set up with a syringe pump that administered styrene into the continuously stirred and aerated bioreactor vessel, containing 50 mL of un-inoculated MSX medium. The flowrate of the syringe pump could be controlled to administer a set volume per h. The calibration compared the styrene flowrate, administered by the syringe pump, with the styrene concentration detected in the aqueous phase of the chemostat. The syringe pump was set to administer styrene into the chemostat aqueous phase at a flowrate of 0.1, 0.5, 1- and 1.5-mL h^{-1}.

The chemostat aqueous phase styrene concentration was much lower than expected for any given flowrate, under the chemostat operation conditions (agitation control 500 rpm and air supply 0.2 L min^{-1}). At a flowrate of styrene 0.1 mL h^{-1} styrene was not detected at all in the chemostat (Figure 5.1). This loss could be because of headspace evaporation or gas stripping due to the aeration and agitation conditions within the chemostat.

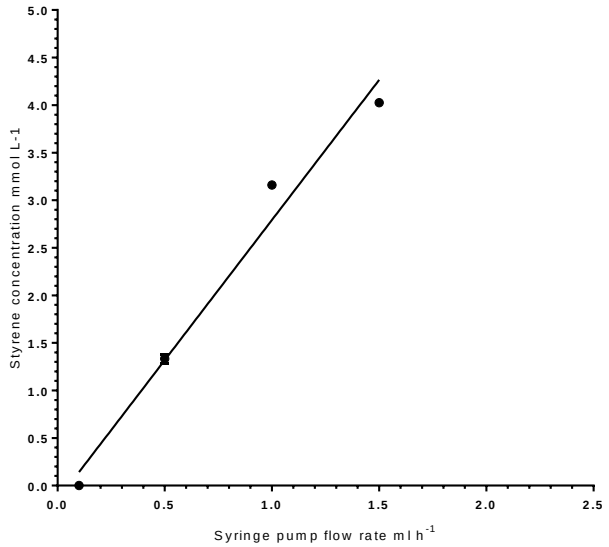


Figure 5.1 Styrene syringe pump flowrate calibration with measured styrene concentration extracted from the ALE styrene chemostat. $R^2 = 0.9496$

In conclusion, the hexane extraction method was reproducible and therefore validated the quantitative determination of styrene in the aqueous phase of the batch and chemostat cultures. Also, the chemostat operational conditions resulted in a > 98% loss of styrene from the aqueous phase. Therefore, to achieve the desired aqueous phase concentration, styrene was added at a flowrate dictated by the calibration to account for significant loss.

5.2.2 Liquid phase styrene as a selection pressure in ALE batch cultures

The aim of this experiment was to validate batch sub-culturing as an ALE method, using liquid phase styrene as the selection pressure. An experiment first tested a range of styrene concentrations on *E. coli* BW25113 growth. Styrene in liquid phase was added at final concentrations of 2.2, 4.3, 13.6 and 24.3 mmol L^{-1}, to *E. coli* BW25113 batch cultures (Figure 5.2). These concentrations represented a range below and up to ≈ 10x the reported *E. coli* BW25113 styrene toxicity threshold concentration (2.9 mmol L^{-1}) (McKenna & Nielsen, 2011). The flasks were inoculated at a starting OD_{600} = 0.1 and then incubated for 48 h. OD_{600} readings were taken at regular time intervals (Figure 5.2).

Table 5.2 shows the specific growth rate (h^{-1}), maximum cell density and lag phase duration (h) of the *E. coli* BW25113 cultures at the concentrations tested. The growth of *E. coli* BW25113 at 2.2 mmol L^{-1} styrene was similar to the control with a slight decrease in the specific growth rate and maximum cell density (Figure 5.2; Table 5.2). At 4.3 - 24.3 mmol L^{-1} styrene concentration, the duration of the lag phase correlated with an increase in styrene concentration, and was significantly longer in comparison to the lag phase of the control culture (Figure 5.2; Table 5.2)

It was expected that growth wouldn't be observed at styrene concentrations 4.3 - 24.3 mmol L^{-1}, as those concentration were up to 10x the toxicity threshold concentration (McKenna & Nielsen, 2011), and therefore lethal to the *E. coli* BW25113 cells.

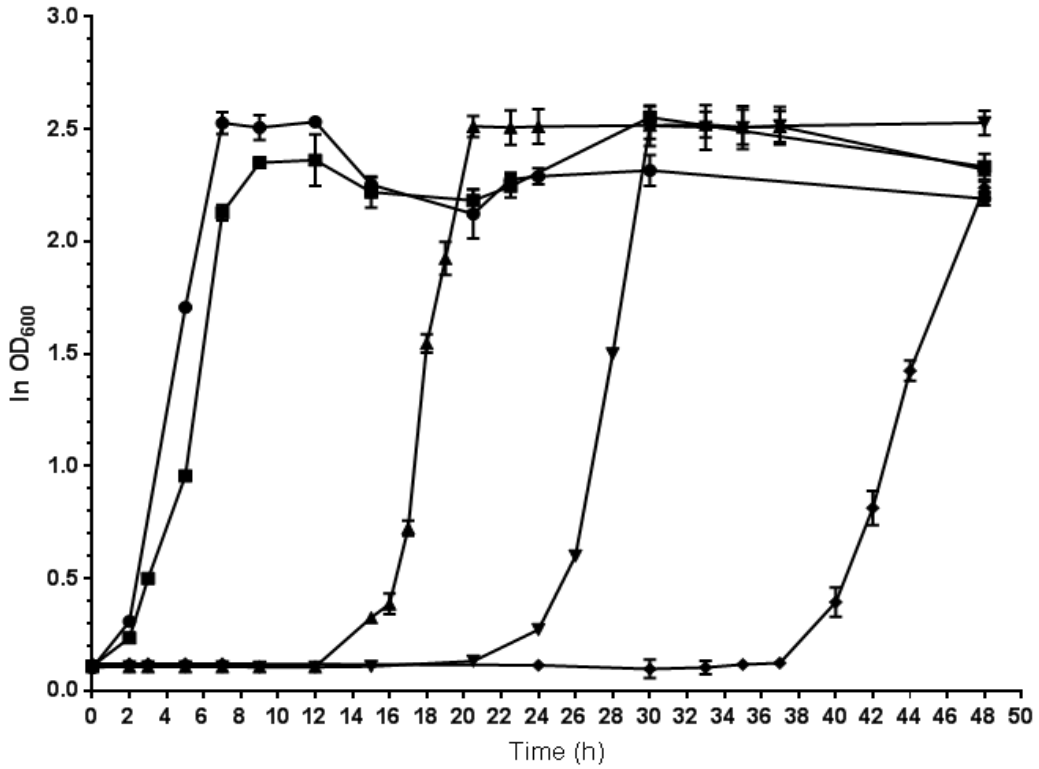


Figure 5.2 Effect of styrene concentration on the growth of *E. coli* BW25113. Growth of *E. coli* in MSX minimal medium (50 mL) in 250 mL shake flasks at 37°C 250 rpm containing styrene at the following concentrations: 0 mmol L^{-1} (●) 2.2 mmol L^{-1} (■) 4.3 mmol L^{-1} (▲) 13.6 mmol L^{-1} (▼) 24.3mmol L^{-1} (♦) The experiments were conducted in triplicate.

Styrene concentration (mmol L^{-1})	Specific growth rate (h^{-1})	Max cell density (OD_{600})	Lag phase duration (h)
0	0.46 ± 0.005	2.5 ± 0.049	1
2.2	0.34 ± 0.003	2.5 ± 0.019	1
4.3	0.41 ± 0.010	2.5 ± 0.023	12
13.6	0.37 ± 0.005	2.5 ± 0.033	24
24.3	0.22 ± 0.020	2.2 ± 0.012	37

Table 5.2 Growth kinetic parameters of *E. coli* at 2.2 – 24.3 mmol L^{-1} styrene concentrations.

However, the growth observed at 4.3 - 24.3 mmol L^{-1} styrene concentration suggested that there may have been a spontaneous mutant generated, which was stress induced at such high styrene concentrations (Figure 5.2) (Ram & Hadany, 2014; Rosenberg et al., 2012). This would mean that there was a constant selective pressure in the batch culture with liquid phase styrene, to induce spontaneous adaptive mutations.

An experiment was set up with an aim to test if the *E. coli* BW25113 cell growth witnessed at 4.3 - 24.3 mmol L^{-1} styrene concentration was due to adaptive mutation. If there was an adaptive mutation present, growth observed in a 4.3 - 24.3 mmol L^{-1} styrene concentration sub-culture should occur after a reduced lag phase than observed in (Figure 5.2; Table 5.2)(Gresham & Dunham, 2014) . This in turn would indicate that there was a constant selective pressure in the batch culture with liquid phase styrene. The 4.3, 13.6 and 24.3 mmol L^{-1} styrene concentration batch cultures (Figure 5.2) were sub-cultured further, as these concentrations induced an extended lag phase. Each of the *E. coli* BW25113 styrene batch cultures were sub-cultured 5x into a concentration of styrene that was the same as the previous sub-culture. Lag duration was monitored by taking regular OD_{600} readings.

Overall, between the 1st and 5th sub-culture the lag phase duration did not decrease significantly across all the styrene concentrations tested (Figure 5.3 – 5.5; Table 5.3). There were some small differences in lag phase durations between the individual sub-cultures. This was notable in the 4th sub-culture at 13.6 mmol L^{-1} styrene, where the lag phase duration decreased to 7 h which was significant from the previous sub-culture which had a lag phase of 24 h (Figure 5.4; Table 5.3). However, in the subsequent culture the lag phase had increased to what was the typical duration as the previous subcultures (Figure 5.4). An explanation for the shortened lag phase in culture 4, could be because possibly an earlier phase of *E. coli* BW25113 cells (late exponential) were taken from the 3rd sub-culture and used as the inoculum in the 4th culture, which enhanced fitness (Figure 5.4) (Gresham & Dunham, 2014).

	Lag phase duration in sub-culture (h)					
	0	1st	2nd	3rd	4th	5th
Styrene concentration mmol L^{-1}						
4.3	12	14	11	13	9	10
13.6	24	16	16	24	7	25
24.3	37	33	25	no growth	no growth	no growth

Table 5.3 Lag phase duration of *E. coli* BW25113 at styrene concentration 4.3 – 24.3 mmol L^{-1} in the 1^{st} – 5^{th} sub-culture experiments.

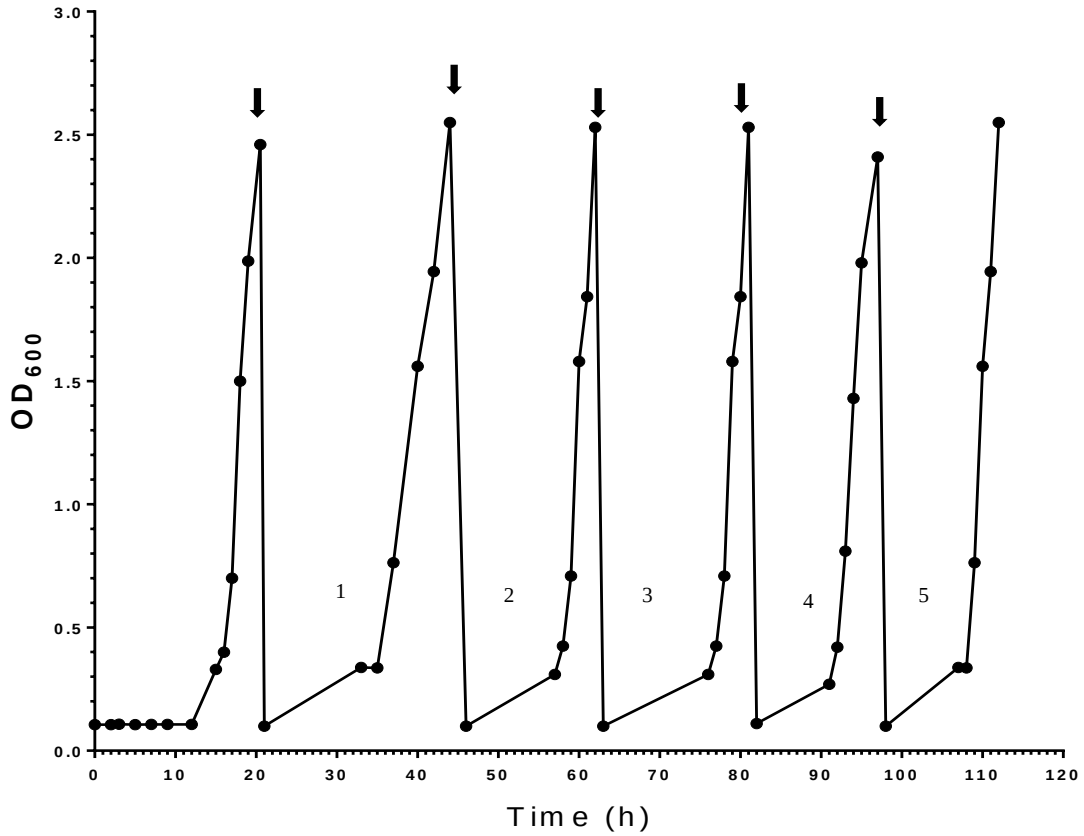


Figure 5.3 Serial *E. coli* BW25113 batch sub-culture experiments at 4.3 mmol L^{-1}. *E. coli* was grown in MSX minimal medium (50 mL) with 4.3 mmol L^{-1} styrene at 37°C and 250 rpm. The arrows represent the times and cell density at which a sample was taken and sub-cultured into a culture containing fresh medium and 4.3 mmol L^{-1} styrene.

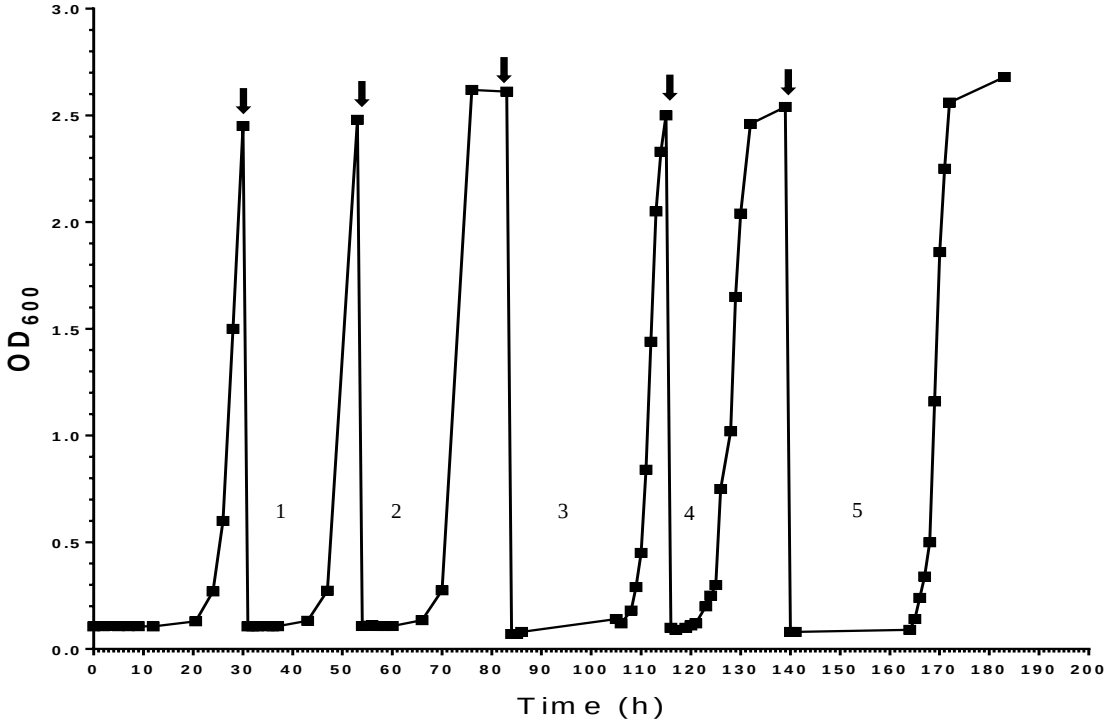


Figure 5.4 Serial *E. coli* BW25113 batch sub-culture experiments at 13.6 mmol L^{-1}. *E. coli* was grown in MSX minimal medium (50 mL) with 13.6 mmol L^{-1} styrene at 37°C and 250 rpm. The arrows represent the times and cell density at which a sample was taken and sub-cultured into a culture containing fresh medium and 13.6 mmol L^{-1} styrene.

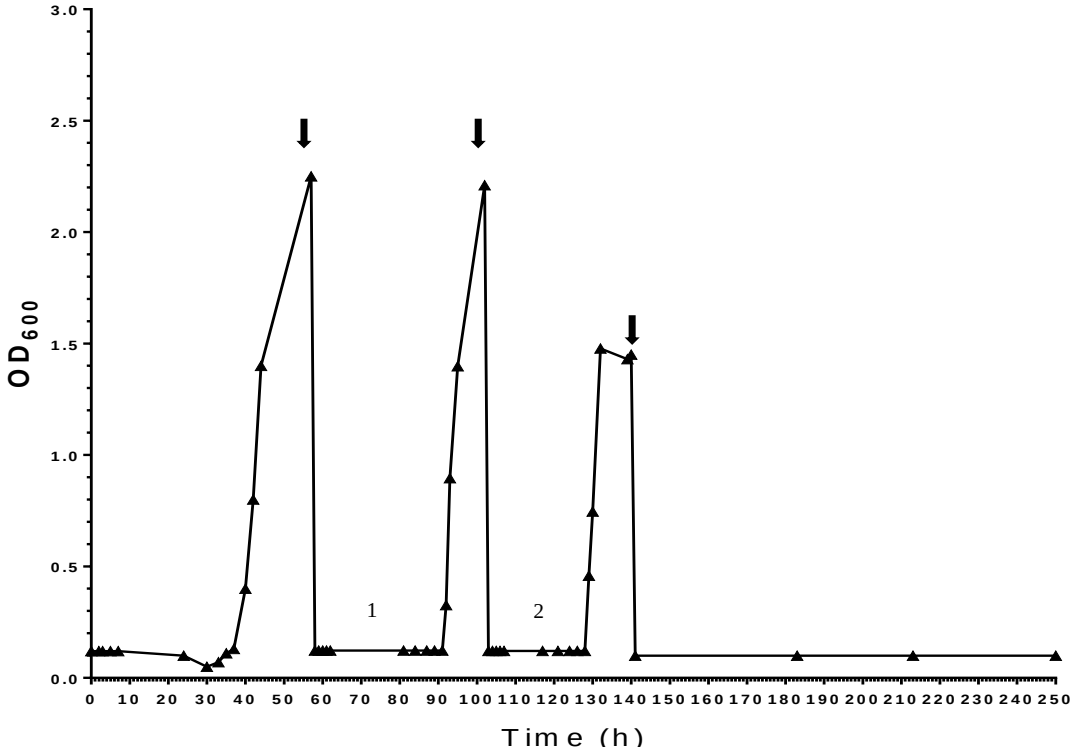


Figure 5.5 Serial *E. coli* BW25113 batch sub-culture experiments at 24.3 mmol L^{-1}. *E. coli* was grown in MSX minimal medium (50 mL) with 24.3 mmol L^{-1} styrene at 37°C and 250 rpm. The arrows represent the times and cell density at which a sample was taken and sub-cultured into a culture containing fresh medium and 24.3 mmol L^{-1} styrene.

At 24.3 mmol L^{-1} styrene concentration, *E. coli* BW25113 growth after an extended lag phase was only seen in the 1st and 2nd sub-culture, which lasted 33 h and 25 h respectively whilst sub-culture 3 did not grow within a 110 h time period (Figure 5.5). Sub-culture 2 reached a lower cell OD than the previous 2 subcultures perhaps due to the stress on the cells, or the deleterious effect of possible adaptive mutations (Sprouffske et al., 2018).

The results from (Figure 5.3-5.5; Table 5.3) suggested that the *E. coli* BW25113 growth observed at high concentration of styrene (4.3 -24.3 mmol L^{-1}) were not of adaptive mutants but non-tolerant wildtype *E. coli* BW25113 cells. This meant that perhaps the styrene aqueous phase concentration in the batch culture did not remain constant and allowed non-tolerant wildtype *E. coli* BW25113 cells to grow after a certain time period.

A test was next conducted with the aim to determine if there was a constant selective pressure over time when styrene was added to the batch culture in the liquid phase. This was done by measuring the aqueous styrene concentration and the biological effect on *E. coli* BW25113 growth and viability. If the selective pressure was constant, then the aqueous styrene concentration should remain constant over the 24 h period and there shouldn't be an increase in cell OD_{600} or viability. If the aqueous styrene concentration does not remain constant, then there isn't a selective environment in the batch culture which meant that nontolerant *E. coli* BW25113 cells could grow.

A batch culture experiment was set up which added styrene at a final concentration of 4.3 mmol L^{-1} to OD_{600} = 0.1 *E. coli* BW25113 batch cultures, which were then incubated and sampled regularly for 24 h. The 4.3 mmol L^{-1} styrene concentration was chosen, because it induced the shortest extended lag time from previous experiments (Figure 5.2), and regular samples for analysis could be taken in a 24 h time period. *E. coli* BW25113 cell samples were taken for measurement of the $_{OD600}$ and serially diluted and plates on LB plates to measure cell viability (CFU/mL). Styrene was extracted from the supernatant of the cell samples taken and analysed by GC-MS.

The results observed showed that styrene concentration in the *E. coli* BW25113 batch culture was relatively constant, then decreased after 12 h. This coincided with the increase in *E. coli* BW25113 cell OD_{600} and viable cells after 12 h (Figure 5.6). This was also an unexpected result as a commonly accepted hypothesis was that styrene

evaporates from the culture medium due to its insolubility and high vapour pressure (Changqing Liu et al., 2018). However, the results suggest that there is an inversely proportional relationship between cell biomass concentration and styrene aqueous phase concentration.

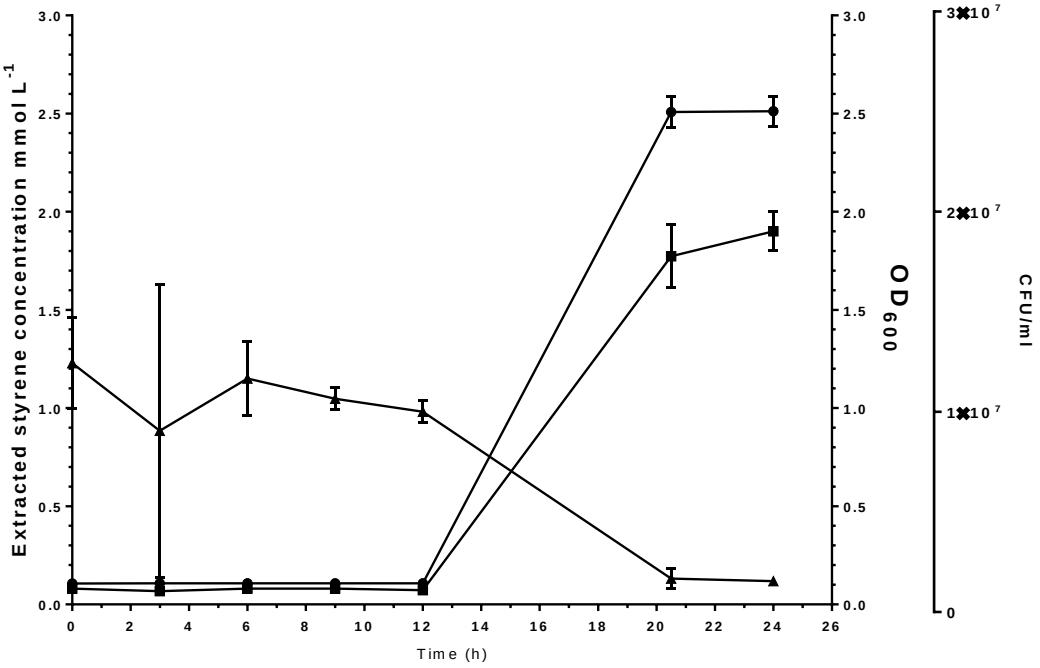


Figure 5.6 Measurement of styrene concentration over time in an *E. coli* BW25113 styrene liquid phase batch culture experiments at 4.3 mmol L^{-1} styrene concentration.
Aqueous styrene concentration from batch culture mmol L^{-1} (▲)
E. coli cell OD_{600} (●)
E. coli cell viability CFU/ml (■)
Incubation conditions were at 37°C and 250 rpm. The experiments were conducted in triplicate.

In either case the results suggest that the presence of *E. coli* BW25113 cells influenced the styrene aqueous phase concentration in the batch culture over time. Batch sub-culturing using liquid phase styrene as the selection pressure could therefore not be performed under a constant selective environment. The results also suggested that a styrene selective environment within a batch culture, was only maintained for the duration of the lag phase it induced on the *E. coli* BW25113 population. For instance, at 4.3 mmol L^{-1} styrene concentration, the extended lag phase that was induced lasted 12 h, which coincided with the styrene aqueous phase concentration that also remained relatively constant for 12 h (Figure 5.6).

In conclusion, liquid phase styrene as a selection pressure in batch culture is limited as an ALE method due to there being a likelihood of false positive "mutant" growth from non-tolerant individuals in the population. However, a singular batch culture test with liquid phase styrene, is suitable to measure the effects of styrene on growth. However, the growth must be observed within a time duration that the styrene selective environment can be maintained. It was decided after this set of experiments, that single batch cultures were to be used in styrene tolerance growth assays. The parameters to conduct the styrene tolerance growth assays were determined in a future experiment in this chapter (5.2.4). There was also potential to perform batch culture tests with styrene in the vapour phase, which was investigated next (5.2.3).

5.2.3 Vapour phase styrene as a selection pressure in batch cultures

Previous findings from 5.2.2 showed that batch sub-culturing as an ALE method with liquid phase styrene was limited because the selection pressure could not be sustained over time. Batch sub-culturing with vapour phase styrene was proposed as a solution to maintain the aqueous concentration of styrene over time.

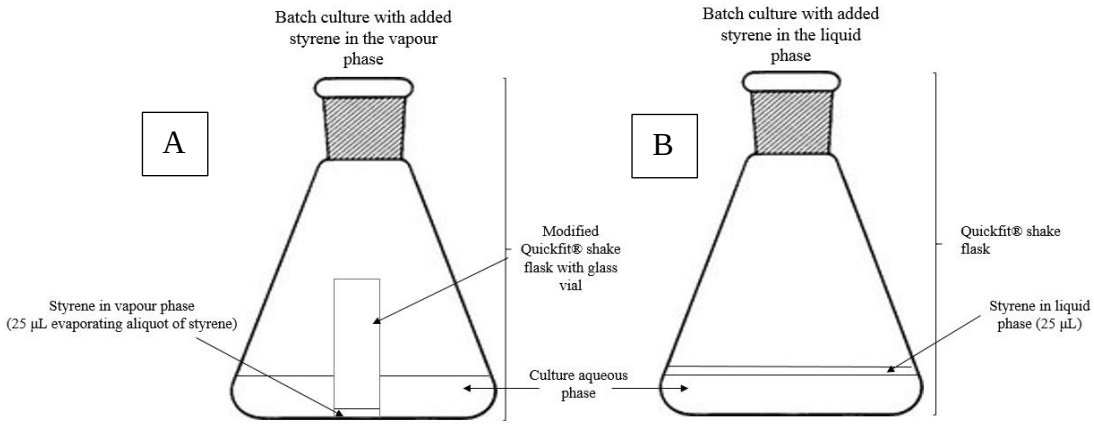


Figure 5.7 Diagram of experiment set up to compare the effects of liquid phase and vapour phase styrene on *E. coli* BW25113. Growth of *E. coli* in MSX minimal medium (50 mL): (A) Styrene batch culture with styrene in vapour phase in 250 mL Quickfit® modified shake flask with 25 µL evaporating aliquot of styrene in glass vial. (B) Styrene batch culture with styrene in liquid phase in Quickfit® shake flask with 25 µL styrene added directly to the culture medium.

An experiment was set up as in 5.2.2 to determine if there was a constant selective pressure over time when styrene was present in batch culture in the vapour phase. This was done by measuring the aqueous styrene concentration and the biological effect on *E. coli* BW25113 growth and viability. If the selective pressure was constant, then the aqueous styrene concentration should remain constant over the 24 h period and there

shouldn't be an increase in cell OD_{600} or viability. If the aqueous styrene concentration does not remain constant, then there isn't a selective environment in the batch culture which meant that non-tolerant *E. coli* BW25113 cells could grow.

The experiment design differed slightly from 5.2.2. To deliver styrene in the vapour phase batch cultures were set up in 250 mL modified Quickfit® glass shake flasks with 50 mL MSX medium. The modification was an additional glass vial attached securely inside (See materials and methods 4.2). A 25 µL evaporating aliquot of styrene was added to the glass vial of the flask (Figure 5.7B). This volume represented an aqueous phase concentration of 4.3 mmol L^{-1} if it had been directly added to the medium. The flask was inoculated with *E. coli* BW25113 at a starting OD_{600} = 0.1, before being sealed and incubated for 24 h. Regular cells samples were taken, for measurement of the OD_{600} and also serially diluted and plated on LB plates to measure cell viability (CFU/mL). Styrene was extracted from the supernatant of the cell samples taken and analysed by GC-MS.

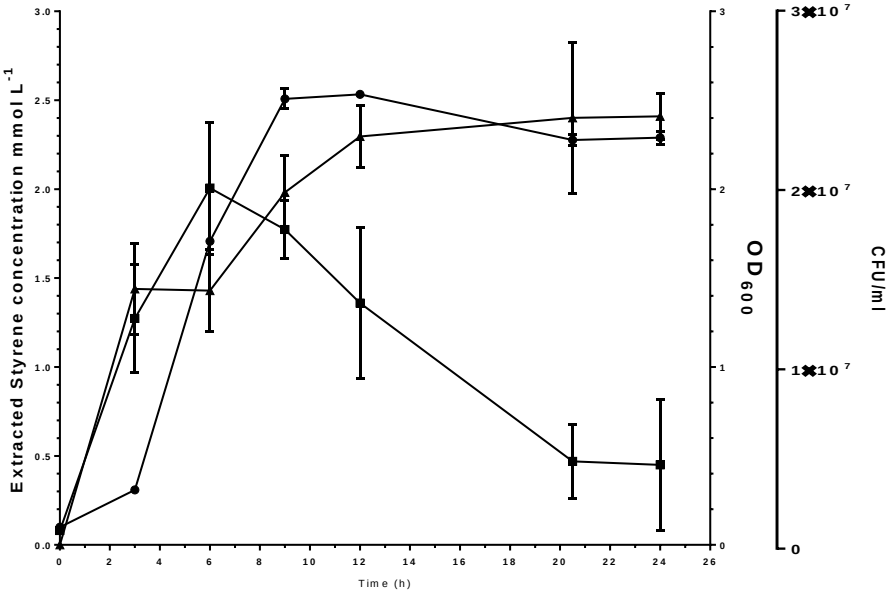


Figure 5.8 Measurement of styrene concentration over time in an *E. coli* BW25113 styrene vapour phase batch culture experiment with 25 µL evaporating aliquot of styrene.
Aqueous styrene concentration from batch culture mmol L^{-1} (▲)
E. coli cell OD_{600} (●)
E. coli cell viability CFU/ml (■)
Incubation conditions were at 37°C and 250 rpm. The experiments were conducted in triplicate.

The results observed showed that the styrene aqueous concentration in the *E. coli* BW25113 batch culture increased between 0 -12 h, presumably because the vapour phase styrene inside the culture vessel was dissolving into the aqueous medium during that period. Between 12 – 24 h, the concentration appeared to remain constant, possibly due to an equilibrium between the vapour and liquid phase being reached. The *E. coli* BW25113 cell OD_{600} increased over time to reach stationary phase. The growth could have been due to the *E. coli* BW25113 cells having a faster rate of growth than the dissolution of the styrene vapour into the aqueous medium. The viable cell counts also increased between 0 – 6 h, but unlike the *E. coli* BW25113 cell OD_{600} decreased after 6 h. This suggested that the aqueous styrene concentration in the styrene vapour batch culture influenced cell viability between 6 – 24 h as it increased (6 – 12 h) and remained constant (12 -24 h) (Figure 5.8). This test suggests that the presence of styrene vapour in batch culture generates a constant selective pressure after a period

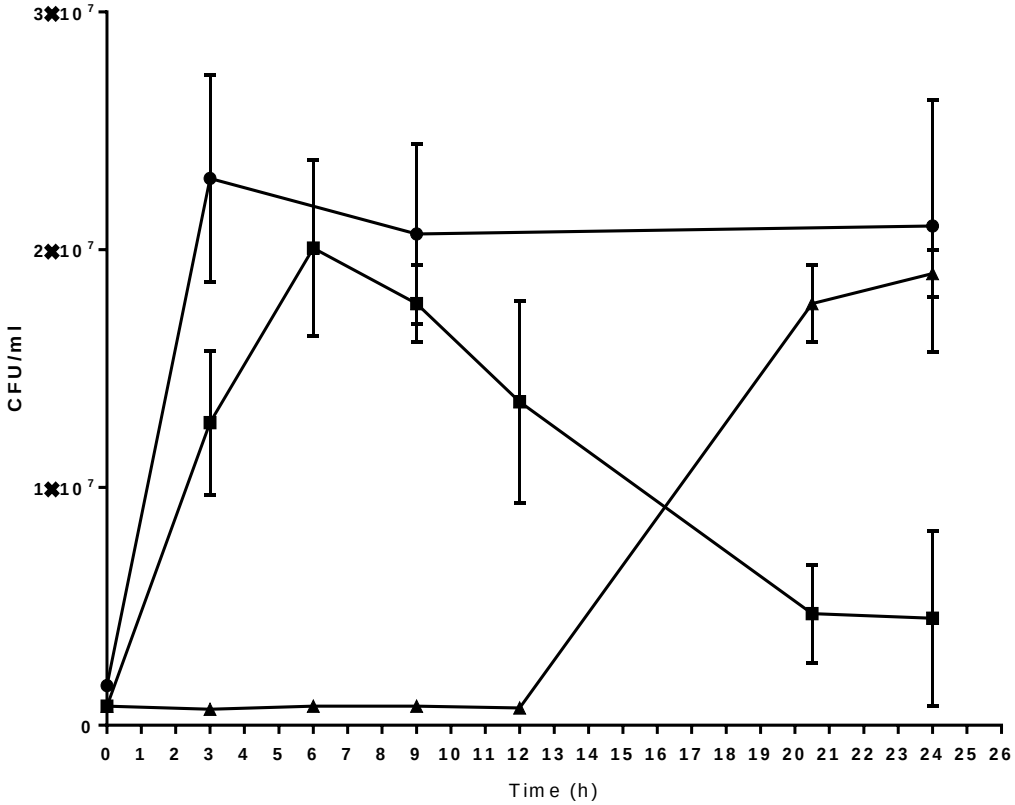


Figure 5.9 Comparison of the effect of styrene concentration in the liquid and vapour phase on the cell viability of *E. coli* BW25113. Growth of *E. coli* of in MSX minimal medium (50 mL) in 250 mL shake flasks containing 25 µL styrene in liquid phase (added to directly to the medium) vapour phase (evaporating aliquot)
Viable count of: Control 0 mmol L^{-1} styrene concentration (●)
Styrene in the vapour phase 25 µL evaporating styrene aliquot (■)
Styrene in the liquid phase 25 µL styrene concentration added to aqueous phase (▲).

of equilibration between the styrene vapour and culture medium of ≥ 12 h. This was

shown by the decrease in cell viability over time in the styrene vapour phase batch culture (Figure 5.8).

A comparison of cell viability over time between styrene liquid and vapour phase batch cultures can be seen in (Figure 5.9) which is indicative of the selective environment that is induced on the population from both phases of styrene. The styrene selective environment in the culture with styrene in the vapour phase could be maintained over time, as evidenced by the decrease in *E. coli* BW25113 cell viability (Figure 5.9).

5.2.4 Liquid phase styrene as a selection pressure in continuous cultures

Chemostat culturing was among the ALE methods that were considered. Previous experiments highlighted the issues with the maintenance of a constant aqueous phase styrene concentration in batch culture, with styrene added in liquid phase (5.2.2). At this stage, running a chemostat culture with a constant flow of styrene to maintain aqueous concentration, appeared to be the best option. An experiment was set up to run a chemostat in the presence of the *E. coli* BW25113 cell biomass and measure the aqueous concentration of styrene over time. The chemostat was set up to comprise of a continuously stirred tank reactor and a styrene pump, which could deliver a constant flow of styrene to the culture. A chemostat *E. coli* BW25113 culture was started in batch and grown to a max cell density of 1.5 (Figure 5.11). After 11 h the MSX medium flow pump was switched on at a dilution rate of 0.1 h^{-1} (Figure 5.11). The styrene pump was then switched on after three consecutive OD_{600} readings were taken over 12 h at a flowrate of 0.5 mL h^{-1}, which corresponded to a styrene aqueous concentration of 1.4 mmol L^{-1} (Appendix C). Four *E. coli* BW25113 cell samples were taken from the chemostat in the 42-h period at regular time points. The styrene extracted from those samples was analysed by GC-MS.

The extracted styrene concentration remained stable and did not decrease over the course of the chemostat run (Figure 5.11). This confirmed that performing ALE with liquid phase styrene in chemostat culture allow greater analytical control over the concentration of styrene than in batch culture (Figure 5.6; Figure 5.11). The results show, ALE with liquid phase styrene in chemostat culture was better at providing a constant styrene selective environment and it was therefore chosen as the ALE method to be used in this study.

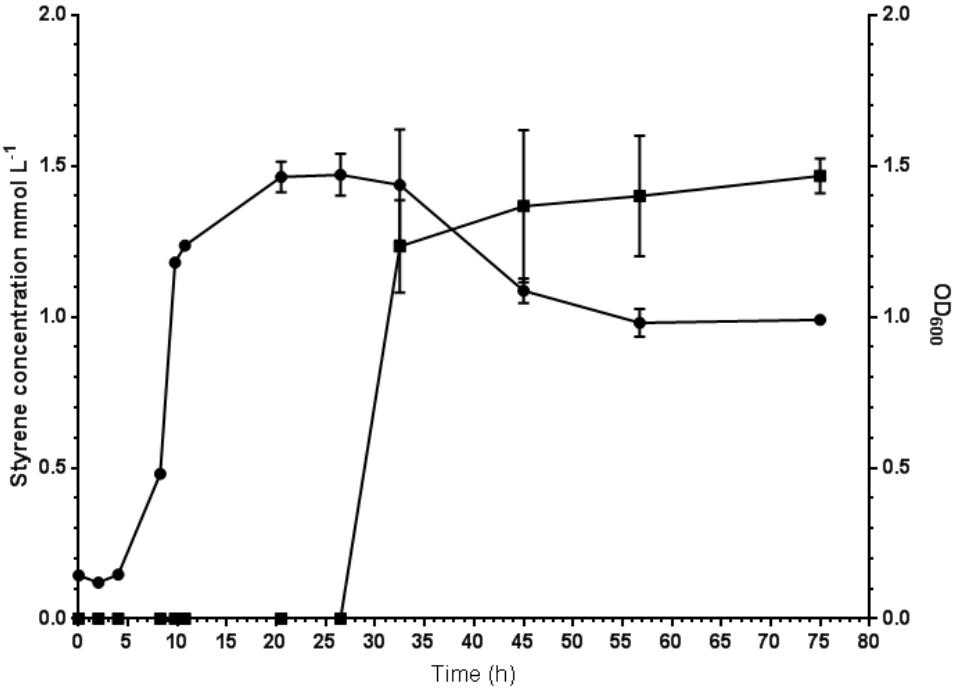


Figure 5.10 Measurement of styrene concentration over time in an *E. coli* chemostat with a pump that administered styrene at a constant flowrate of 0.5 ml h^{-1}. The styrene pump was switched on after 32.5 h.
Styrene concentration in mmol L^{-1} (■)
E. coli cell OD_{600} (●)
The experiments were conducted in triplicate.

5.2.5 The effect of styrene on *E. coli* BW25113 cells

This experiment aims to determine the experimental parameters that can identify an *E. coli* BW25113 styrene tolerant mutant in a styrene tolerance assay. As results from 5.2.2 showed, single batch cultures with styrene in liquid phase can be used to determine the effect of styrene on growth.

It appeared that the events that were occurring in the lag phase to reinitiate growth, were not because of spontaneous mutation. Therefore, to observe an adaptive mutagenic event it was necessary to observe the growth rate in shorter time, at a styrene concentration that would not extend the lag phase. An experiment was set up with the aim to measure the effect of styrene on growth rate at a concentration range less than the reported toxicity threshold (2.9 mmol L^{-1}) (McKenna & Nielsen, 2011).

An overnight seed culture containing wildtype *E. coli* BW25113 was used to inoculate an *E. coli* BW25113 growth tolerance assay batch culture and grown to $OD_{600} \approx 0.25$. Styrene liquid was then added to the culture to achieve a final concentration within a

range of 2.3 - 2.9 mmol L^{-1}. This range was chosen to represent concentrations that are in short range of the reported toxicity threshold concentration (2.9 mmol L^{-1}) (McKenna & Nielsen, 2011). OD_{600} readings were taken at regular time intervals from when the styrene was added for a total time of 5 h.

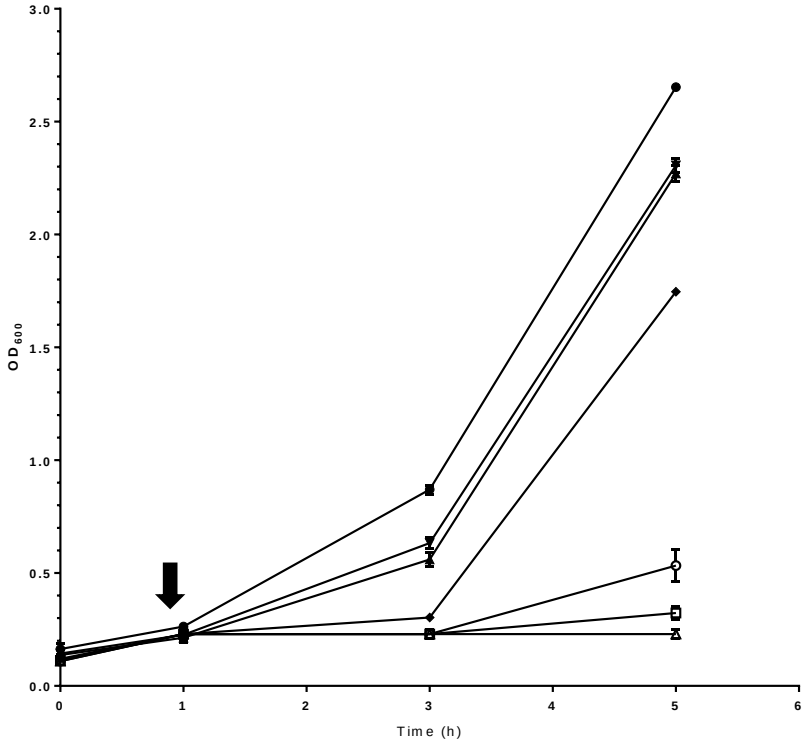


Figure 5.11 Effect of styrene concentration on the growth of *E. coli* BW25113. Growth of *E. coli* of in MSX minimal medium (50 mL) containing Styrene at
Control 0 mmol L^{-1} (●), 2.3 mmol L-1 (▲), 2.4 mmol L^{-1} (▼), 2.6 mmol L^{-1} (♦), 2.7 mmol L^{-1} (○) 2.8 mmol L^{-1} (⧋) 2.9 mmol L^{-1} (△) using conical shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Styrene was added at the time-point highlighted by the arrow.

Strain	Styrene concentration mmolL^{-1}	Maximum cell OD_{600} reached	Specific growth rate h^{-1}
E. coli BW25113 wildtype	0	2.65 ± 0.006	0.58 ± 0.014
	2.3	2.27 ± 0.034	0.59 ± 0.024
	2.4	2.31 ± 0.031	0.58 ± 0.030
	2.6	1.75 ± 0.012	0.51 ± 0.020
	2.7	0.53 ± 0.072	0.21 ± 0.041
	2.8	0.32 ± 0.031	0.09 ± 0.046
	2.9	0.23 ± 0.02	0

Table 5.4 Growth kinetic parameters of *E. coli* at 2.3 – 2.9 mmol L^{-1} styrene concentrations.

The results of the styrene tolerance assay batch culture showed growth to be slowed down at styrene concentrations between 2.2 - 2.6 mmol L^{-1} (Figure 5.9). Above 2.6 mmol L^{-1} styrene concentrations, *E. coli* BW25113 growth was completely inhibited due to an extended lag phase of 2 h after styrene addition. There was also a large difference in specific growth rate between styrene concentration 2.6 and 2.7 mmol L^{-1} which was 0.51 ± 0.020 and 0.21 ± 0.041 respectively (Table 5.4). The styrene toxicity threshold concentration could therefore be defined in this study at 2.7 mmol L^{-1}, because at 2.6 mmol L^{-1} styrene concentration the *E. coli* BW25113 growth lag was not seen (Figure 5.9).

Next, styrene toxicity was estimated as a function of *E. coli* BW25113 cell viability rather than its effect on *E. coli* BW25113 growth. The method used to determine *E. coli* BW25113 cell viability was measuring the viable count of *E. coli* BW25113 cells over time at a styrene concentration of 2.7 mmol L^{-1}. This concentration was chosen because it had been identified as the toxicity threshold concentration of styrene on *E. coli* BW25113 in this study. An overnight seed culture was again used to inoculate a batch culture, which was grown to OD600 ≈ 0.25 before styrene addition.

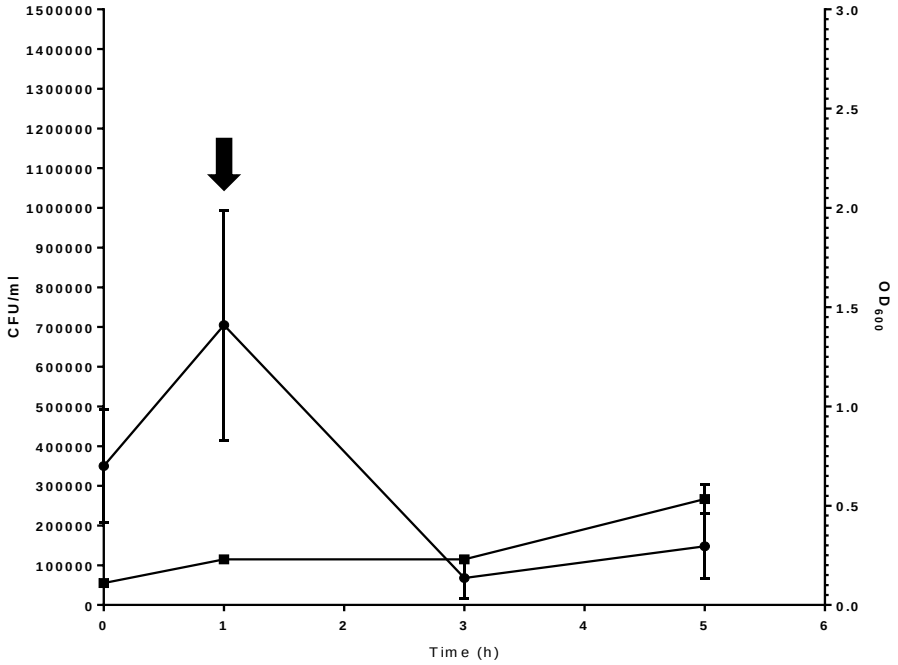


Figure 5.12 Effect of styrene concentration on the cell viability of *E. coli* BW25113. *E. coli* in MSX minimal medium (50 mL) containing Styrene at 2.7 mmol L^{-1} Viable count (●) OD_{600} (■). The experiments were conducted in triplicate. Styrene was added at time-point highlighted with a black arrow.

Samples were taken from the batch cultures at 2 h time intervals after styrene addition. Figure 5.10 shows the *E. coli* BW25113 viable count over time at 2.7 mmol L^{-1} styrene concentration. The results revealed a great decrease in the *E. coli* BW25113 viable cell count at 2.7 mmol L^{-1} styrene within 2 h after styrene addition. (Figure 5.10).

From the results, it was possible to determine the experimental parameters to identify an *E. coli* BW25113 styrene mutant in a batch culture after the addition of liquid phase styrene. It was defined as observed growth within 2 h, after styrene in the liquid phase was added to a batch culture to final concentration of 2.7 mmol L^{-1}. This was later used as a screening parameter in Chapter 6 to isolate mutants with enhanced tolerance to styrene.

5.2 Conclusion

The conclusions from this chapter outlined the ALE methods and parameters that could be used to generate an *E. coli* BW25113 styrene tolerant mutant, under a constant styrene selective environment. A styrene selective environment can be defined in this context as providing a constant aqueous phase concentration of styrene over time.

Batch sub-culturing and chemostat culturing, where styrene is added in the liquid phase, were considered as potential ALE methods to be used in this study.

It was concluded that batch sub-culturing with liquid phase styrene was not a method to be used in this study. This is because the results suggested that the presence of *E. coli* BW25113 cells influenced the styrene aqueous phase concentration in the batch culture over time. This effect could be due to the highly hydrophobic nature of styrene interacting strongly with the hydrophobic cores of the *E. coli* BW25113 membranes (McKenna & Nielsen, 2011). This could be also due to the fact that styrene gets degraded / metabolised by *E. coli* (Panke et al., 2000). In either case, this would have made performing repeated batch sub-culturing tests, over many generations very difficult for the analyst; as they would have had to ensure they were sub-culturing cells within a time window where the styrene selective environment was sustained.

Single batch culture tests with liquid phase styrene were suitable however to measure the effects of styrene on *E. coli* growth. Results from these test defined the *E. coli* BW25113 styrene toxicity threshold concentration in this study as 2.7 mmol L^{-1}. The addition of liquid phase styrene to a final concentration of 2.7 mmol L^{-1} induced

an extended lag phase of 2 h on a growing population of *E. coli* BW25113 wild type cells. Styrene tolerant mutant *E. coli* cells could be identified by growth within 2 h in the presence of 2.7 mmol L^{-1}.

E. coli BW25113 cell viability in batch culture, with styrene in liquid and vapour phase, was compared to assess the stability of styrene tolerance selection. The addition of an evaporating volume of styrene that supplied styrene in the vapour phase decreased cell viability over time, in comparison to the same volume of styrene liquid added directly to the medium. This method was chosen to be used as a screening technique to isolate *E. coli* tolerant mutants in this study.

Finally, the aqueous styrene concentration was measured in an ALE chemostat culturing method, where a constant flowrate of styrene liquid was fed to a steady state *E. coli* chemostat. The aqueous concentration of styrene remained constant over the duration of the experiment. The styrene ALE chemostat method was therefore successful in maintaining a styrene selective environment conducive for the selection of a tolerant mutant and was chosen as the ALE method to be used in this study.

Chapter 6

The application of Adaptive Laboratory Evolution to enhance *E. coli* tolerance to styrene

6.1 Introduction

This chapter reports on the attempt to generate and isolate styrene tolerant *E. coli* BW25113 mutants using ALE. Chemostat culturing was chosen as the ALE method over batch sub-culturing, based on the results from chapter 5 which revealed that selection for styrene tolerance is more easily maintained in chemostat culture. Findings from chapter 5 also highlighted the methods used to isolate *E. coli* styrene tolerant mutants and the growth parameters for mutant detection in tolerance assays. Supplying vapour phase styrene in batch culture was the method used to isolate styrene tolerant mutants, from the *E. coli* BW25113 ALE chemostat cell populations. Enhanced styrene tolerance was characterised as the detection of growth within 2 h at 2.7 mmol L^{-1} styrene concentration (5.2.4). Figure 6.1 outlines the experimental plan used in this chapter.

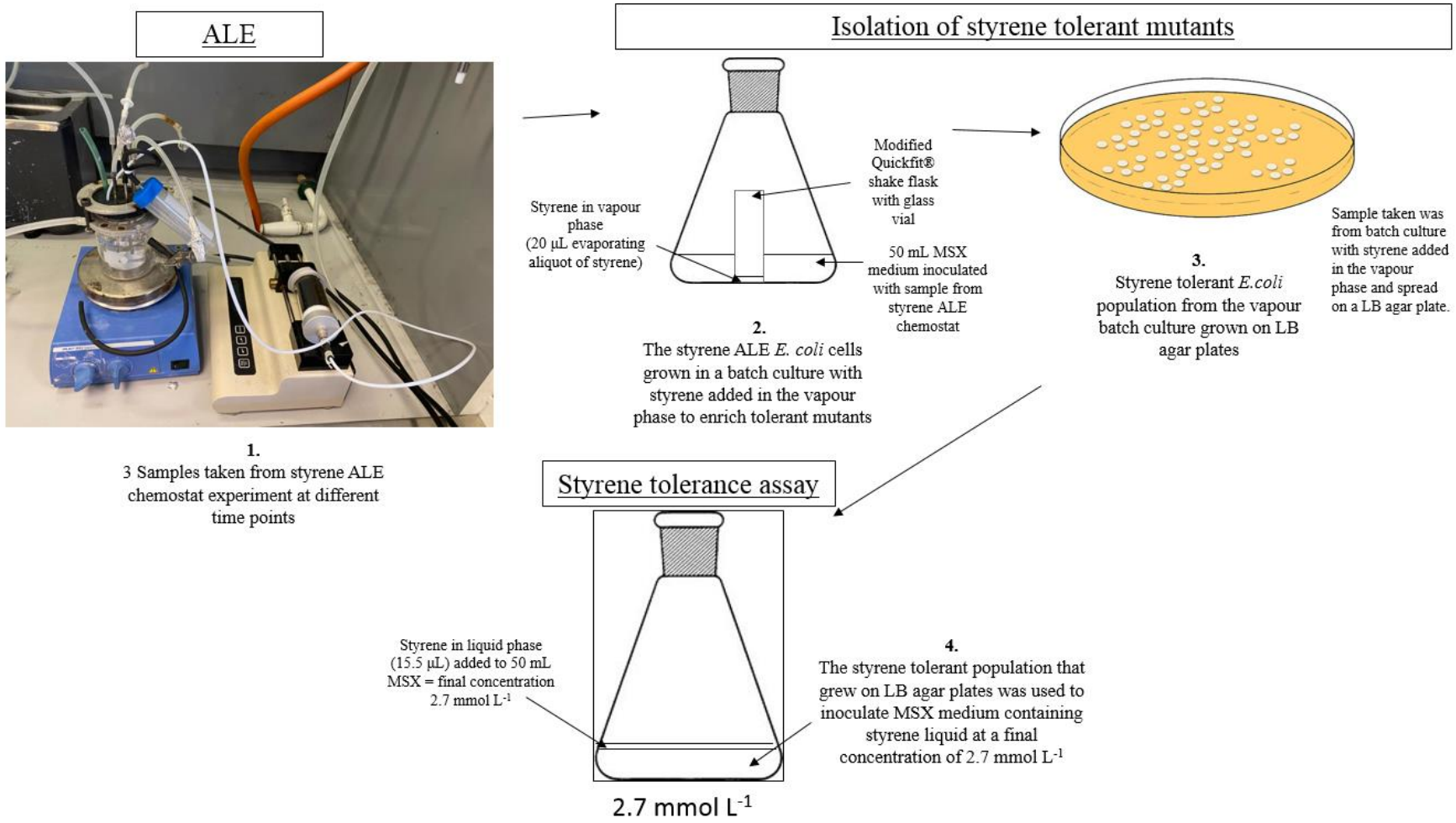


Figure 6.1 Summary of the experimental plan used in the ALE and mutant isolation in chapter 5. Showing the 1) ALE styrene evolution chemostat culture. 2 & 3) Styrene tolerant mutant isolation. 4) Styrene tolerance assay.

6.2 Results and Discussion

6.2.1 ALE of the *E. coli* BW25113 cells with an increasing concentration of styrene

The ALE experiments were carried out under aerobic conditions in a stirred chemostat bioreactor using an MSX medium supplemented with 1 g L^{-1} glucose. This concentration of glucose was confirmed as the limiting nutrient because it was the highest glucose concentration that led to a decrease in *E. coli* biomass yield (Appendix E). The system was maintained at steady state with a constant volume of 57 mL and a dilution rate of 0.1 h^{-1}. Steady state was defined as at least 2 consistent OD_{600} readings obtained over a 2.1 day time period which accounts for 5 x volume changes in the reactor vessel. A syringe pump controlled the flow - rate of styrene into the bioreactor, ranging from 0.6 – 1.6 mL h^{-1}. Previous calibration data was used to calculate the aqueous styrene concentration from the flowrate of the syringe pump (Appendix C). The styrene concentration in chemostat was increased stepwise from 1.7 – 4 mmol L^{-1} each time steady state was established at a given concentration.

The ALE chemostat with increasing concentration of styrene (Figure 6.2) was started off as a batch culture and grown up to a cell OD of 1.32. After steady state was established, styrene was introduced into the chemostat at a flow-rate of 0.6 mL h^{-1}, which corresponded with an aqueous phase concentration of 1.7 mmol L^{-1}, which was kept constant from day 2 – 5 (Figure 6.2). Within this 3-day period there was an initial decline in OD_{600} followed by a period of recovery (Figure 6.2). This was unexpected because in batch culture, the *E. coli* growth was relatively unaffected at a styrene concentration of 2.2 mmol L^{-1}. The styrene flowrate was increased to a styrene chemostat concentration of 3.15 mmol L^{-1} on days 6-8, and a dramatic decrease in cell concentration was observed.

Cell growth did not recover until styrene concentration was decreased back to 1.7 mmol L^{-1} between days 9-12. After this recovery, the styrene concentration was increased by smaller increments from 1.7 to 3.15 mmol L^{-1}. At each concentration increment there was a period of decrease in *E. coli* cell concentration, followed by the subsequent recovery to a steady cell density. The pattern of adaptation was most distinct at 2.1 mmol L^{-1} styrene concentration, where the

re was a characteristic dip in cell density followed by recovery back to pseudo steady state over course of 6 days (Figure 6.2). The most stable steady state achieved was at styrene concentration 2.1 mmol L^{-1} where stable cell OD_{600} readings were

observed for 3 consecutive days which occurred after a previous dip in cell density (Figure 6.2). Despite the trend in *E. coli* cell density decline and then subsequent growth being witnessed, overall, the steady state cell concentration declined between styrene concentrations 2.4 – 4 mmol L^{-1}. At 4 mmol L^{-1} the *E. coli* cells were washed out of the system, evidenced by the constant decline in cell OD_{600} without any recovery. The chemostat run was stopped on day 40 when the styrene concentration in the chemostat was 4 mmol L^{-1} (Figure 6.2). It is likely that the fall and rise in cell density after increase in styrene concentration could be due to the selection of strains with the ability to tolerate styrene, and grow at a rate equal to or greater than the dilution rate (Dragosits & Mattanovich, 2013; W. Harder et al., 1977).

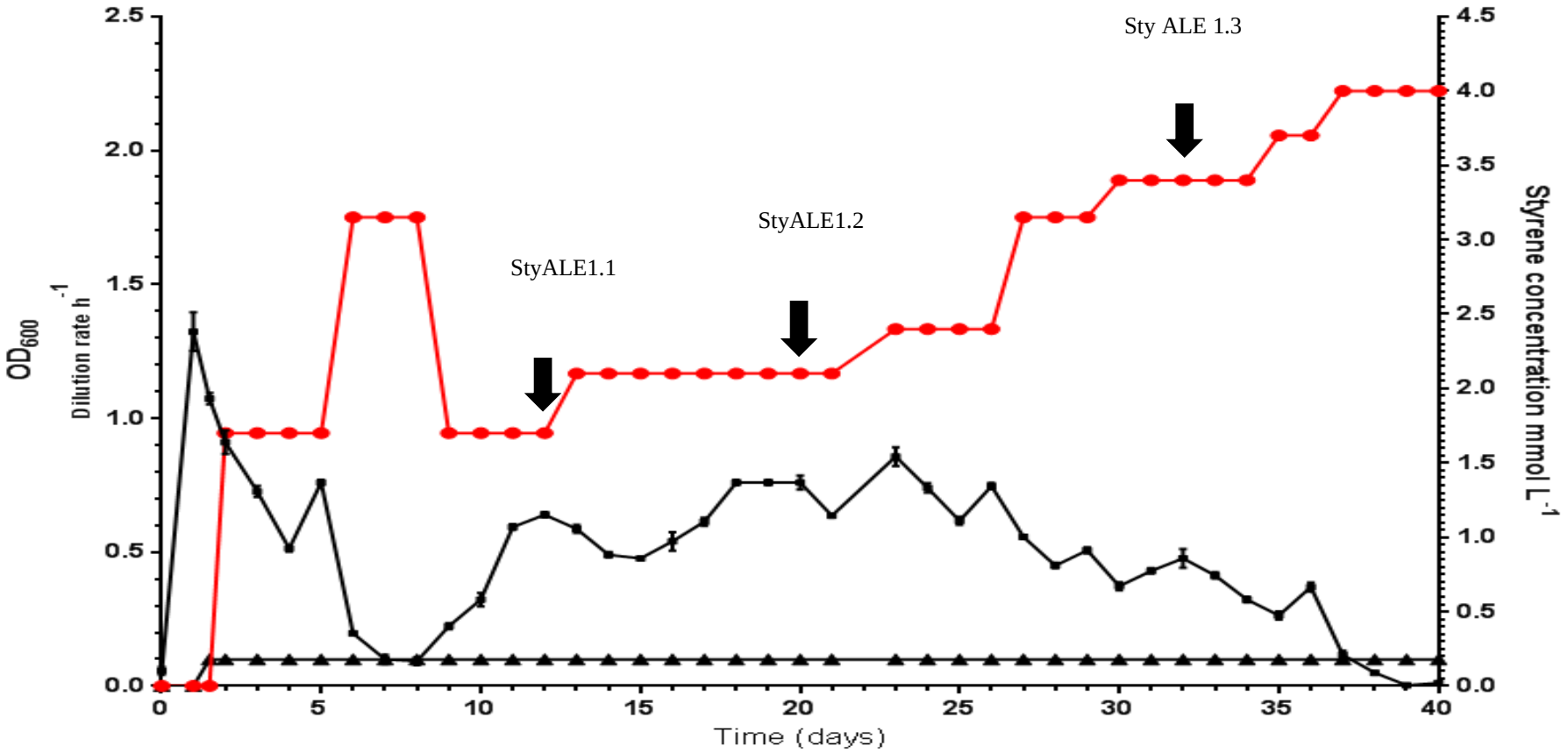


Figure 6.2. ALE and selection of styrene tolerant *E. coli* BW25113 in a chemostat with an increasing concentration of styrene. A Chemostat culture of *E. coli* in a mini-bioreactor (50 mL working volume) was grown in MSX minimal medium containing 1 g L^{-1} glucose at 37°C and aeration rate of ~ 0.2 L h^{-1} with styrene concentration (●) gradually increased from 1.7 to 4 mmol L-1 and dilution rate (▲) 0.1 h^{-1}. Cell density (■) was reported as OD at 600 nm. Samples for mutant isolation were taken at various points highlighted by the black arrows.

6.2.2 Isolation of styrene tolerant mutants from ALE chemostat with increasing concentration of styrene

During the ALE chemostat with increasing concentration of styrene, the *E. coli* cells were sampled from the chemostat culture on day 12, 20 and 32 named Sty ALE 1.1, 1.2 and 1.3 respectively, as marked by arrows on Figure 6.2. Sty ALE 1.1 – 1.3 samples were used to inoculate 50 mL MSX batch cultures in modified Quickfit® shake flasks, with a 20 μL evaporating aliquot of styrene (Figure 6.1). This method was the screening technique from 5.2.3 using vapour phase styrene as a selective pressure in batch culture; intended to achieve an aqueous styrene concentration that would enrich the chemostat population and yield viable cells that were styrene tolerant.

A control *E. coli* BW25113 batch culture that had not under-gone ALE was prepared in the same way for comparison. The inoculated batch cultures were sealed and incubated at 37 °C for 24 h. After 24 h, all the *E. coli* cultures that were inoculated from the ALE chemostat with increasing concentration of styrene, grew under a styrene vapour selection, and achieved a maximum cell density as highlighted in (Table 6.1). A sample from the ALE chemostat with increasing concentration of styrene culture and the control was spread on LB agar plates and incubated at 37 °C (Figure 6.1;6.3). Viable colonies were present on the LB agar plates from the ALE chemostat with increasing concentration of styrene samples, after growth under styrene vapour, whilst the control cells formed no viable colonies (Figure 6.3). The colonies observed had variable morphology and size most likely due to variation among liquid culture replicates.

Strain	Viable cells after styrene vapour screen.	Maximum cell OD_{600} reached in tolerance assay (2.7 mmol L^{-1} styrene)	Specific growth rate h^{-1} in tolerance assay (2.7 mmol L^{-1} styrene)
Control	-	0.53 ± 0.072	0.32 ± 0.016
Sty ALE 1.1	+	0.51 ± 0.012	0.32 ± 0.004
StyALE 1.2	+	0.51 ± 0.010	0.31 ± 0.014
StyALE 1.3	+	0.28 ± 0.010	0.19 ± 0.011

+ Viable colonies observed – No viable colonies observed

Table 6.1 A table summarising the mutant isolation results of the *E. coli* BW25113 wildtype and Sty ALE chemostat 1 samples.

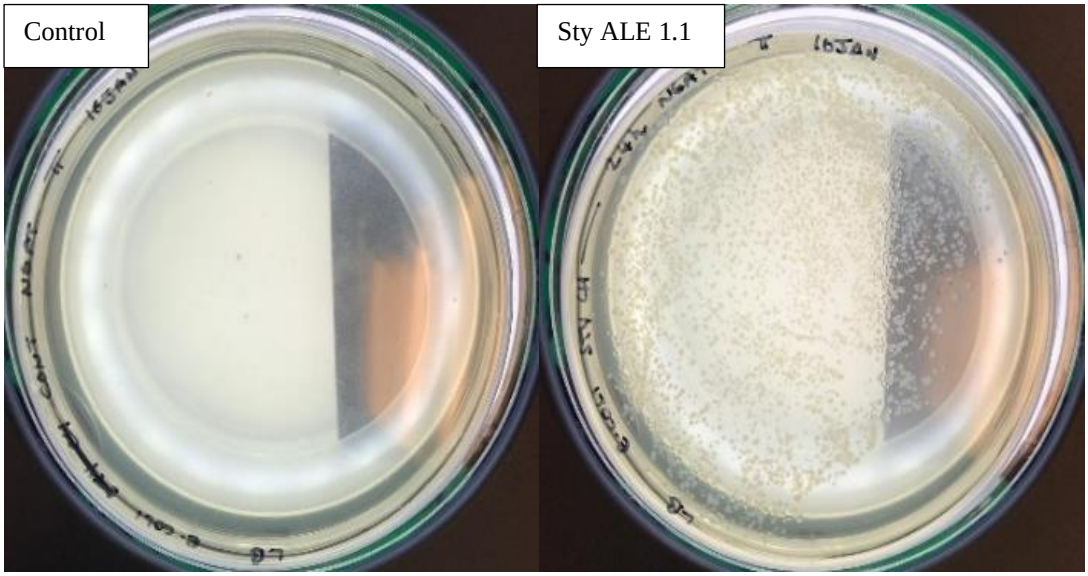


Figure 6.3 Photograph LB agar plates used to test *E. coli* survival after growth under 3.4 mmol L^{-1} styrene vapour. An aliquot was taken from the cell cultures of Sty ALE 1 *E. coli* cell cultures grown in modified shake flasks with 20 µL styrene added to the central well. 20 µL of the culture was spread on LB agar plates and incubated at 37 °C for 24 h. Pictured above are LB agar plates of the control and Sty ALE 1.1. *E. coli* growth on LB plates was observed in the styrene chemostat samples but are not pictured.

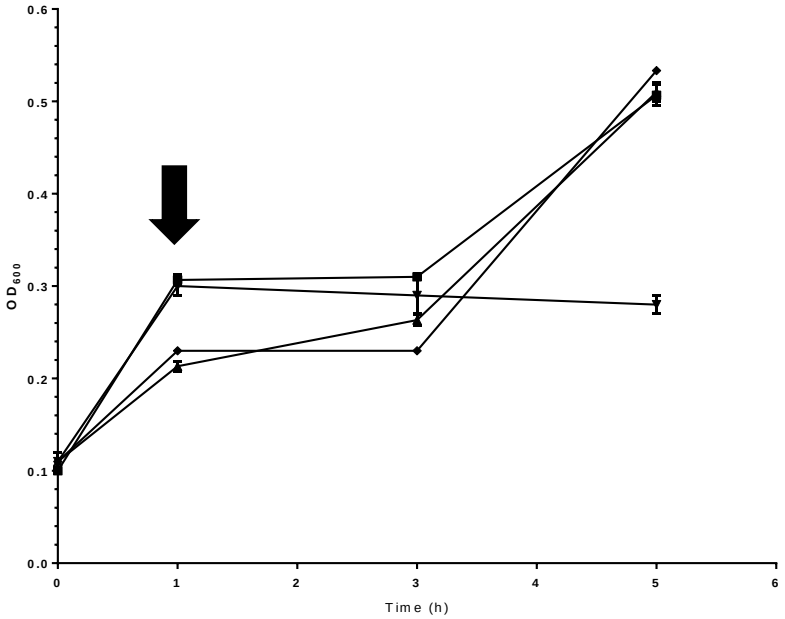


Figure 6.4 Styrene tolerance assay batch culture on samples from Sty ALE 1 observing the effect of 2.7 mmol L^{-1} styrene concentration on the growth of *E. coli.* Control (♦) Sty ALE 1.1 (■) Sty ALE 1.2 (▲) Sty ALE 1.3 (▼) using conical shake flasks (250 mL) at 37°C 250 RPM. The experiments were conducted in triplicate. Styrene was added at the time point highlighted by the arrow.

The viable colonies observed on the LB agar plates from the ALE chemostat with increasing concentration of styrene samples were tested for tolerance. The growth of the *E. coli* population isolated on the LB agar plates was tested in a styrene tolerance assay at 2.7 mmol L^{-1} styrene concentration. As defined in chapter 5, a styrene tolerant mutant could be identified if growth was detected within a 2 h time period from when styrene was added to a final concentration of 2.7 mmol L^{-1} (Figure 6.1). These mutant growth parameters were determined in 5.2.4 when tests were conducted to characterise the effect of styrene on the *E. coli* cells. To accelerate isolation the *E. coli* cell population consisting of several *E. coli* colonies were taken from the Sty ALE 2.1- 2.3 LB agar plates and used to inoculate fresh MSX medium of a 50 mL batch culture. The batch cultures were grown up to $OD_{600} \approx 0.25$ and at this point liquid styrene was added to make a final concentration of 2.7 mmol L^{-1}. OD_{600} readings were taken every 2 h for a total time of 5 h. The results from the styrene tolerance assay batch culture is shown in (Figure 6.4). Sty ALE 1.1 and 1.2 samples displayed similar growth and failed to display enhanced styrene tolerance in comparison to the control (Figure 6.4; Table 6.1). Sty ALE 1.3 did not grow at all in the duration of the experiment, which was surprising because the sample was taken when the aqueous styrene concentration in the chemostat was 3.4 mmol L^{-1} (Figure 6.2; Table 6.1) which inferred it was tolerant to this concentration of styrene. This may have happened due to the presence of a weakened mutant population due to prolonged exposure to a lethal amount of styrene chemical stress (Swings et al., 2017)

6.2.3 ALE of the *E. coli* BW25113 cells with a constant flowrate of styrene

An ALE chemostat was set up in the same way as the chemostat in 6.2.1, but the syringe pump administered a constant flowrate of styrene at 1 mL h^{-1}. Previous calibration data was used to calculate the aqueous styrene concentration from this flowrate of the syringe pump (Appendix). The styrene concentration in the chemostat was kept at 3.15 mmol L^{-1}. This approach was used, after the failure to generate styrene tolerance in the *E. coli* cells from the ALE chemostat with increasing concentration of styrene (Figure 6.2). A significant decline in OD_{600} was observed overall at 3.15 mmol L^{-1} styrene concentration in the ALE chemostat with increasing concentration of styrene, despite some small peaks of recovery (Figure 6.2). It was proposed that if steady state could be maintained at 3.15 mmol L^{-1} over multiple volume changes, it might increase the likelihood of generating a styrene tolerant mutant. From day 1-3,

the ALE chemostat with constant flowrate of styrene was started off in batch and then the medium pump switched on at a dilution rate of 0.1 h^{-1} to establish steady state (Figure 6.5). On day 3, the syringe pump was switched at a flowrate of 1 mL h^{-1} which was kept at this flowrate for 29 days which was equal to 14 volume changes.

There was an initial sharp decrease in *E. coli* cell density from steady state OD_{600} what was it to 0.1 between days 2-4. The *E. coli* cell density then remained at 0.1 between days 4-6 and the chemostat was closely monitored during that time to prevent cell washout. From day 7 – 11, the *E. coli* cell density experienced an increase to a cell density of 0.86 which was the highest OD_{600} recorded on this chemostat run (Figure 6.5). However, this was followed by a decrease in OD_{600} from 0.86 to 0.33 between days 12-19 (Figure 6.4). As the decrease in OD_{600} occurred after the first cell sample was taken, it is possible that the sampling at this point may have perturbed the cell growth equilibrium, making it rapidly decrease. The cell density then appeared to recover and fluctuated around $OD_{600} \approx 0.5$ for 12 days (Figure 6.5). During this period the *E. coli* cells appeared to be maintaining a steady rate of growth at the dilution rate set and at 3.15 mmol L^{-1} styrene concentration (Figure 6.5).

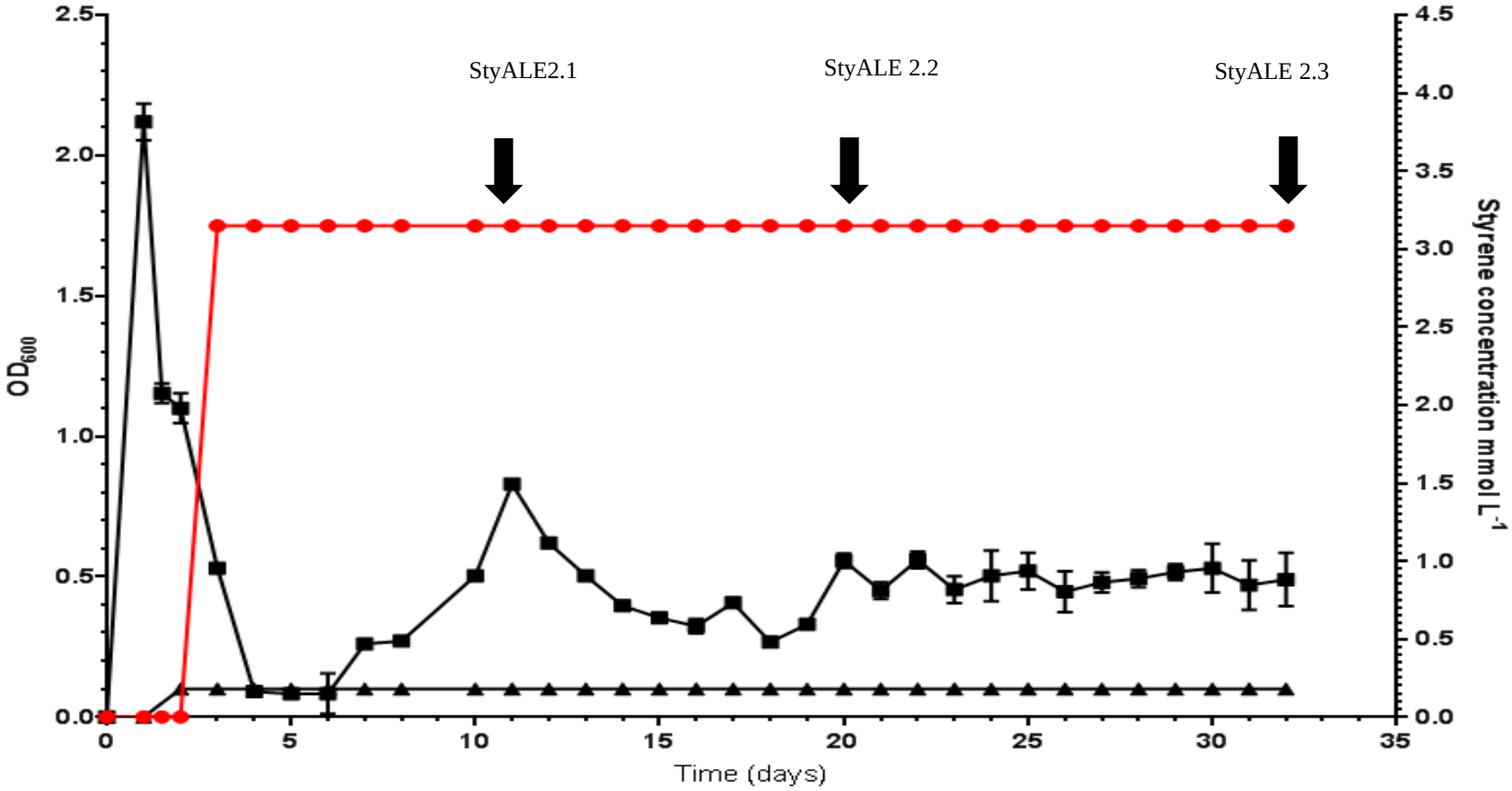


Figure 6.5 ALE and selection of styrene tolerant *E. coli* in a chemostat with a constant concentration of styrene. A Chemostat culture of *E. coli* in a mini-bioreactor (50 mL working volume) was grown in MSX minimal medium containing 1 g L^{-1} glucose at 37°C and aeration rate of ~ 0.2 L h^{-1} with styrene concentration (●) at 3.15 mmol L-1 and dilution rate (▲) 0.1 h^{-1}. Cell density (■) was reported as OD at 600 nm. Samples for mutant isolation were taken at various points highlighted by the black arrows.

6.2.4 Isolation of styrene tolerant mutants from ALE chemostat with constant concentration of styrene

During the ALE chemostat with constant flowrate of styrene, the *E. coli* cells were sampled from the chemostat on day 11, 20 and 33 representing Sty ALE samples 2.1, 2.2 and 2.3 respectively (Figure 6.5). The samples taken from this chemostat were subjected to the same mutant isolation conditions in 6.2.2 (grown in a 50 mL batch culture for 24 h under 20 μL evaporating aliquot of styrene vapour and then plated on LB agar plates).

The results from the ALE chemostat with constant flowrate of styrene were similar to the results of the ALE chemostat with increasing concentration of styrene. The samples grew under styrene vapour selection to maximum cell density and viable colonies grew on LB agar plates (Figure 6.6; Table 6.2). The growth of the *E. coli* population isolated on the LB agar plates was tested in a styrene tolerance assay at 2.7 mmol L^{-1} styrene concentration. Styrene tolerance was again identified by the growth of the *E. coli* cells within a 2 h time period from styrene was added to a final concentration of 2.7 mmol L^{-1} (Figure 6.1). These mutant growth parameters were determined in 5.2.4, when tests were conducted to characterise the effect of styrene on the *E. coli* cells. The *E. coli* cell population consisting of several *E. coli* colonies were taken from the Sty ALE 2.1-2.3 LB agar plate and used to inoculate fresh MSX medium of a 50 mL batch culture.

Strain	Viable cells after styrene vapour screen.	Maximum cell OD_{600} reached in tolerance assay (2.7 mmol L^{-1} styrene)	Specific growth rate h^{-1} in tolerance assay (2.7 mmol L^{-1} styrene)
Control	-	0.53 ± 0.072	0.32 ± 0.016
Sty ALE 2.1	+	0.59 ± 0.085	0.34 ± 0.003
StyALE 2.2	+	0.37 ± 0.028	0.25 ± 0.002
StyALE 2.3	+	0.50 ± 0.014	0.30 ± 0.031

+ Viable colonies observed – No viable colonies observed

Table 6.2 Table summarising the mutant isolation results of the *E. coli* BW25113 wildtype and Sty ALE chemostat 2 samples.

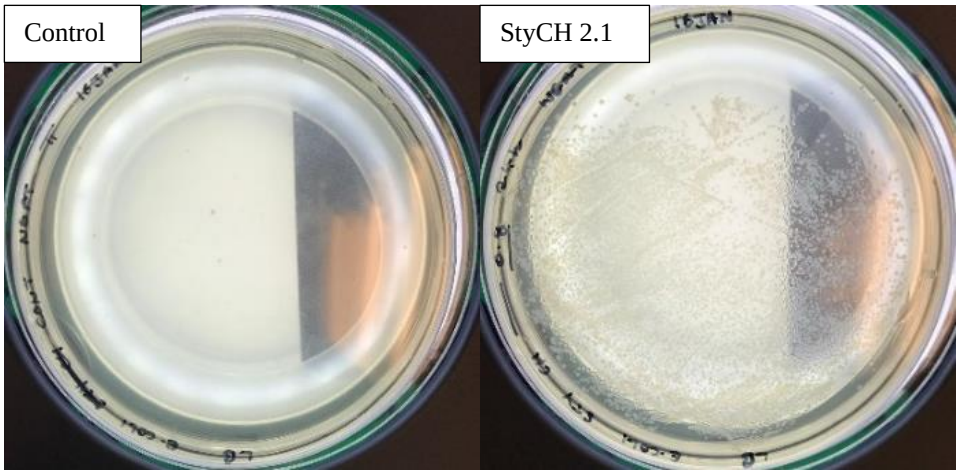


Figure 6.6 Photograph LB agar plates used to test *E. coli* survival after growth under 3.4 mmol L^{-1} styrene vapour. An aliquot was taken from the cell cultures of the Styrene ALE chemostat 2 *E. coli* cell cultures grown in modified Quickfit® shake flasks with 20 µL styrene added to the central well. 20 µL of the culture was spread on LB agar plates and incubated at 37 °C for 24 h. Pictured above are LB agar plates of the control and StyCH 2.1. *E. coli* growth on LB plates was observed in the styrene chemostat samples but are not pictured.

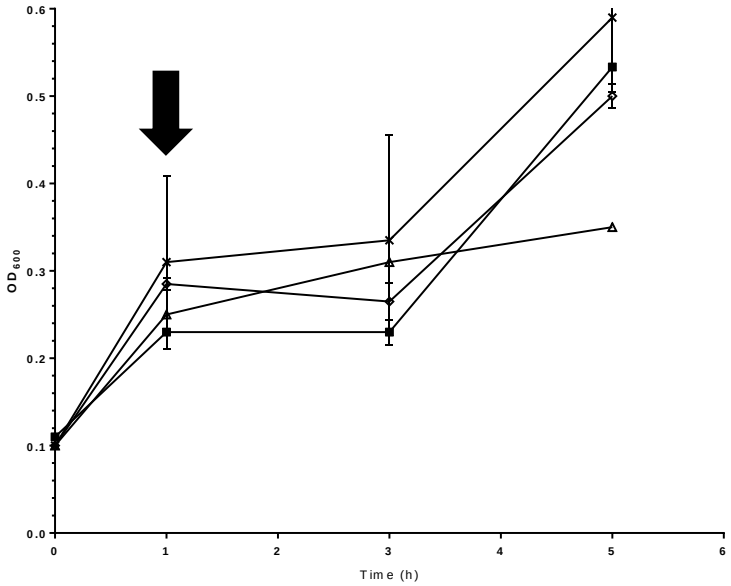


Figure 6.7 Styrene tolerance assay batch culture on samples from Sty ALE 2 observing the effect of 2.7 mmol L^{-1} styrene concentration on the growth of *E. coli.* Control (■) Sty ALE 2.1 (✕), Sty ALE 2.2 (△) Sty ALE 2.3 (◊) using conical shake flasks (250 mL) at 37°C 250 RPM. The experiments were conducted in triplicate. Styrene was added at the time point highlighted by the arrow.

The batch cultures were grown up to $OD_{600} \approx 0.25$ and at this point liquid styrene was added to make a final concentration of 2.7 mmol L^{-1}. OD_{600} readings were taken every 2 h for a total time of 5 h. The results from the styrene tolerance assay batch culture is shown in (Figure 6.7). Sty ALE 2.1 and 2.3 samples displayed similar growth to the

control and failed to display enhanced styrene tolerance (Figure 6.7; Table 6.2). Once again, a sample from the chemostat (Sty ALE 2.3) displayed lower tolerance to styrene than the control and did not growth within 5 h of the addition of styrene at 2.7 mmol L^{-1}.

6.3 Conclusion

In conclusion, the chemostat evolution approach developed in this chapter was not successful in achieving the aim of using ALE to generate and isolate a styrene tolerant *E. coli* mutant. Adaptive growth patterns were seen in the two different methods used to feed styrene into the chemostat, which inferred that styrene tolerant mutants were being generated. However, the *E. coli* culture samples from both the styrene chemostats did not show any improved tolerance in comparison to the control.

The main hypothesis proposed for this failure is because styrene is too toxic to the *E. coli* cells, which do not possess inherent resistance to solvents. The use of a highly toxic chemical as a selection pressure in ALE runs the risk of complete extinction of the population or generating weak mutants, which cannot survive and/or be isolated (Swings et al., 2017). It is possible that the *E. coli* adaptive mutant population were not able to survive or were severely weakened by the presence of styrene. There are two constituents of the styrene compound: the aromatic ring and the vinyl substituent ($CH=CH_2$). The aromatic ring contributes to hydrophobicity related toxicity effects of styrene mainly at the membrane (Mukhopadhyay, 2015). Further toxic action is likely due to the presence of a double bond in the vinyl substituent (Harder et al., 2003; LoPachin et al., 2019; Messer & Petrov, 2013; Schwöbel et al., 2011). Gram-negative bacterial species have been showed to be more sensitive to vinyl phenols than their phenolic acid precursors (Licandro-Seraut et al., 2013). The proposed mechanism of toxicity was that the vinyl derivatives of phenolic compounds did not induce the upregulation of efflux pump systems and therefore accumulated in the cytoplasm (Licandro-Seraut et al., 2013). The double bond on styrene's vinyl may also increase the electrophilic reactivity of the compound towards biological targets that protect the *E. coli* cell against toxicity effects (A. Harder et al., 2003; LoPachin et al., 2019; Messer & Petrov, 2013; Schwöbel et al., 2011).

A potential solution that was proposed, would be to use a two-stage approach to: first adapt the *E. coli* BW25113 strain to evolve resistance to the toxicity effects relating to the hydrophobicity of the aromatic ring and then the vinyl group. The next stage in the study led us to implement this experimentally by conducting the same ALE experiments with a chemical that is structurally similar to styrene but without the vinyl group. Then an additional styrene selection stage was implemented to select for enhanced resistance to the toxicity effects of styrene's vinyl group.

Chapter 7

The application of Adaptive Laboratory Evolution to enhance *E. coli* tolerance to ethylbenzene

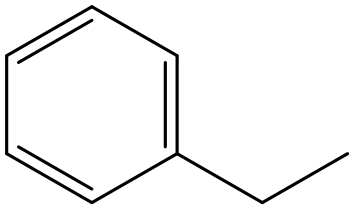
7.1 Introduction

This chapter reports on the two stage ALE strategy used to evolve the *E. coli* BW25113 strain; first to the toxicity effects associated with the hydrophobicity of the aromatic ring; secondly to the electrophilic reactivity of the styrene double bond. Results in chapter 6 shaped the approach of this chapter, as the results failed to generate a styrene tolerant mutant.

A conclusion drawn from Chapter 6 highlighted that styrene may perhaps have additional toxicity effects in *E. coli*, due to the double bond in the vinyl aromatic ring side group. Ethylbenzene was identified as an ideal chemical to use as a selection pressure to evolve resistance to toxicity effects associated with the aromatic ring structure. Ethylbenzene is structurally related to styrene but with a saturated alkyl side chain. Styrene and ethylbenzene compounds are both activated toward electrophilic aromatic substitution because they possess substituents that donate electrons to the aromatic ring (Butler, 2007). However, styrene can be chemically defined as more reactive as there is a greater electron density on the aromatic ring from the donated bonded electron pair, which makes it more readily 'displaced into new bonding patterns' (LoPachin et al., 2019). This potentially contributes to ethylbenzene being less toxic to the *E. coli* BW25113 strain in comparison to styrene.

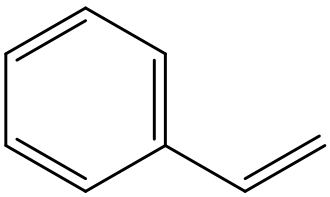
As ethylbenzene has very similar physicochemical properties to styrene, an assumption was made that the developed ALE and mutant isolation methods from chapter 6 could also be applied (Table 7.1). Additional steps were added to the mutant isolation process to facilitate the two stage ALE strategy (Figure 7.1). Ethylbenzene tolerant *E. coli* mutants were isolated from the ethylbenzene ALE chemostat. The ethylbenzene tolerant population were then further evolved under styrene vapour selection to isolate styrene tolerant colonies from within population. Fig 7.1 outlines the experimental plan used in this chapter.

A



ethylbenzene

B



styrene

Figure 7.1 Chemical structures of A) ethylbenzene B) styrene drawn using ChemBioDraw Ultra 14.0 software.

Log P_{ow}	2.9	3.1
Solubility limit in water 25 °C (g L^{-1})	0.30	0.17
Vapour pressure (mmHg)	5	7

Table 7.1 Chemical properties of styrene and ethylbenzene. Chemical structures drawn using ChemBioDraw Ultra 14.0 software.

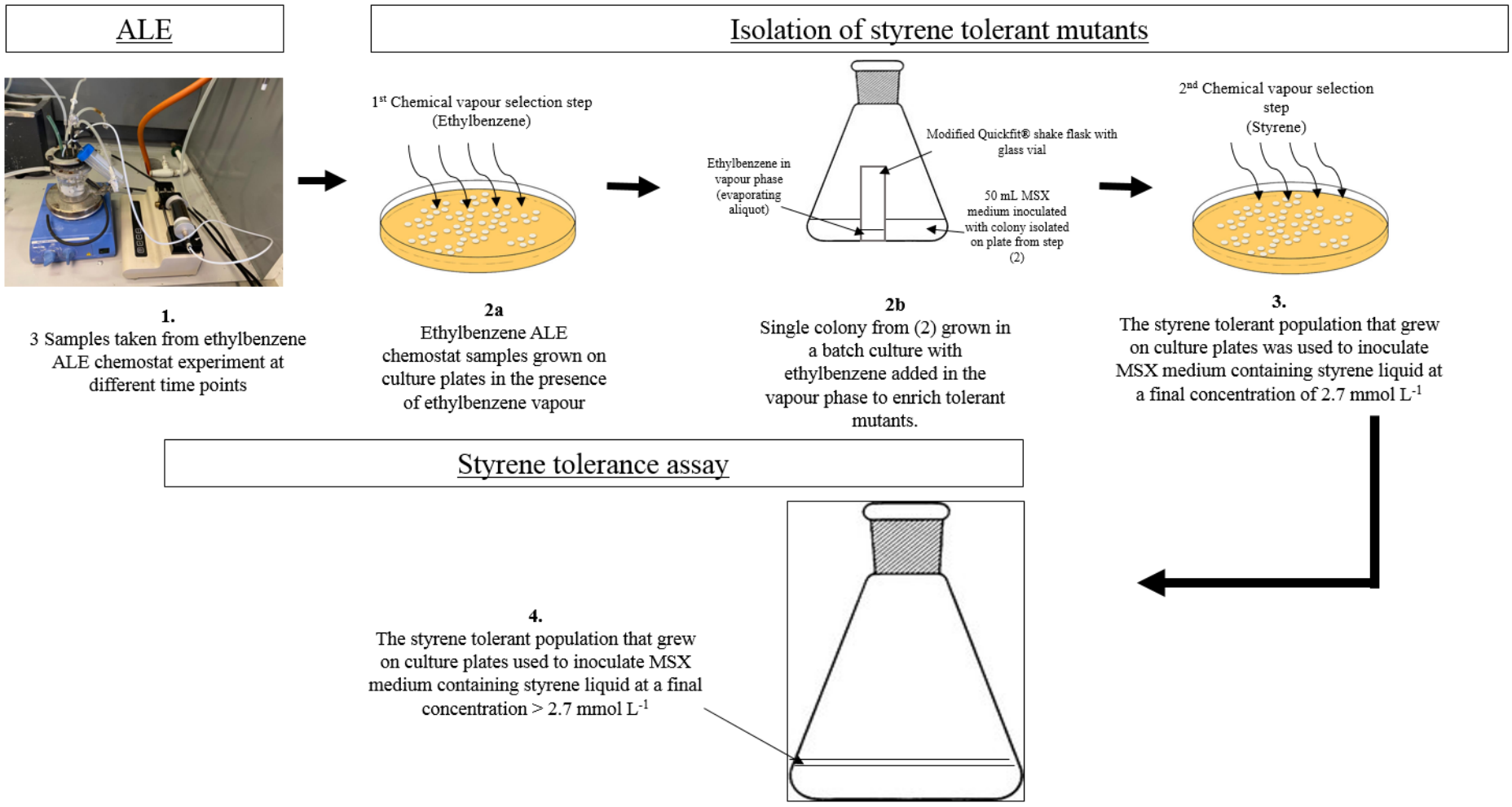


Figure 7.2 Summary of the experimental plan showing the two stage ALE strategy 1) ALE ethylbenzene evolution chemostat culture. 2a/b & 3) Two stage mutant isolation 4) Styrene tolerance assay.

7.2 Results and Discussion

7.2.1 Analytical determination of ethylbenzene in the aqueous phase.

The same method was used to validate the quantitative determination of ethylbenzene as in (5.2.1) Ethylbenzene was extracted with hexane from working standard solutions with ethylbenzene added to a final concentration of 1 – 10 mmol L^{-1}. The range for ethylbenzene was larger due to the higher experimental concentrations used later on. The organic hexane phase was then analysed by GC-MS. The quantitative determination of ethylbenzene was validated, by assessment of how reproducible the ethylbenzene recovery percentage was across the samples.

Ethylbenzene concentration mmol L^{-1}	Recovery (%)
1	26.38 ± 0.048
2	32.10 ± 0.100
4	37.80 ± 0.190
5	37.93 ± 0.229
10	41.85 ± 0.360

Table 7.2 Recovery (%) of ethylbenzene extracted with hexane solvent styrene working standard solutions. 3 x 5 mL hexane extractions were performed on 15 mL sample taken from the ethylbenzene working standard solutions containing 1- 5 mmol L^{-1} ethylbenzene. Percentage recovery = $\frac{[ethylbenzene\ standard\ ]}{ethylbenzene\ \ reference]} \times 100$. The results and standard deviations are the average of triplicate experiments.

The recovery percentage of ethylbenzene extracted from the aqueous phase was relatively low, but reproducible across the range of ethylbenzene working standard solutions (Table 7.1). A standard curve was constructed from the data obtained to quantitatively determine ethylbenzene concentration in the aqueous phase of the batch cultures (Appendix B). A separate calibration was again constructed to quantitatively determine the ethylbenzene concentration in the aqueous phase of the chemostat culture (Appendix D).

7.2.2 The effect of ethylbenzene on *E. coli* BW25113

The aim of this experiment was to characterise the toxicity effects of ethylbenzene on *E. coli* BW25113. The determination of the ethylbenzene toxicity threshold concentration would allow the successful detection of an ethylbenzene tolerant mutant. It was important to characterise this first stage of tolerance in the two stage ALE strategy that was being implemented.

A tolerance growth assay was set up in batch culture to observe the effect of ethylbenzene on *E. coli* BW25113. Liquid phase ethylbenzene was added to 50 mL MSX medium to a final concentration range of 1 – 4 mmol L^{-1}. This concentration range was higher than the concentrations used in the styrene tolerance assay batch culture previously (Chapter 6) because I expected that ethylbenzene would be less toxic to the *E. coli* BW25113 strain than styrene due to the difference in structure. However, it was unknown what the toxicity threshold concentration was, as it had not been previously reported. It was also unknown if ethylbenzene would induce a similar length of extended lag phase as was seen with styrene. The cultures were incubated for 6 h and samples were taken at regular time intervals to measure the OD_{600}.

Batch cultures showed growth to not be inhibited at ethylbenzene concentrations of 2.2 – 3 mmol L^{-1} (Figure 7.2; Table 7.3). At 4 mmol L^{-1} ethylbenzene concentration the *E. coli* specific growth rate was inhibited by > 50% (Table 7.3) which led an extended lag phase of at least 5 h after ethylbenzene addition. This result established the ethylbenzene toxicity threshold to be at 4 mmol L^{-1}, because below this concentration, growth was observed (Figure 7.2; Table 7.3).

In conclusion, the minimum target for an adaptive mutant with enhanced ethylbenzene tolerance would be to display growth at 4 mmol L^{-1} within 5 h after exposure to ethylbenzene.

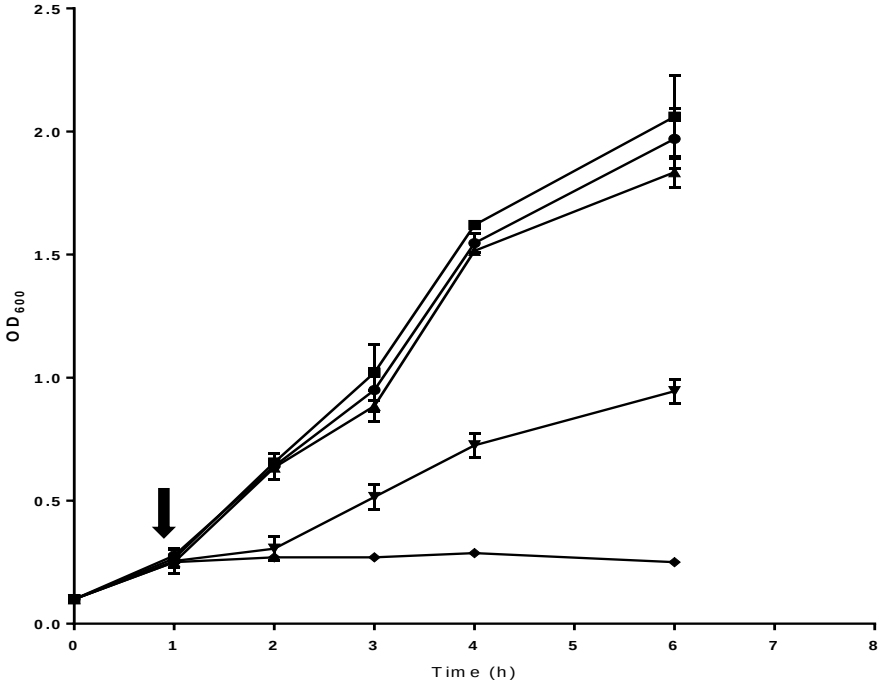


Figure 7.3 Effect of ethylbenzene concentration on the growth of *E. coli*. Growth of *E. coli* of in MSX minimal medium (50 mL) containing ethylbenzene at Control 0 mmol L^{-1} (■)1 mmol L^{-1} (●) 2 mmol L^{-1} (▲) 3 mmol L^{-1} (▼) 4 mmol L^{-1} (♦) using Quickfit® flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Ethylbenzene was added at the time-point highlighted by the arrow.

Strain	Ethylbenzene concentration $mmolL^{-1}$	Maximum cell OD_{600} reached	Specific growth rate h^{-1}
E. coli BW25113 wildtype	0	1.97 ± 0.122	0.68 ± 0.006
	1	2.06 ± 0.169	0.67 ± 0.032
	2	1.84 ± 0.064	0.66 ± 0.033
	3	0.95 ± 0.050	0.46 ± 0.063
	4	0.25 ± 0.006	0.17 ± 0.030

Table 7.3 Growth kinetic parameters of *E. coli* at 1 – 4 mmol L^{-1} ethylbenzene concentrations.

7.2.3 ALE of the *E. coli* BW25113 cells with an increasing concentration of ethylbenzene

ALE experiments with ethylbenzene were carried out in a stirred chemostat using MSX medium supplemented with 1 g L^{-1} glucose under aerobic conditions. The system was maintained at steady state at a constant working volume of 57 mL and a dilution rate of 0.1 h^{-1}. The ethylbenzene chemostat had a syringe pump which controlled the flow of ethylbenzene into the reactor. The flowrate of ethylbenzene going into the chemostat was between 1-3.2 mL h^{-1} which was calibrated to achieve a final concentration range that increased stepwise between 2.3 – 7.2 mmol L^{-1} (Appendix D). These concentrations were above the ethylbenzene solubility limit in water but were reported in mmol L^{-1}, for comparison to other reported toxicity threshold values. The ethylbenzene chemostat was started off as a batch culture and grown up to a cell density of OD_{600} = 3.1. At this point, the medium pump was switched on to set up a steady cell equilibrium (Figure 7.3). After steady state was established, the ethylbenzene syringe pump flow rate was set at 1 mL h^{-1} that set an aqueous phase concentration of 2.3 mmol L^{-1}. The concentration was maintained over a 2.1 day time period, which accounted for 5 volume changes in the reactor vessel (Figure 7.3). Within this period, there was an initial decrease followed by a rapid increase in *E. coli* cell OD_{600}. The ethylbenzene concentration was then increased to 3.4 mmol L^{-1} via increased flow to the chemostat, however there was a sharp decline in OD_{600} with no recovery. The OD_{600} continued to decline over a period of the next 7 days which accounted for 16.8 bioreactor volume changes.

On day 15, the cell density was critically low and so to prevent complete cell washout, the ethylbenzene flow-rate was decreased to lower the aqueous concentration back to a tolerable concentration of 2.3 mmol L^{-1}, which allowed the *E. coli* cells to recover. On day 19, there was a technical fault with the tubing that supplied the flow of media into the chemostat. This fault stopped the flow of medium into the reactor, causing the dilution rate to decrease and as a result the ethylbenzene concentration in the reactor increased. The OD_{600} dropped rapidly to $OD_{600} \approx 0.1$. To re-establish experimental conditions, both the ethylbenzene and medium pumps were switched off allowing the *E. coli* cell density to recover between days 19 -22 (Figure 7.3).

On day 22, the pumps were switched back on, and the ethylbenzene flowrate into the chemostat was set to deliver an aqueous concentration of 2.9 mmol L^{-1}. There was a decrease in the *E. coli* cell density and subsequent recovery which occurred over

a shorter period of 2.5 volume changes. This rapid *E. coli* cell recovery at an ethylbenzene concentration of 2.9 mmol L^{-1} indicated that there may have been tolerant mutants present, which allowed the *E. coli* cells to recover at an accelerated rate. On day 31**,** the ethylbenzene concentration was increased from 2.9 mmol L^{-1} to 3.2 mmol L^{-1} and the subsequent OD response from the *E. coli* cells also increased. This indicated evidence of a faster adaptive response which had not been previously seen when the ethylbenzene concentration was increased (Figure 7.3)

On day 33, the ethylbenzene concentration was further increased to 3.6 mmol L^{-1} and the OD_{600} decreased before remaining steady for 5 days. Between days 41 – 91, ethylbenzene concentration was increased by smaller increments of ≈ 0.45 mmol L^{-1} going from 4.1 – 7.2 mmol L^{-1}. At each ethylbenzene concentration, despite some fluctuation in cell density, steady state cell concentration was established. However, overall there appeared to be an overall net decrease in cell concentration. The ethylbenzene chemostat was stopped after 91 days. At this point, the syringe pump was delivering ethylbenzene to achieve an aqueous concentration of 7.2 mmol L^{-1} (Figure 7.3).

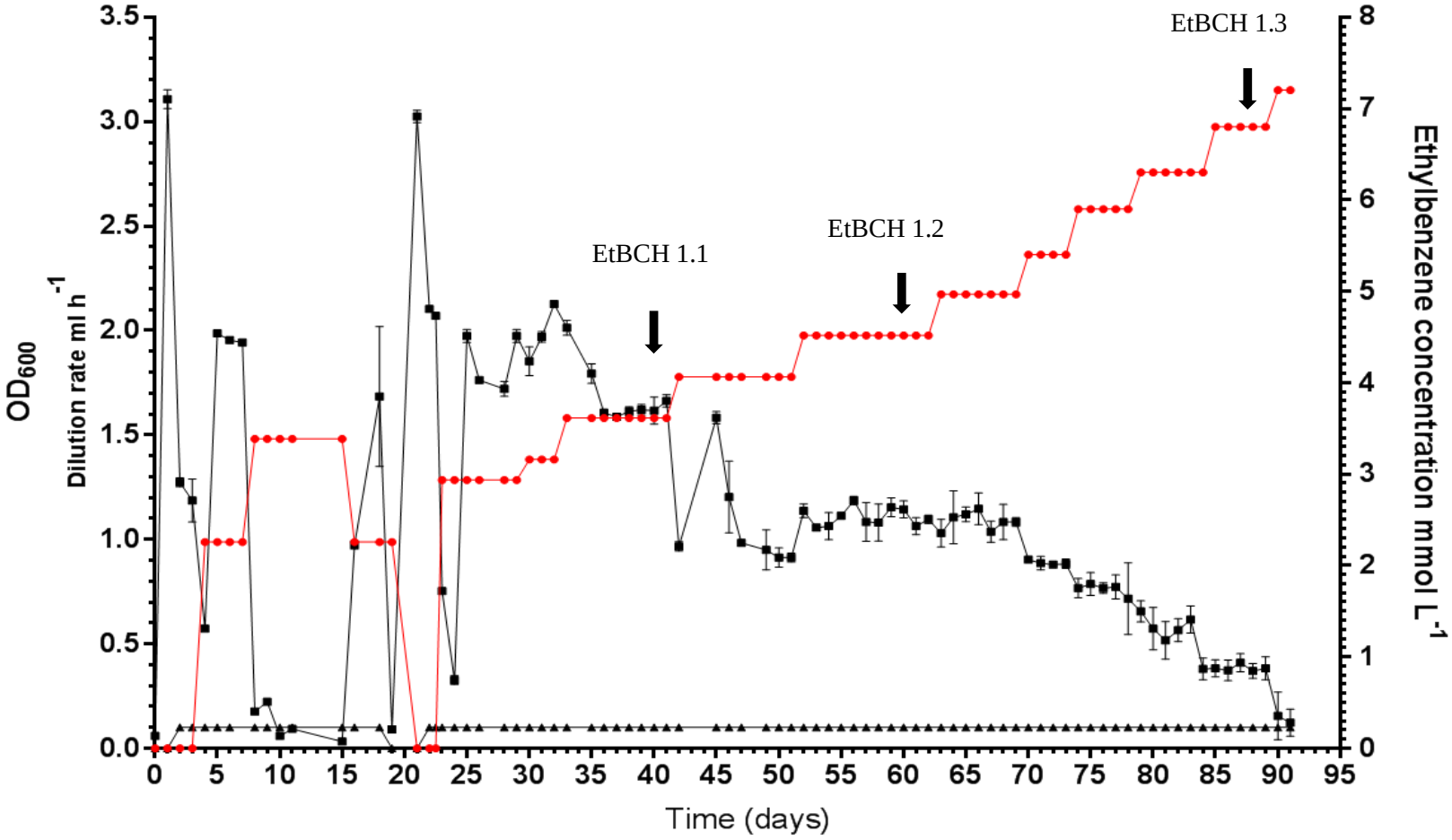


Figure 7.4 ALE and selection of ethylbenzene tolerant *E. coli* in a chemostat. A Chemostat culture of *E. coli* in a mini-bioreactor (50 mL working volume) was grown in MSX minimal medium containing 1 g L^{-1} glucose at 37°C and aeration rate of ~ 0.2 L h^{-1} with ethylbenzene concentration (●) gradually increased from 1.7 to 4 mmol L^{-1} and dilution rate (▲) 0.1 h^{-1}. Cell density (■) was reported as OD at 600 nm. Samples for mutant isolation were taken at various points highlighted by the black arrows.

7.2.4 Isolation of an ethylbenzene tolerant *E. coli* BW25113 mutant

The aim of the experiment was to isolate mutant colony isolates from *E. coli* cell samples taken from the ALE chemostat under ethylbenzene selection (Figure 7.3) and test for tolerance to ethylbenzene. Cell samples EtBCH 1.1, EtBCH 1.2 and EtBCH 1.3 were taken at ethylbenzene concentration 3.6, 4.5 and 6.8 mmol L^{-1} respectively (highlighted by arrows on Figure 7.3). The EtBCH 1.1- 1.3 samples taken from the ALE ethylbenzene chemostat were immediately screened to isolate the ethylbenzene tolerant population.

Figure 7.4 shows a detailed diagram of the mutant isolation process carried out to fulfil the two stage ALE strategy. The method took samples from the evolved population from the ethylbenzene ALE chemostat and isolated the ethylbenzene tolerant population on solid medium in an ethylbenzene vapour saturated environment (Figure 7.4 steps 1-4). A single colony was picked from the plate and used to inoculate a batch culture, which was subjected to more rounds of ethylbenzene vapour selection via batch sub-culturing (Figure 7.4 step 5). Single colony isolates from the ethylbenzene vapour batch culture were isolated on solid medium in the presence of a saturating concentration of styrene vapour (Figure 7.4 step 6). Styrene vapour was used here as the selection pressure to isolate styrene tolerant individuals from the evolved ALE ethylbenzene chemostat population. The single colony isolates from (Figure 7.4 step 6) were picked and used to inoculate a growth competition tolerance assay to test the growth of the isolated mutants at various concentrations of ethylbenzene and styrene (Figure 7.4 step 7).

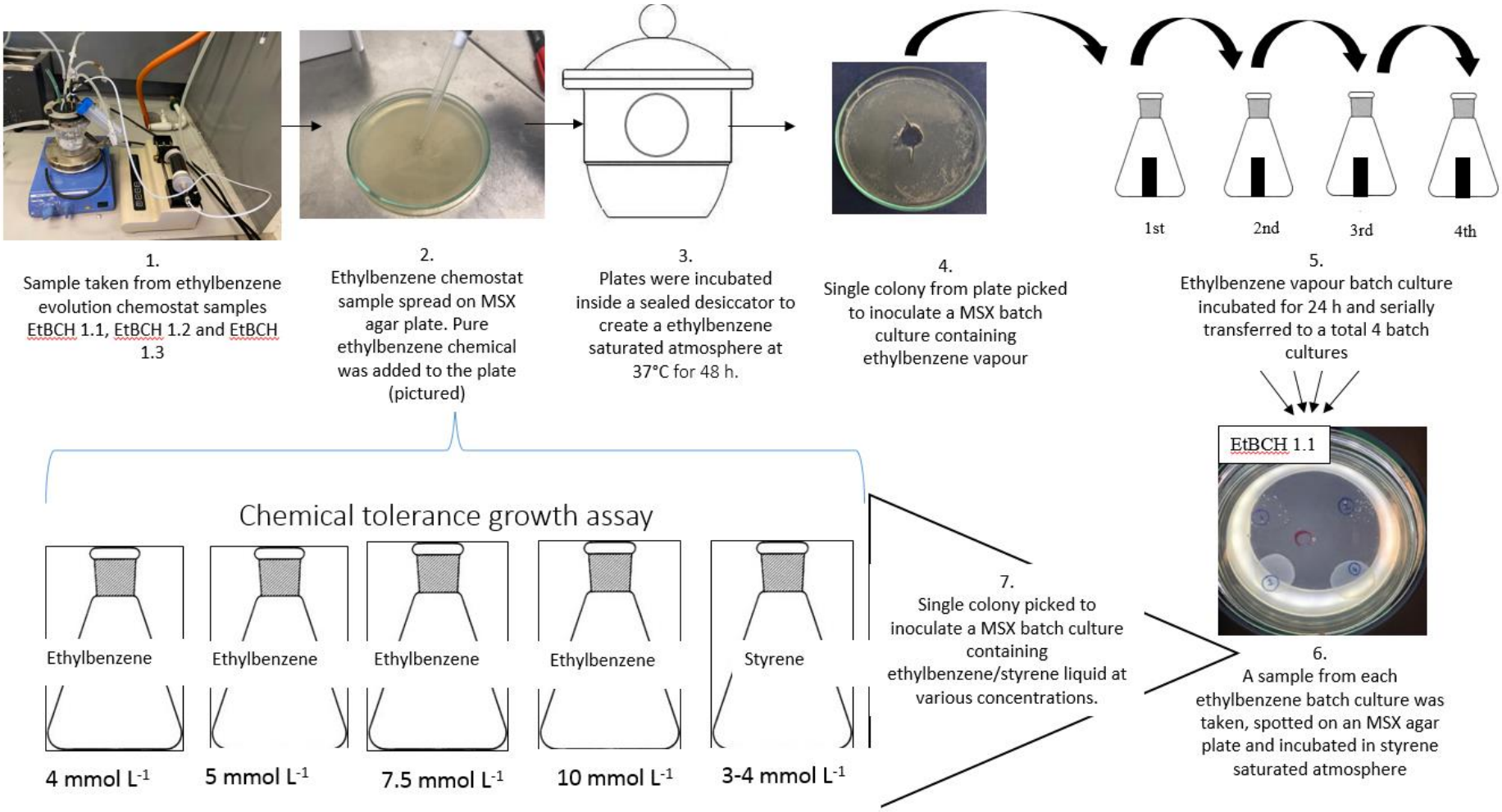


Figure 7.5 Schematic diagram of the ethylbenzene mutant isolation process showing the two stage ALE strategy.

The results following the screening process to isolate ethylbenzene tolerant mutants were as follows. Single colonies from the evolved population samples taken from the ethylbenzene chemostat EtBCH 1.1-1.3 (Figure 7.3) were isolated on MSX agar plates (Figure 7.4 step 2-4). The colony isolates grew in all 4 batch sub-cultures containing ethylbenzene vapour (Figure 7.4 step 5). For comparison, the *E. coli* BW25113 wildtype controls that were subjected to the same conditions did not grow in the presence of ethylbenzene vapour on plates or in batch culture.

The next step (Figure 7.4 step 6) took samples from the 4 ethylbenzene vapour sub-cultures from each colony isolates (EtBCH 1.1-1.3) and plated on a MSX agar plate and incubated at 37 °C under a styrene vapour atmosphere for 48 h (Figure 7.4 step 6). Figure 7.5 shows an image of the MSX plate with *E. coli* growth, spotted 1st – 4th, from all 4 batch subcultures containing styrene vapour. The colony growth and density increased from the 1st – 4th batch culture (Figure 7.5). This indicated that the frequency of the tolerant mutations increased with each cycle of *E. coli* batch sub-culture growth. This experiment suggested that a stable styrene tolerant mutant had been isolated. For comparison, a wild type control sample was also spotted on an MSX plate and grown under ethylbenzene vapour but yielded no growth (Figure 7.5).

Single colonies were isolated from a streak plate of the 4th batch sub-cultures from each sample of EtBCH 1.1-1.3 sample and named mEtBCH 1, mEtBCH 2, and mEtBCH 3. The isolated mutants were tested in ethylbenzene tolerance assays at ethylbenzene concentrations > 4 mmol L^{-1} to monitor growth over 12 h period from ethylbenzene addition. An overnight pure culture of mutant samples mEtBCH 1, mEtBCH 2, and mEtBCH 3 were used to inoculate the MSX medium (50 mL), at a starting $OD_{600} \approx 0.1$. All 3 batch cultures were grown up to $OD_{600} \approx 0.2$ and, at this point, ethylbenzene was added to make a final concentration of between 5 -10 mmol L^{-1}. The batch culture was incubated and OD_{600} readings were taken at regular time intervals. Colonies were observed with variable morphology and size possible due to the growth rate variation in amongst the culture population.

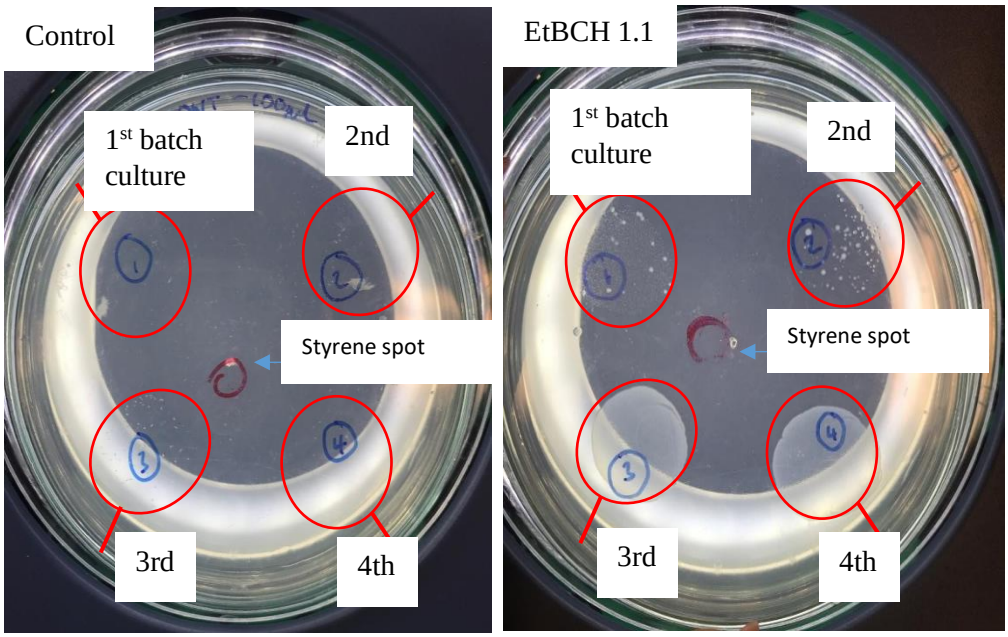


Figure 7.6 Photograph of MSX agar plates used to test *E. coli* survival growth in a styrene vapour saturated atmosphere after serial batch culturing with styrene vapour. Left – wild type control (non-evolved *E. coli* cells) Right – EtBCH 1.1 A 10 µL sample was taken from ethylbenzene chemostat samples EtBCH 1.1, EtBCH 1.2 and EtBCH 1.3 which had been 4x batch sub-cultured with ethylbenzene vapour. The sample was spotted on the labelled numbered location of the plate. Pure styrene was spotted in the centre of the plate, and with the plate lid cover created a saturated vapour environment. The plates were then incubated in a desiccator at 37 °C for 48 h.

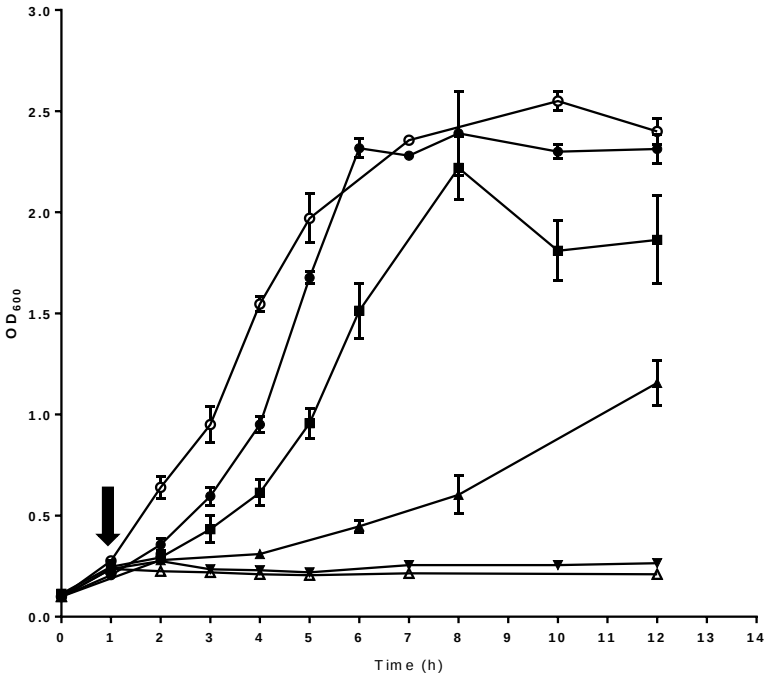


Figure 7.7 Ethylbenzene tolerance assay batch culture on *E. coli* mutant mEtBCH 1 from Ethylbenzene ALE chemostat observing the effect of 7.5 and 10 mmol L^{-1} ethylbenzene concentration on the growth. Wild type 0 mmol L^{-1} (○); Mutant mEtBCH 1 0 mmol L^{-1}(●); Wild type 7.5 mmol L^{-1} (△) Mutant mEtBCH 1 7.5 mmol L^{-1} (■); Wild type 10 mmol L^{-1} (▼); Mutant mEtBCH 1 10 mmol L^{-1}(▲) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Ethylbenzene was added at the time point highlighted by the arrow.

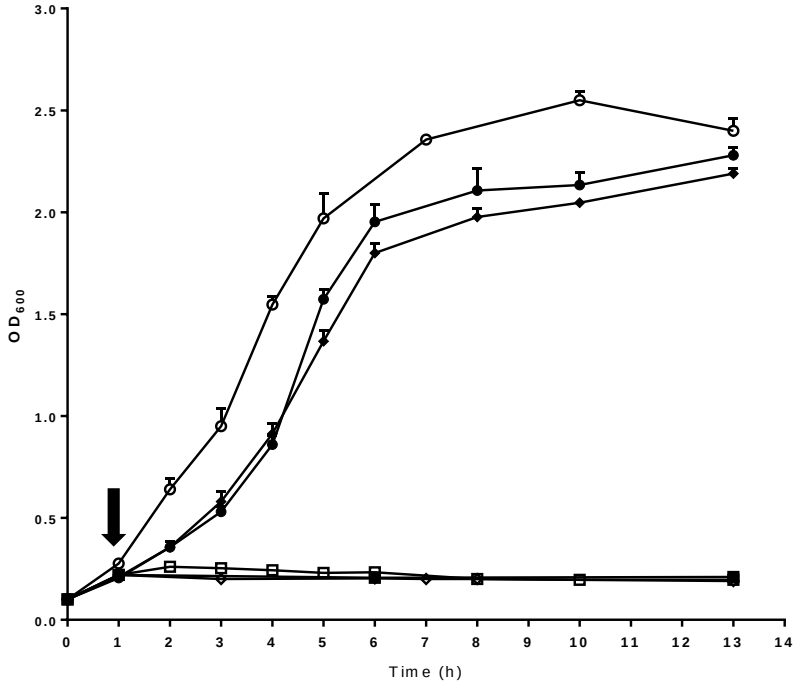


Figure 7.8 Ethylbenzene tolerance assay batch culture on *E. coli* mutant mEtBCH2 from Ethylbenzene chemostat observing the effect of 7.5 and 10 mmol L^{-1} ethylbenzene concentration on the growth. Wild type 0 mmol L^{-1} (○); Mutant mEtBCH 1 0 mmol L^{-1}(●); Wild type 5 mmol L^{-1} (◇); Mutant mEtBCH 1 5 mmol L^{-1} (◆); Wild type 7.5 mmol L-1 (□)Mutant mEtBCH 1 7.5 mmol L^{-1} (■) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Ethylbenzene was added at the time point highlighted by the arrow.

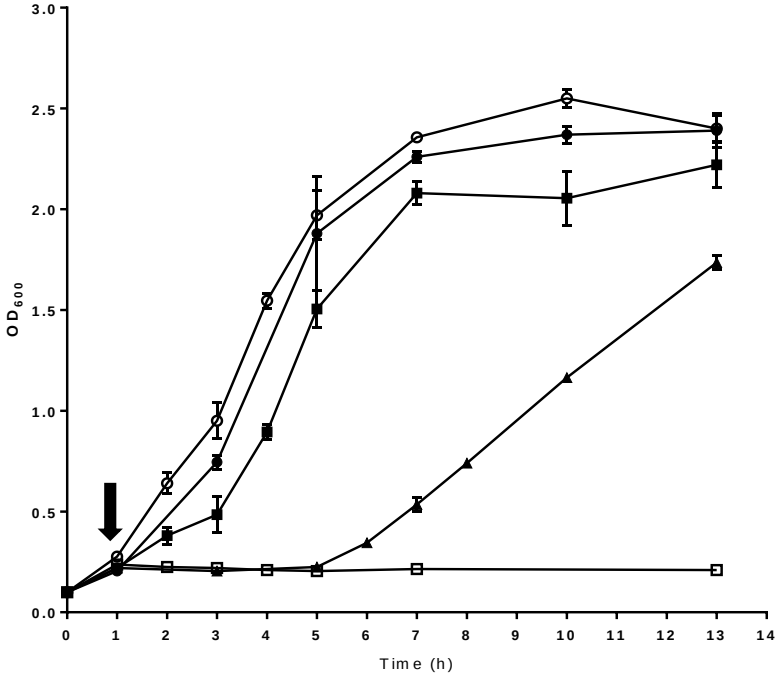


Figure 7.9. Ethylbenzene tolerance assay batch culture on *E. coli* mutant mEtBCH 3 from Ethylbenzene chemostat observing the effect of 7.5 and 10 mmol L^{-1} ethylbenzene concentration on the growth. Wild type 0 mmol L^{-1} (○) Mutant mEtBCH3 0 mmol L^{-1} (●) Mutant mEtBCH3 7.5 mmol L^{-1}(■) Wild type 7.5 mmol L^{-1} (□) Mutant mEtBCH3 10 mmol L^{-1} (▲) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Ethylbenzene was added at the time point highlighted by the arrow.

Strain	Ethylbenzene concentration mmolL^{-1}	Maximum cell OD_{600} reached	Specific growth rate h^{-1}
E. coli BW25113 wildtype	0	2.40 ± 0.062	0.57 ± 0.038
	7.5	0.20 ± 0.001	0
	10	0.21 ± 0.001	0
E. coli BW25113 mutant mEtBCH 1	0	2.34 ± 0.036	0.53 ± 0.030
	7.5	1.86 ± 0.217	0.37 ± 0.021
	10	1.05 ± 0.110	0.16 ± 0.018
E. coli BW25113 mutant mEtBCH 2	0	2.28 ± 0.04	0.50 ± 0.037
	5	2.19 ± 0.026	0.52 ± 0.020
	7.5	0.20 ± 0.005	0
E. coli BW25113 mutant mEtBCH 3	0	2.39 ± 0.084	0.57 ± 0.053
	7.5	2.22 ± 0.113	0.52 ± 0.038
	10	1.74 ± 0.035^{L}	0.29 ± 0.016^{L}

^{L} = Growth after Lag phase

Table 7.4 Growth kinetic parameters of *E. coli* BW25113 wildtype and mutants isolated from ethylbenzene ALE chemostat at 5 – 10 mmol L^{-1} ethylbenzene concentrations.

The results from the ethylbenzene tolerance assay showed that the mutants isolated, mEtBCH 1, mEtBCH 2, and mEtBCH 3 were all able to grow at a higher ethylbenzene concentration in comparison to the wildtype (Figures 7.6 – 7.8; Table 7.4). Out of the 3 *E. coli* mutants, mEtBCH 2 was the weakest because the highest ethylbenzene concentration growth was observed at was 5 mmol L^{-1}. The mEtBCH 1 and mEtBCH 3 mutants were able to grow at 7.5 and 10 mmol L^{-1} ethylbenzene concentration (Figures 7.6-7.8; Table 7.4). The mEtBCH 3 mutant had a higher specific growth rate that the mEtBCH 1 mutant at 7.5 mmol L^{-1} ethylbenzene but the mEtBCH 1 mutant could grow at 10 mmol L^{-1} without an extended lag phase (Figures 7.6 – 7.8; Table 7.4)

7.3 Conclusion

A two stage ALE strategy whereby an *E. coli* BW25113 population was evolved under ethylbenzene selection, and then screened for enhanced tolerance to styrene was successful in isolating 3 ethylbenzene tolerant *E. coli* BW25113 mutants, mEtBCH 1-

3. The mutants displayed different fitness in the presence of ethylbenzene. All the mutants isolated could grow at 5 mmol L^{-1} which is above the experimental ethylbenzene toxicity threshold concentration of 4 mmol L^{-1}. mEtBCH 1 and 3 could grow at 7.5 mmol L^{-1} and 10 mmol L^{-1} ethylbenzene concentration, but mEtBCH 3 displayed an extended lag phase of 5 h at 10 mmol L^{-1}.

Chapter 8

Isolation and characterisation of a styrene tolerant *E. coli* BW25113 mutant

8.1 Introduction

This chapter reports on the characterisation of the styrene tolerant *E. coli* BW25113 mutants, which were generated by the two stage ALE strategy by chemostat under ethylbenzene selection and then isolated under styrene selection. The *E. coli* BW25113 mutants were tested for tolerance to a final styrene concentration of ≥ 2.9 mmol L^{-1}.

8.2 Results and Discussion

8.2.1 Isolation of a styrene tolerant *E. coli* BW25113 mutant

The aim of this experiment was to test the mutants' mEtBCH 1-3 which had been isolated under styrene vapour selection (Figure 7.5) after being generated in an ALE chemostat (Figure 7.3) under ethylbenzene selection. The tolerance assay batch culture with ethylbenzene, showed that all 3 mEtBCH 1-3 mutants that had been previously isolated from styrene vapour selection could grow in the presence of 5 mmol L^{-1} ethylbenzene concentration. However, there were varying levels of fitness at concentrations above 5 mmol L^{-1} ethylbenzene concentration (Figure 7.7-7.9; Table 7.4).

An overnight pure culture of mutant samples mEtBCH 1, mEtBCH 2, and mEtBCH 3 were used to inoculate the MSX medium (50 mL), at a starting OD600 $\approx$ 0.1. All 3 batch cultures were grown up to OD600 $\approx$ 0.2 and, at this point, styrene was added to make a final concentration of 3, 3.75 and 4 mmol L^{-1}. The batch culture was incubated and OD600 readings were taken at regular time intervals.

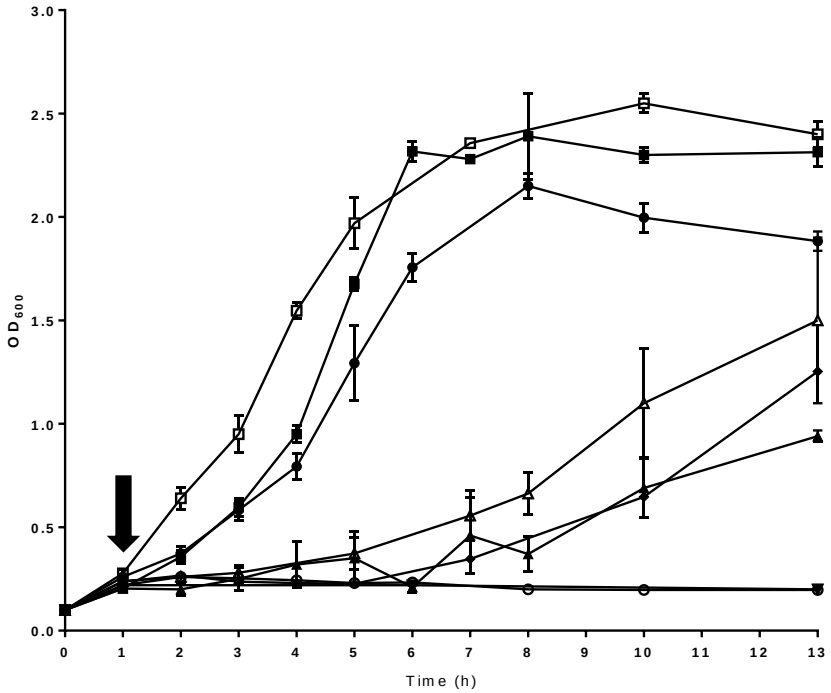


Figure 8.1 Styrene tolerance assay batch culture on E. coli mutant mEtBCH 1 from Ethylbenzene chemostat observing the effect of 3, 3.75 and 4 mmol L^{-1} styrene concentration on the growth Wild type 0 mmol L^{-1} (△), Mutant mEtBCH 1 0 mmol L^{-1} (■), Wildtype 3 mmol L^{-1} (♦) Mutant mEtBCH 1 3 mmol L^{-1}(●), Wildtype 3.75 mmol L^{-1} (▼), Mutant mEtBCH 1 3.75 mmol L^{-1} (△) Wildtype 4 mmol L^{-1} (○), Mutant mEtBCH 1 4 mmol L^{-1} (▲) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Styrene was added at the time point highlighted by the arrow.

Strain	Styrene concentration mmolL^{-1}	Maximum cell OD_{600} reached	Specific growth rate h^{-1}
E. coli BW25113 wildtype	0	2.55 ± 0.062	0.57 ± 0.038
	3	1.25 ± 0.021	0.24 ± 0.010^{L}
	3.75	0.20 ± 0.010	0
	4	0.26 ± 0.007	0
E. coli BW25113 mutant mEtBCH 1	0	2.39 ± 0.208	0.51 ± 0.025
	3	2.15 ± 0.062	0.48 ± 0.051
	3.75	1.50 ± 0.400	0.20 ± 0.032
	4	0.94 ± 0.028	0.19 ± 0.032
E. coli BW25113 mutant mEtBCH 2	0	2.28 ± 0.040	0.47 ± 0.015
	3	1.37 ± 0.045	0.15 ± 0.012
	3.75	0.26 ± 0.017	0
	4	0.21 ± 0.013	0
E. coli BW25113 mutant mEtBCH 3	0	2.39 ± 0.085	0.55 ± 0.029
	3	2.00 ± 0.070	0.39 ± 0.031
	3.75	1.11 ± 0.014	0.15 ± 0.0239^{L}
	4	0.2 ± 0.007	0

L = Growth after Lag phase

Table 8.1 Growth kinetic parameters of *E. coli* BW25113 wildtype and mutants isolated from ethylbenzene ALE chemostat at 3 – 4 mmol L^{-1} styrene concentrations.

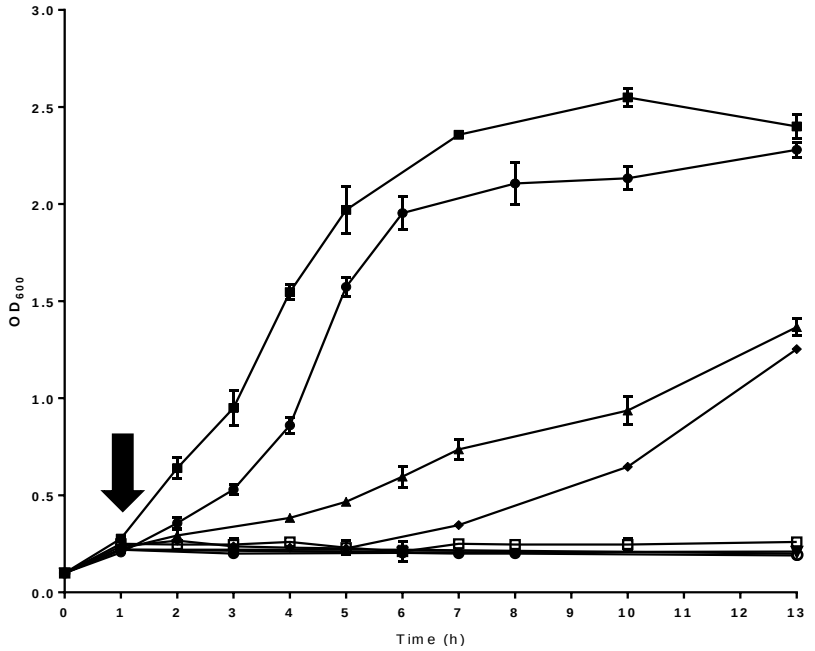


Figure 8.2 Styrene tolerance assay batch culture on *E. coli* mutant mEtBCH 2 from Ethylbenzene chemostat observing the effect of 3, 3.75, 4 mmol L-1 styrene concentration on the growth Wild type 0 mmol L^{-1} (■), Mutant mEtBCH2 0 mmol L^{-1} (●), Wild type 3 mmol L^{-1} (♦), Mutant mEtBCH2 3 mmol L^{-1} (▲) Wildtype 3.75 mmol L^{-1} (▼); Mutant mEtBCH2 3.75 mmol L^{-1} (□) Wildtype 4 mmol L^{-1} (▼) mEtBCH2 4mmol L^{-1} (O) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Styrene was added at the time point highlighted by the arrow.

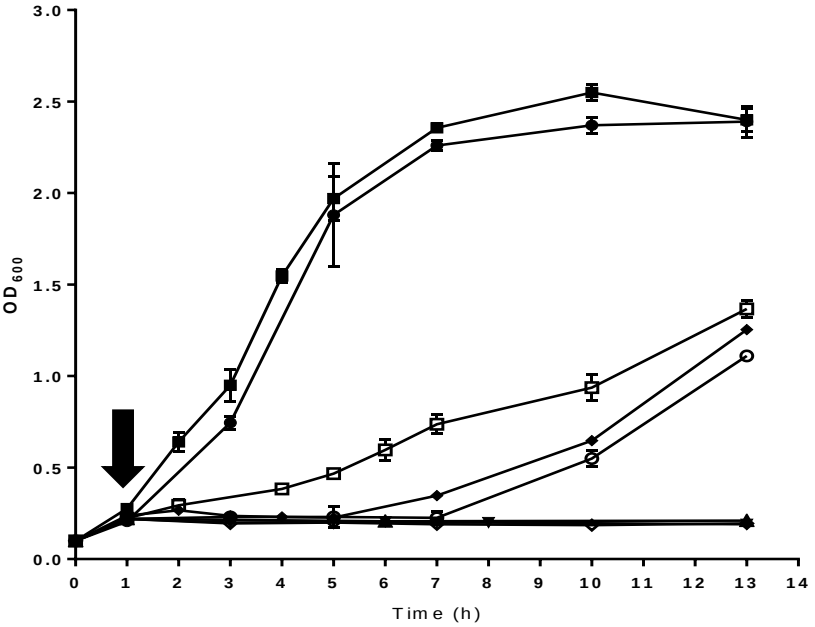


Figure 8.3 Styrene tolerance assay batch culture on *E. coli* mutant mEtBCH 3 from Ethylbenzene chemostat observing the effect of 3, 3.75, 4 mmol L-1 styrene concentration on the growth Wild type 0 mmol L^{-1} (■), Mutant mEtBCH3 0 mmol L^{-1} (●), Wild type 3 mmol L^{-1} (♦), Mutant mEtBCH3 3 mmol L^{-1} (◬), Wild type 3.75 mmol L^{-1} (▲),

Mutant mEtBCH3 3.75 mmol L^{-1} (○), Wild type 4 mmol L^{-1} (▼), Mutant mEtBCH3 4 mmol L^{-1} (◇) using Quickfit® shake flasks (250 mL) at 37°C 250 rpm. The experiments were conducted in triplicate. Styrene was added at the time point highlighted by the arrow.

The results showed all three mutants (mEtBCH 1-3) were able to grow at a 3 mmol L^{-1} styrene concentration with varying degrees of growth inhibition in comparison to the specific growth rate in the absence of styrene (Figure 8.1 -8.3; Table 8.1). mEtBCH 2 displayed the lowest tolerance to styrene as the mutant was unable to grow at a styrene concentration of 3.75 and 4 mmol L^{-1} (Figure 8.2; Table 8.1). mEtBCH 3 could grow at a 3.75 mmol L^{-1} after an extended lag phase of around 7 h but was unable to grow at a styrene concentration of 4 mmol L^{-1} (Figure 8.3; Table 8.1) mEtBCH 1 was observed to be the fittest mutant as it displayed the highest specific growth rate at 3 mmol L^{-1} styrene concentration (Table 8.1). The mEtBCH 1 mutant could also grow at the higher concentrations of styrene (3.75, 4 mmol L^{-1}) without an extended lag phase (Figure 8.1) The *E. coli* wild type could grow at 3 mmol L^{-1} after an extended lag phase of 5 h, but did not grow at 3.75 and 4 mmol L^{-1}.

Conclusion

The mutants isolated displayed different levels of fitness in the styrene tolerance assay batch culture but had enhanced tolerance to styrene in comparison to the wild type. The two stage ALE approach, whereby a population is evolved under ethylbenzene and then screened for enhanced tolerance to styrene was successful in isolating mutants with enhanced styrene tolerance. The mEtBCH 1 *E. coli* mutant was observed as the fitter mutant based on having a higher specific growth rate at all the styrene concentrations tested (3- 4 mmol L^{-1}) without an extended lag phase. The mEtBCH 1 was further analysed to determine the genetic mutations it has acquired in comparison to the wild type.

8.2.2 DNA sequencing results

A styrene and ethylbenzene tolerant *E. coli* BW25113 mutant, mEtBCH 1, was isolated from a population that had been evolved and subjected to rounds of ethylbenzene and styrene selection (Figure 7.3; 7.5). mEtBCH 1 could grow at a higher concentration of styrene and ethylbenzene, in comparison to both the *E. coli* wildtype strain and the other mutants isolated.

The Illumina Miseq platform was used for the resequencing analysis of mEtBCH 1. SNPs and indels were called by aligning reads against the *E. coli* BW25113 wild type that had not undergone ALE. Variant analysis identified 42 mutations including: 27 SNPs, 2 stop gained and 13 indel mutations. No large genome rearrangements were identified in the mEtBCH 1 mutant. Figure 8.4; Table 8.2 shows all the mutations that were present in mEtBCH 1. The locations of the mutations were studied on published structures of AcrB (PDB ID: 2DHH), AcrR (PDB ID: 3BCG), PhoQ histidine kinase phosphotransfer domain in complex with ATP (PDB ID: 1ID0). Rendered images of the protein structures were generated using Jmol.

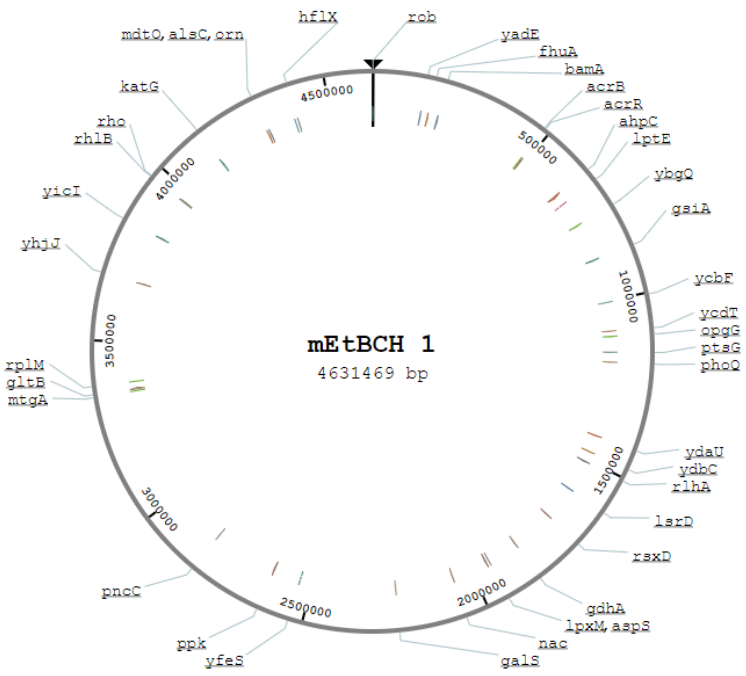


Figure 8.4 Chromosome map of mutations discovered through whole genome resequencing of the styrene tolerant *E. coli* strain mEtBCH1

There were mutations located in central metabolic pathway genes that were most likely generated as a response to nutrient limitation conditions. The stop gained mutation in the *gdhA* gene caused a truncation at the Trp311 residue in the NADP specific glutamate dehydrogenase enzyme (GDH). The GDH enzyme is involved in the synthesis of glutamate by the assimilation of nitrogen using α-KG. Although it is unknown exactly what a mutation at this location would have caused, the GDH pathway is favoured during glucose limitation due to lower ATP requirements (Kumar & Shimizu, 2010). mETBCH 1 had a mutation present in *gltB*, part of another glutamate production pathway that has higher energy requirement (Kumar & Shimizu, 2010). This maybe perhaps linked to cellular attempts to save ATP if this mutation caused a decrease in GS-GOGAT pathway activity. This scenario is further supported by the mutation found in the *nac* gene that controls expression activity of *gdhA* which again may lead to the upregulation of the GDH pathway (Kumar & Shimizu, 2010; Muse & Bender, 1998). As glutamate may play an important role as an osmoprotectant in chemical resistant *E. coli* strains, these mutations are of some significance. (Wang et al., 2013).

There was a great proportion of mutations found in genes encoding cell membrane related components. This is to be expected as it is established that the main toxic action of hydrophobic chemicals is membrane related, therefore alterations to membrane function is likely to occur under chemical stress (Minty et al., 2011). mEtBCH 1 had a mutation present in the *lptE, lptM and bamA* gene, which encode proteins that are part of the component assembly of the Gram-negative outer membrane. LptE is part of a complex with seven other proteins, LptABCDEFG. The LptABCDEFG complex spans the cell membrane envelope, facilitating LPS transport from the periplasm to the cell surface (Chng et al., 2010; Malojčić et al., 2014). LptDE form an outer membrane complex which acts as a recognition site for LPS binding (Chng et al., 2010; Malojčić et al., 2014). The missense mutation in mEtBCH 1 caused an amino acid substitution, Glu139Lys, located in a region of the protein that is highly conserved among LptE homologs in proteobacteria (Malojčić et al., 2014). This region maps to the loop connecting the β4 with the amphipathic helix α2 helix. Basic residues in this region are crucial for binding to negatively charged portions of lipid A in the individual LPS molecules (Malojčić et al., 2014) Therefore, it is possible that the substitution of the negatively charged glutamate amino acid for the positively charged lysine amino acid provides an additional interaction for LPS binding. *E. coli* LptE

substrate binding site mutants that had basic residues replaced at position 136 in LptE were more sensitive to vancomycin and rifampin (Malojčić et al., 2014).

mEtBCH 1 had a deletion in the *lpxM* gene which codes for myristoyl-transferase (LpxM), a lipid A biosynthetic enzyme. Lipid A is a highly conserved component of lipopolysaccharide (LPS) comprising of 6 saturated hydrophobic fatty acid chains connected by a glucosamine and phosphate head group (Clifton et al., 2016; Ebbensgaard et al., 2018; Ma et al., 1996). The lipid A moiety helps to anchor the polysaccharide chain portion of the LPS to the outer membrane (Emiola et al., 2015; Walker et al., 2004). The presence of LPS molecules are reported to protect the organism against toxic hydrophobic compounds by forming a dense hydrophilic layer on the cell surface (Clifton et al., 2016; Ebbensgaard et al., 2018; Ma et al., 1996). Alterations in the activity of LpxM would most likely have an effect on the abundance of LPS molecules. Further studies should aim to determine the effect of the deletion mutation in the LpxM gene. Increase in LpxM activity would generate more LPS molecules on the cell surface, providing greater resistance to hydrophobic compounds (Sikkema et al., 1995; Weber & de Bont, 1996). The effect of the deletion mutation in *lpxM* will have to be more carefully analysed to determine the effect it has on chemical tolerance.

A mutation was found in the *fhuA* gene which encodes a siderophore transporter (FhuA), which binds and transports ferrichrome-iron across the outer membrane. The overall structure of the FhuA is a 747 residue protein consisting of a β-barrel comprising 22 anti-parallel β strands (residues 161-747) and a "plug" domain inside the β-barrel, made up of a four-stranded β-sheet and four short α - helices (residues 1-160) (Ferguson et al., 1998; Locher et al., 1998). The β-barrel channel is "unplugged" via binding of the energy transducing TonB protein to an extracellular loop region of the β strand. The stop mutation would have resulted in a protein truncation at Tyr554 located in the β14 strand. There are amino acid residues present between 555- 747 that are needed for binding to the ferrichrome-ion. The aromatic Phe702 residue on β21 strand and the Ala577 located on periplasmic β strand connector loop, binds to the "plug" domain via hydrophobic interactions (Endriß et al., 2003; Ferguson et al., 1998). Therefore, it is likely a truncated FhuA would have affected these interactions with the "plug" domain and ultimately the ferrichrome-iron transporting ability. Disruptions to the Ala577 and the "plug" domain hydrophobic interactions reduced *E. coli* cell sensitivity to albomycin which are structural

analogues to ferrichrome-iron (Endriß et al., 2003). It is possible that mEtBCH 1 possession of a truncated FhuA protein could have enhanced tolerance to styrene/ethylbenzene in the same way. Studies have also demonstrated that mutation in iron-related genes are a common in *E. coli* as an adaptive response to different environmental stressors (Dragosits et al., 2013).

The final mutation found associated with outer membrane assembly, was present in the *bamA* gene which codes for a subunit of the outer membrane protein assembly complex, involved in assembly and insertion of beta-barrel proteins into the outer membrane (Storek et al., 2019). The mutation caused a Ala41Val amino acid substitution, which is unlikely to have had a major effect because of the similar physicochemical properties of the amino acids (Creixell et al., 2012).

The resequencing of the mEtBCH 1 mutant revealed several mutations in genes that have been associated with bacterial chemical tolerance.

There were mutations found in genes that encode ribosomal proteins, which were previously highlighted in other genomic studies to be linked to tolerance (Jiang et al., 2019; Minty et al., 2011). The mEtBCH 1 mutant had a mutation present in the *rhlB* and *rplM* genes. *rhlB* encodes the ATP-dependent RNA DEAD box helicase RhlB that degrades double stranded RNA (Tseng et al., 2015) whilst *rplM* encodes an early assembly protein of the 50S ribosomal subunit (Herold & Nierhaus, 1987). The mutation found in the *rhlB* was potentially of greater significance, due to the translated protein mutation present in the Q motif region. This is a functional region that controls ATP binding and hydrolysis (Tanner et al., 2003). Gene mutations in other ATP-dependent RNA helicases, such as *deaD* and *hrpA*, and *rspB* which codes the 30S ribosomal subunit protein S2, were found in several lineages of isobutanol tolerant *E. coli* strains (Minty et al., 2011).

The mEtBCH 1 strain had a missense mutation in the *rho* gene, that encodes the Rho transcriptional regulator protein which is a global regulator of gene expression (Dombroski et al., 1988; Krishna Leela et al., 2013). The protein structure is a homo-hexamer with two RNA binding sites and an ATP binding domain (Dombroski et al., 1988). The missense mutation resulting in a Met245Ile substitution may not have had any effect on the overall protein structure, as the two amino acids have similar physicochemical properties (Creixell et al., 2012). Previous gene enrichment studies have shown transcriptional regulator genes to be heavily represented in chemical resistant *E. coli* mutants. However, this mutation was highlighted as important as

mutations in transcriptional termination genes, are often found in chemical resistant *E. coli* strains (J. D. Winkler et al., 2016). *E. coli* strains with a mutation present in the *rho* regulatory gene have been linked to solvent and biofuel resistant phenotypes (J. D. Winkler et al., 2016)

The mEtBCH 1 mutant also possessed some more notable mutations commonly linked with solvent tolerance. Mutations were found in the *acrB, acrR, rob* and *phoQ* genes, which are involved in the expression of the AcrAB-TolC efflux pump. The *acrB* gene encodes AcrB, the efflux pump component of the AcrAB-TolC complex (Hobbs et al., 2012; Ma et al., 1996; Murakami et al., 2006; Watanabe & Doukyu, 2012). *acrR* and *rob* express protein products that repress the transcription of *acrAB* (Ma et al., 1996; Watanabe & Doukyu, 2012). AcrB has three distinct domains, a transmembrane domain, a "pore" domain and a TolC docking domain (Murakami et al., 2006) (Figure 2.12; 8.5A). mEtBCH 1 had two mutations in the *acrB* gene causing amino acid substitutions at Phe178Leu and Thr489Ile located in the pore and transmembrane domain respectively. The Phe178 residue is located in the substrate

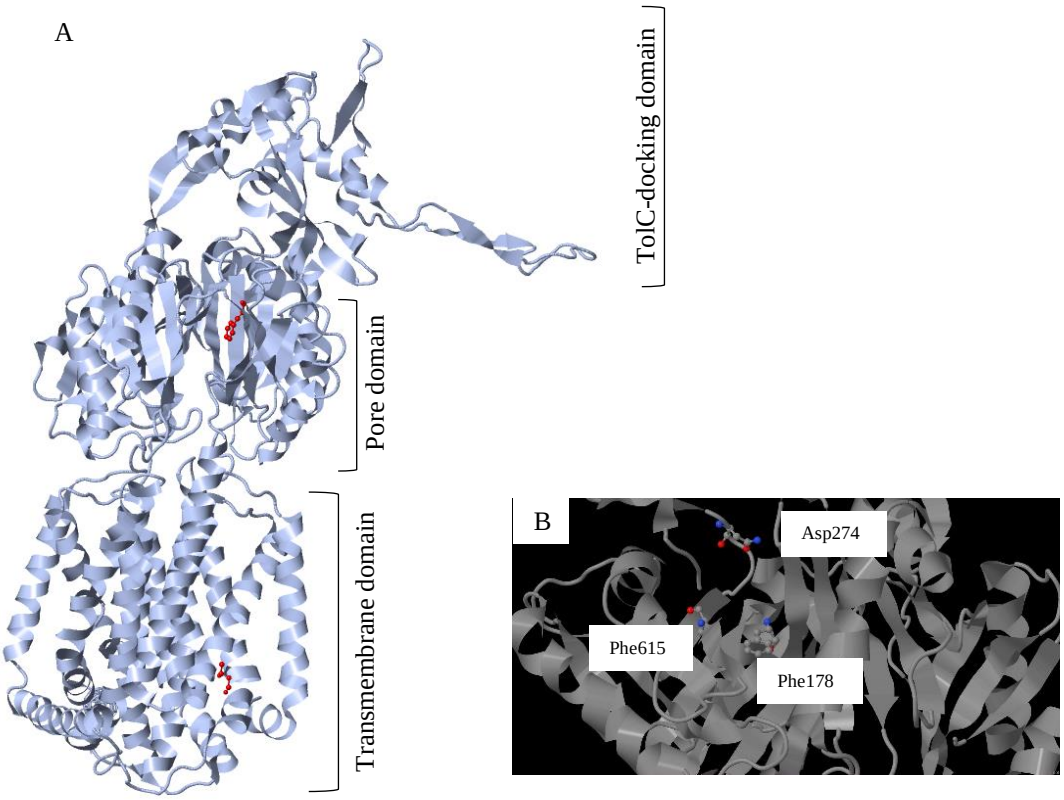


Figure 8.5 A) Structure of AcrB showing three distinct domains; Tol-C docking domain, Pore domain, Transmembrane domain. Locations of the mutations in the pore domain (Phe178Leu) and the transmembrane domain (Thr489Ile) are highlighted in yellow. B) Close up of the Phe residues present in the binding pocket of the pore domain. Rendered images of the protein structures were generated using Jmol.

binding pocket which contains many Phe residues that protrude into the binding pocket and form hydrophobic bonds with the substrate (Murakami et al., 2006) (Figure 8.5B). There are some polar residues in this area, such as Asn 274 and Gln 176, possibly forming hydrogen bonds with a bound substrate (Murakami et al., 2006). A substitution from an aromatic Phe residue to an Ile may have improved the steric arrangement for styrene export.

It is not clear what effect the second Thr489Ile mutation in AcrB may have had, as this residue due has not been highlighted to be of interest in literature. There was a nucleotide deletion mutation present in *acrR* which caused a frameshift in the protein sequence of AcrR at Val29. AcrR represses the transcription activity of *acrAB*; genetic mutations that inactivate the *E. coli acrR* gene have been shown to enhance solvent tolerance (Jellen-Ritter & Kern, 2001; Luhe et al., 2012; Rodionov et al., 2001; Watanabe & Doukyu, 2012). A Val29Gly mutation was identified in an ALE *E. coli* mutant that had been evolved under an antibiotic selection pressure and also in antibiotic resistant clinical isolates (Shuster et al., 2016). The Val29 residue is in the linker region between the first two α- helices (Figure 8.6). This typical helix turn helix motif is present in the DNA binding domain and therefore a mutation in this location may have influenced binding activity of AcrR to the *acrAB* operon (Figure 8.6).



Figure 8.6 A) Structure of AcrR, transcriptional regulator of *acrAB* operon. Val29 residue highlighted in yellow located in the linker region between the first two helices. Mutations at Val29 has been linked to enhanced antibiotic tolerance and was also present in the mEtBCH 1 strain. Rendered images of the protein structures were generated using Jmol.

A missense G → A mutation was present in the *rob* gene which expresses Rob, a global transcriptional regulator protein. Rob controls the genes linked to several multi-drug/solvent resistance and oxidative stress systems in *E. coli*. The missense mutation in mEtBCH 1 resulted in an amino acid substitution Arg156His located in the C-terminal of the protein. The C-terminal domain has a role in the Rob activation mechanism, by releasing it from its inactive self-sequestered form into an active single protein that can bind to DNA (Duval & Lister, 2013; Griffith et al., 2009). The Arg156 residue may be important for the interactions between the Rob protein molecules, therefore changes could reduce the sequestration effect releasing more active Rob protein. This mutation may confer the upregulation of stressor resistance systems and contribute to the tolerance phenotype seen in mEtBCH 1.

A further gene mutation of interest found in mEtBCH 1 was in phoQ which is a membrane enzyme that controls the phosphorylation of the transcription factor, PhoP (Marina et al., 2001; Monsieurs et al., 2005; Véscovi et al., 1997; Yuan et al., 2017). The active phosphorylated form of PhoP is said to repress *acrA* transcription as the DNA binding site is located 32 bp upstream from the acrA promoter (Monsieurs et al., 2005). AcrA functions in the complex as the periplasmic protein that connects the AcrB efflux pump with the TolC outer membrane channel (Anes et al., 2015; Machas et al., 2019). The structure of PhoQ is a prototypical histidine kinase containing a periplasmic sensor domain, a transmembrane 4 helix bundle, a cytoplasmic HAMP domain and a kinase domain (Figure 8.7A). The mutation was in the PhoQ kinase domain causing an amino acid substitution at Met466, mapping specifically to the β-hairpin region at the back of the nucleotide binding site (Marina et al., 2001) (Figure 8.7B). Met466 functional importance was inferred from the suggestion that ATP binding induces a shift in the position of the β-hairpin, to allow Met466 to interact with the histidine phosphotransfer region (Marina et al., 2001) (Figure 8.7A). The *phoQ* mutation causing an amino acid substitution at Met466Ile could therefore be of some significance because of its direct link to PhoP phosphorylation and therefore the deregulation of acrA expression. Additional experiments will be necessary to address these possibilities.

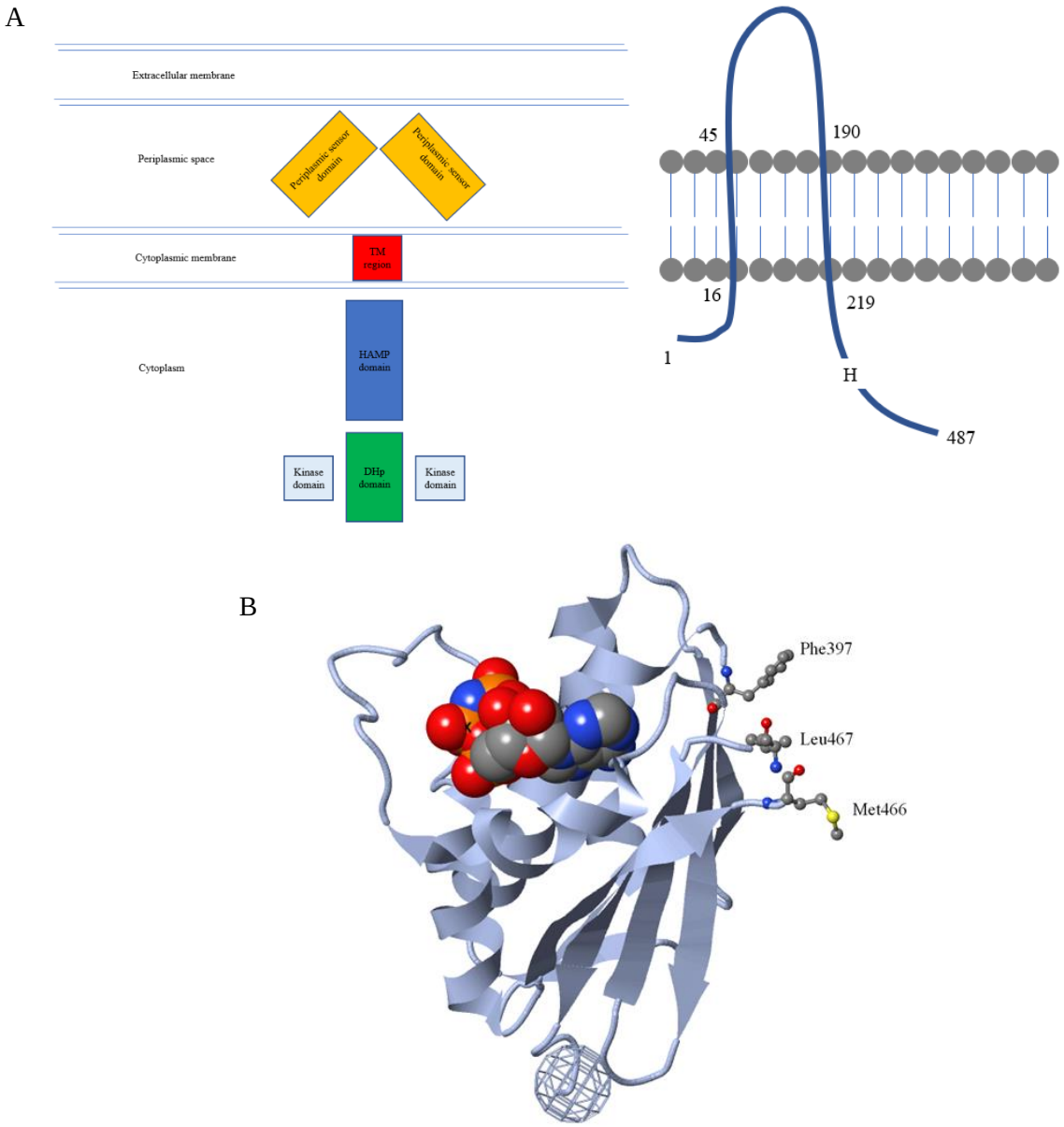


Figure 8.7 A) Topology of the PhoQ protein featuring 2 TM regions (aa 16-45; 190-219) a periplasmic sensor domain (aa 45-190) and a cytoplasmic histidine kinase phosphotransfer and the ATP-binding kinase domain (aa 219-487) B) Structure of the histidine kinase phosphotransfer domain in complex with ATP; hydrophobic residues (Phe397, Leu467 and Met466) with functional importance are highlighted. Rendered images of the protein structures were generated using Jmol.

	Gene(s)	Gene description	Molecular function	Cellular Location	Genomic Coordinate	Nucleotide Change	Protein Change
1	*bamA*	Outer membrane protein assembly factor BamA	Outer membrane protein assembly factor BamA	Outer membrane	194536	C>T	Ala41Val
2	*gsiA*	Glutathione import ATP-binding protein GsiA	Glutathione import ATP-binding protein GsiA	Periplasm	863870	Deletion: C	Frameshift
3	*yghQ*	Inner membrane protein YghQ	Inner membrane protein YghQ	Inner membrane	3125148	A>T	Tyr207Phe
4	*alsC*	D-allose transport system permease protein AlsC	D-allose transport system permease protein AlsC	Membrane	4298403	G>A	Gly295Ala
5	*lptE*	LPS-assembly lipoprotein LptE	Lipopolysaccharide binding and assembly	Inner membrane	667228	G>A	Glu139Lys
6	*fhuA*	Ferrichrome outer membrane transporter/phage receptor	Iron ion homeostasis	Outer membrane	165632	T>A	Truncated at Tyr554
7	*acrB*	Multidrug efflux pump subunit AcrB	Transmembrane efflux transporter	Inner membrane	478394	C>T	Thr489Ile
8					479328	T>C	Phe178Leu
9	*ptsG*	PTS system glucose specific EIICB component	Glucose transmembrane transport	Inner membrane	1153362	G>C	Gly13Ala
10	*phoQ*	Sensor protein PhoQ	Sensor protein PhoQ	Membrane	1183835	G>A	Met466Ile
11	*opgG*	Glucans biosynthesis protein G	Glucans biosynthesis protein G	Periplasm	1105524	Deletion: G	Frameshift
12	*lsrD*	Autoinducer 2 import system permease protein LsrD	Autoinducer 2 import system permease protein LsrD	Membrane	1598898	G>A	Val199Met

Table 8.2 Mutations found in genome resequencing of the *E. coli* BW25113 mutant mEtBCH 1

	Gene(s)	Gene description	Molecular function	Cellular Location	Genomic Coordinate	Nucleotide Change	Protein Change
13	*rsxD*	Ion-translocating oxidoreductase complex subunit D	transmembrane transport	Membrane	1703665	Duplication: C	Frameshift
14	*lpxM*	Lipid A biosynthesis myristoyltransferase	Lipopolysaccharide biosynthetic process	Inner membrane	1934433	Deletion: A	Frameshift
15	*mtgA*	Biosynthetic peptidoglycan transglycosylase	Peptidoglycan biosynthetic process	Outer membrane	3343079	Deletion: G	Frameshift
16	*ycdT*	Probable diguanylate cyclase DgcT	Integral component of membrane	Outer membrane	1088808	Deletion: G	Frameshift
17	*pncC*	Nicotinamide-nucleotide amidohydrolase PncC	Nicotinamide-nucleotide amidohydrolase PncC	Cytoplasm	2817513	A>C	Ile65Leu
18	*orn*	Oligoribonuclease	RNA catabolic process	Cytoplasm	4381728	C>T	Pro103Leu
19	*acrR*	HTH-type transcriptional regulator AcrR	acrAB operon transcription regulator	Cytoplasm	481296	Deletion: G	Frameshift
20	*nac*	Nitrogen assimilation regulatory protein Nac	Nitrogen assimilation regulatory protein nac	Cytoplasm	2054835	G>A	Asp194Asn
21	*galS*	HTH-type transcriptional regulator GalS	HTH-type transcriptional regulator GalS	Cytoplasm	2234615	dupCAAC	Frameshift
22	*rlha*	rRNA modification	rRNA modification	Cytoplasm	1501725	G>A	Ala230Thr
23	*rplM*	50S ribosomal protein L13	rRNA modification	Cytoplasm	3371589	A>G	Asp141Gly
24	*rho*	Transcription termination factor Rho	Transcription termination factor Rho	Cytoplasm	3960511	G>A	Met245Ile
25	*ydbC*	Pyridoxine 4-dehydrogenase	Pyridoxine 4-dehydrogenase	Cytoplasm	1469274	C>A	Ala266Asp
26	*aspS*	Aspartate--tRNA ligase	Aspartate--tRNA ligase	Cytoplasm	1944602	G>A	Ala60Thr
27	*rhlB*	ATP-dependent RNA helicase RhlB	ATP-dependent RNA helicase RhlB	Cytoplasm	3958909	Deletion: A	Frameshift
28	*hflX*	GTPase HflX	GTPase HflX	Cytoplasm	4391159	C>T	Ala224Val

Table 8.2 cont Mutations found in genome resequencing of the *E. coli* BW25113 mutant mEtBCH 1

	Gene(s)	Gene description	Molecular function	Cellular Location	Genomic Coordinate	Nucleotide Change	Protein Change
29	*gdhA*	NADP-specific glutamate dehydrogenase	Glutamate biosynthetic process	Cytoplasm	1837560	G>A	Truncated at Trp311
30	*gltB*	Glutamate synthase [NADPH] large chain	Glutamate biosynthetic process	Cytoplasm	3352116	G>A	Gly1345Ser
31	*ahpC*	Alkyl hydroperoxide reductase C	Detoxifies hydrogen peroxide (H2O2)	Periplasm	634825	G>C	Gly142Ala
32	*katG*	Catalase-peroxidase	Detoxify hydrogen peroxide (H2O2)	Periplasm	4125686	T>G	Phe642Val
33	*rob*	Multi-drug/solvent resistance genes transcription regulator	Multi-drug/solvent resistance genes transcription regulator	Cytoplasm	4624661	G>A	Arg156His
34	*ppk*	Polyphosphate kinase					
35	*ydaU*	Uncharacterized protein YdaU	Phage or Prophage Related	Cytoplasm	1415888	Duplication:C	Frameshift
36	*yfeS*	Uncharacterised conserved protein YfeS	Uncharacterised	Unknown	2531450	G>A	Gly115Ser
37	*ybgQ*	Uncharacterized outer membrane usher protein YbgQ	Putative fimbrial usher protein	Cell outer membrane	747187	Duplication:C	Frameshift
38	*yhjJ*	Uncharacterised protein YhjJ	Uncharacterised	Periplasm	3674052	T>C	Tyr417His

Table 8.2 cont Mutations found in genome resequencing of the *E. coli* BW25113 mutant mEtBCH 1

		Gene(s)	Gene description	Molecular function	Cellular Location	Genomic Coordinate	Nucleotide Change	Protein Change
40		*yicL*	Uncharacterized inner membrane transporter YicL	Uncharacterised inner membrane transporter YicL	Membrane	3825769	T>C	Val710Ala
41		*ycbF*	Uncharacterized fimbrial chaperone YcbF	Putative fimbrial chaperone	Periplasm	999609	Duplication: G	Frameshift

Table 8.2 cont Mutations found in genome resequencing of the *E. coli* BW25113 mutant mEtBCH 1

8.3 Conclusions

This study was able to identify mutations in the *E. coli* BW25112 mEtBCH 1 mutant which may have contributed to the overall styrene tolerance phenotype. mEtBCH 1 could grow at a higher concentration of styrene and ethylbenzene, in comparison to both the *E. coli* wildtype strain and the other mutants isolated. Variant analysis identified 42 mutations including 27 SNPs, 2 stop gained and 13 indel mutations. There were mutations located in central metabolic pathway genes, seemingly unrelated to tolerance and were most likely generated as a result of glucose limited ALE conditions. Other gene mutations identified speculatively to contribute towards styrene tolerance included mutations present in genes that encode ribosomal proteins and global transcription regulators. However, mutations present in genes involved in the expression of the AcrAB-TolC were highlighted as significant in the strain, due to the role of efflux pumps in chemical tolerance. The presence of an *acrR*, *acrB*, *rob* and *phoQ* mutation in the mEtBCH 1 suggests the potential role of the AcrAB-TolC system in styrene tolerance.

Chapter 9

Discussion and further work

The main result from this project generated an *E. coli* BW25113 mutant strain that was tolerant to a styrene concentration above the reported toxicity threshold. The mEtBCH 1 mutant was able to grow at a concentration of 4 mmol L^{-1}, which is a 38% higher concentration than the wild type. This is a significant result in the field as there have not been any reports of an *E. coli* strain that can grow at concentrations higher than 2.9 mmol L^{-1} (McKenna & Nielsen, 2011). The implications of this result contribute to the overall body of research to investigate ways to increase the productivity of a styrene bioprocess. As toxicity remains to be the biggest hindrance in achieve industrially significant titres, an increase in tolerance by even a marginal amount could alleviate the toxicity issues faced in fermentations (Claypool et al., 2014). The spontaneous separation of styrene from water above 3.2 mmol L^{-1} could mean that the generated strain would remain productive at fermentation operating conditions.

Variant analysis from resequencing data of the mEtBCH 1 mutant showed that it had acquired 42 mutations during the ALE process. The key mutations that were speculatively identified as contributing to overall tolerance were present in *acrR*, *acrB, rob* and *phoQ* genes. The presence of these mutations suggests the potential role of the AcrAB-TolC efflux system in styrene tolerance. The AcrAB-TolC efflux pump has been recognised as an important cellular mechanism in bacterial chemical tolerance. This system is often a target of optimisation to enhance chemical tolerance (Hobbs et al., 2012; Ma et al., 1996; Mingardon et al., 2015; Watanabe & Doukyu, 2012; White et al., 1997). Rational attempts targeting the AcrAB-TolC system demonstrated the involvement of the efflux pump upon *E. coli* exposure to styrene. *acrAB* gene deletion mutants were at a growth disadvantage in comparison to the wildtype (Mingardon et al., 2015). The absence of the pump in the styrene production *E. coli* strain was significantly higher when the pump expression is induced (Mingardon et al., 2015).

PhoQ indirectly affects the expression of AcrA, the linker periplasmic protein connecting AcrB to TolC (Anes et al., 2015; Machas et al., 2019). The catalytic kinase domain of the PhoQ protein controls the phosphorylation state of PhoP, which reportedly functions as a transcriptional regulator of *acrA* (Monsieurs et al., 2005). In mEtBCH 1 Met466 in the PhoQ kinase domain was substituted for another hydrophobic residue however, it is unclear if this mutation made an overall difference

in protein function. The Asn202 residue has been identified to play a crucial role in switching PhoQ between kinase and phosphatase states(Goldberg et al., 2010; Yuan et al., 2017). A similar strategy could be undertaken to examine the role Met466 plays in PhoQ function; perhaps by using a plasmid borne mutation process to incorporate bases in primers at the positions that correspond to codon 466 in *phoQ* (Regelmann et al., 2002). Kinase activity assays may then be set up to analyse in greater detail how changes at Met466 affect the phosphorylation activity of PhoQ.

The frameshift mutation found in acrR supported the hypothesis for the involvement of the AcrAB-TolC efflux pump in styrene tolerance. Mutations in AcrAB regulatory genes *marR, soxR,* and *acrR* have been shown to increase the expression level of the AcrAB-TolC pump (Jellen-Ritter & Kern, 2001; H. Wang et al., 2001; Watanabe & Doukyu, 2012). The position of the mEtBCH 1 *acrR* mutation at Val29 has also been highlighted as being present in clinical isolates of antibiotic resistant *E coli* strains (Shuster et al., 2016). This finding is of particular interest as the AcrAB-TolC efflux pump has also been implicated as a vital contributor in the enhanced resistance of *E. coli* to antibiotic compounds (Du et al., 2014; Müller & Pos, 2015). Preliminary experiments were conducted to test if the styrene tolerant *E. coli* mutants generated in this project exhibited enhanced tolerance to antibiotics. The mEtBCH 1 strain showed enhanced tolerance to high concentrations of chloramphenicol in comparison to the wild type strain. Although this was not reported in the results section, it did highlight how the ALE process generated mutants that displayed cross resistance to different stressors. Studies investigating cross stressor resistance in *E. coli* ALE experiments, have found that certain adaptive mutations can be beneficial across different stressor environments (Dragosits et al., 2013; Horinouchi, Sakai, et al., 2017). It would be worth further investigating the effect of the *acrR* Val29 mutation has on the expression of AcrA and AcrB.

The *acrB* mutation found in the mEtBCH 1 traced to a Phe178Leu substitution in the substrate binding pocket of the AcrB pump protein. The Phe178 residue and other aromatic residues have a functional purpose as important for hydrophobic binding (Murakami et al., 2006). Furthermore, in the proposed method of efflux, the AcrB binding site expands to allow entry of the substrate molecule, before shrinking to push the substrate into the upper domains (Murakami et al., 2006). Perhaps substitutions in this location, enabled a substrate efflux path that facilitated an increase in the active export of styrene. The efficiency of substrate binding has been attributed

to the volume and side chain arrangements of the binding pocket (Murakami et al., 2006). The second Thr489Ile mutation in AcrB is in the α-helix 6 transmembrane portion of the efflux pump. Thr489 has not previously been identified in literature to be key for activity, but is present in a location particularly important for entry in/out of the binding pocket (Murakami et al., 2006). The export mechanism in AcrB is driven by proton motive force, exporting chemical molecules from the outer leaflet of the cytoplasmic membrane out of the cell (Anes et al., 2015; Machas et al., 2019). The proton translocating pathway runs through transmembrane portion of AcrB facilitated by three positively charged residues in the region, Asp 407, Asp 408 and Lys 940 (Murakami et al., 2006). Changes to the steric arrangement of these positively charged residues due to a neighbouring Thr978Ala residue substitutions reportedly resulted in the loss of the chemical resistance phenotype (Murakami et al., 2006). As Thr489 is also in proximity the proton binding residues, it may be a functionally important residue yet to be identified.

Although this work provided unique insights on the genetic basis of styrene tolerance from genome resequencing data; further insight could be attained from other omics analysis. Transcriptomics analysis can measure the changes in gene expression from the strain of interest relative to the parental strain under certain conditions (Hoeijmakers et al., 2013; Lowe et al., 2017). It would have been of great interest to understand how the mutations present in mEtBCH 1 altered the gene expression in comparison to the wildtype. Firstly, by looking at the changes in the transcriptome of the parental strain upon exposure to styrene; and comparing the number of differentially upregulated/downregulated genes to the styrene tolerant *E. coli* strains. This could be done by RNA sequencing the mRNA transcripts, generated from the parental and mutant strains grown in the presence of styrene (Hoeijmakers et al., 2013; Horinouchi et al., 2010; Tomas et al., 2004). RNA sequence read counts can be reported as fold changes relative to the wildtype strain and other styrene tolerant strains (Lowe et al., 2017). The transcriptomic profile of the different styrene tolerant mutants could also be compared with each other to confirm whether there were any common genes that were differentially expressed. This ultimately would give additional insight of the response required to enable growth in the presence of styrene.

To aid in the characterisation of the mutations present in mEtBCH 1, enrichment analysis could also be used to group functionally related differentially expressed genes.

The mEtBCH 1 strain had mutations present in genes that were involved in common GO terms based on biological process, molecular function or cellular component. Further work can use GO enrichment analysis to identify the most significantly enriched GO term and which associated genes are differentially expressed. The mEtBCH 1 strain had mutations in different transcription factors; transcriptomic analysis could have been effectively used to determine the effect of these mutations on the expression of genes under their regulatory control. *E. coli* expresses 304 transcription factors which regulate almost half the genes in the organism (Pérez-Rueda et al., 2015). Transcription factors that regulate adaptive response mechanisms, are often differentially expressed in chemical tolerant microbial strains (Pattrick et al., 2019). Further work could explore what effect the presence of these mutations has on the expression of the mEtBCH 1 transcription factors and genes under their regulation. Some transcriptional regulator gene mutations were in *acrR* and *rob* that control the expression of multidrug resistance related genes in *E. coli* (Jellen-Ritter & Kern, 2001; Luhe et al., 2012; Ma et al., 1996). Gene heat maps could provide a way to visualise which genes under the regulatory control of *acrR* and *rob* are differentially expressed, relative to the parental strain in the presence of styrene chemical.

An analysis of the mEtBCH 1 proteome would have provided a greater understanding of styrene dependent global changes in protein abundance. Elucidating the proteomic profile of the styrene tolerant mutants, will have perhaps confirmed if the mutants had a higher abundance of proteins involved in tolerance systems. Information from other omics analysis suggested there was perhaps an increase in the expression of proteins involved in the AcrAB-TolC efflux system because of mutations present in the *acrB*, *acrR* and *rob*. Studies looking at the proteome changes of evolved chemical tolerant *E. coli* mutants; confirmed the differential abundance of proteins associated with stress defence mechanisms and membrane transport proteins (Pattrick et al., 2019). Further work could perhaps use similar methods, such as 2D gel electrophoresis, to resolve all the membrane proteins that were differentially expressed followed by MALDI-TOF MS identification. This type of proteomic analysis could provide a meaningful correlation between genotype and phenotype if integrated with prior omics data. Proteomic analysis may also reveal changes in the abundance of non-vital proteins in the mEtBCH 1 strain which may indicate cellular adjustments in resource allocation (Gauger et al., 2007). The reduced growth rate in the presence of chemicals is often

linked to a cellular metabolic diversion away from growth and reproduction evidence by a downregulation of protein involved in cell membrane biogenesis, cell motility and chemotaxis (Pattrick et al., 2019).

The combination of all the omics data should ideally provide insight to identify mutagenesis targets for styrene tolerant mutants to be constructed. Genome integration of the mutations acquired by mEtBCH 1 into the parental strain, especially the ones found in *acrR*, *acrB*, *phoQ* and *rob*, could help verify if these mutations play a role in styrene tolerance. Equally transcriptomic insights into which genes are differentially expressed in the presence of styrene may also shed light on styrene tolerance mechanisms. One of the ways this could be done is from the construction of single gene knockout strains of the AcrAB-TolC associated mutated genes found in mEtBCH 1 to measure the effect on growth in the presence of styrene.

The ALE method followed by screening under selective pressure was chosen in this project to generate styrene tolerant *E. coli* mutants. As microbial tolerance is often controlled by multiple genetic factors, gTME could also have been utilised in this project. However, since ALE did provide the initial non-intuitive generation of adaptive mutations, gTME may have had the greatest impact if introduced secondary to ALE. So far, *E. coli* chemical tolerance has been enhanced by gTME of σ^{70} (*rpoD)* and CRP (Alper et al., 2007; Chong et al., 2014; Zhang et al., 2015). Transcriptional regulatory genes which were identified to be mutated in mEtBCH 1 could have perhaps served as gTME targets.

Although ALE was successful overall in generating an *E. coli* styrene tolerant mutant; there were some challenges experienced. In chapter 6, when styrene was added to batch culture medium in liquid phase, a styrene selective environment could not be sustained. The results suggested that the presence of *E. coli* cells influenced the styrene aqueous phase concentration in the batch culture over time (Figure 5.6). One of the several hypotheses put forward for this phenomenon was it being due to the membrane absorption of styrene, lowering the concentration in the aqueous medium. Styrene has a Log P_{ow} value that suggests favourable binding to the hydrophobic portions of the membrane (Ramos et al., 2002; Sikkema et al., 1995). It is therefore possible that styrene is sequestered in the membrane in a non-homogenous manner. This could mean that not all cells are exposed to the same concentration of styrene, allowing the

growth of *E. coli* wild type cells that are not tolerant. The accumulation of styrene in the membrane and the resulting decrease in styrene concentration in the medium occurs over a period. The time dependent effect of membrane sequestration means that the growth of non-tolerant *E. coli* cells is detected after an extended lag phase. This lag phase extension takes place after styrene addition to early exponential phase cultures, completely halting growth for a period of time. Once styrene from the medium is sequestered in the membranes of a cell subpopulation, other cells that did not contact styrene will be present in a selective environment where styrene is not at sub-lethal concentration. A non-adapted sub-population will therefore grow without mutation occurring. Further testing should aim to determine styrene membrane interactions and how much styrene accumulates in the membrane. The determination of the membrane buffer coefficient could perhaps deliver more useful insight. This parameter can be used to accurately quantify the aqueous concentrations of styrene in microbial cell cultures (Barnoud et al., 2014). This could be done *in vivo* or perhaps using model membrane systems consisting of a specific composition of phospholipids (Barnoud et al., 2014; De Young & Dill, 1988; J Sikkema, de Bont, & Poolman, 1994; Sikkema et al., 1995). The inversely proportional relationship of *E. coli* BW25113 cell biomass concentration with styrene could also indicate that styrene is being utilised as a substrate for growth. Such substrate consumption kinetics has been reported in mixed bacterial cultures containing styrene degrading strains like the *Pseudomonas sp*. (Hartmans et al., 1990; Portune et al., 2015; Przybulewska et al., 2006). Styrene degrading strains possess a special class of mono-oxygenase enzymes which *E. coli* is not known to possess naturally (Panke et.; 2002; Panke.; 2000). To test this theory, further experiments could be conducted by testing for styrene degradation products, which are typically styrene side chain oxygenated compounds. The microbial metabolism of styrene converts it to styrene oxide and then 2-phenylethanol as a by-product from further reduction (Hartmans et al., 1990; Panke et al., 2000; Straathof et al., 2002).

Overall, perhaps the most direct way to solve issues with *E. coli* toxicity may have been to use an alternative microbial host with a greater inherent styrene tolerance. Many *Pseudomonas. spp* strains for instance have a higher chemical tolerance than *E. coli* (Nijkamp et al., 2005; Verhoef et al., 2009; Weber et al., 1993; Wierckx et al., 2005). In particular, the *Pseudomonas putida* S12 strain is able to withstand high

concentrations of toxic aromatic compounds by means of RND efflux pump extrusion from the cell (Isken & De Bont, 1996; Kieboom et al., 1998; Rojas et al., 2001)

However, the availability of a phenylalanine overproducing *E. coli* strain from previous metabolic engineering of the shikimate justifies the use of an *E. coli* platform. Previous attempts to engineer *P. putida* in a similar way to *E. coli*, via the over expression of feedback resistant shikimate pathway enzymes, did not lead to improved phenylalanine availability (Nijkamp et al., 2005). Also unlike the Pseudomonads, *E. coli* also lacks the ability to catabolise shikimate pathway derived compounds making it an ideal choice (Polen et al., 2005).

Prior to the initiation of this project, the generations of a phenylalanine overproducing *Pseudomonas* strain had not been achieved. However, more recently there have been reports of a *Pseudomonas taiwanensis* phenylalanine overproducer generated by deletion of its degradation pathway (Otto et al., 2019). The engineering efforts to increase flux towards phenylalanine included the deletion of three genes involved in phenylalanine catabolism in addition to the overexpression of feedback resistant shikimate pathway enzymes (Otto et al., 2019). The strain was also engineered to express PAL from *A. thaliana* and could produce trans-cinnamic acid(at yields of 23% from a glucose feedstock (Otto et al., 2019). Although styrene production has not been engineered in a *Pseudomonas* strain platform this data could present an exciting future prospect. This result supports the feasibility of engineering a similar trans-cinnamic acid pathway in a pseudomonas strain. Further engineering will be required to include a PADC enzyme to carry out the decarboxylation reaction to styrene. The issue of toxicity related productivity issues may be less of a hindrance as *P. putida* can withstand and utilise styrene at concentrations of 1% v/v; for comparison the threshold concentration in *E. coli* equates to 3.45×10^{-4} % v/v.

Chapter 10

Conclusions

ALE is an effective strategy to generate *E. coli* cells with enhanced tolerance to styrene. In this study, the aim of generating a styrene tolerant mutant was achieved. Styrene provided a case study framework to develop robust ALE methods that can be used with other chemicals with similar physiochemical properties. Batch sub-culturing whereby styrene is added in the liquid phase to microbial cultures was shown to not be an effective method to select styrene tolerant mutants. Conducting ALE under a styrene vapour pressure in batch culture proved to be an effective method for screening to isolate tolerant mutants. The addition of an evaporating volume of styrene that supplied styrene in the vapour phase decreased cell viability over time, in comparison to the same volume of styrene liquid added directly to the medium. This method also has potential to be developed for ALE batch sub-culturing. ALE in chemostat was proved experimentally that a styrene selective environment can be maintained over time via a constant styrene flowrate into the reactor system. This study highlighted that the potential limitations of the use of liquid phase styrene in ALE chemostats due to gas stripping can be overcome by adjustment of styrene flowrate with aqueous concentration method rather than theoretical calculation. This study overcame toxicity challenges of conducting ALE under styrene selection, by the use of a two stage approach to: first adapt the *E. coli* BW25113 strain to evolve resistance to the toxicity effects relating to the hydrophobicity of the aromatic ring and then the reactive double bond. The method used, ethylbenzene, a structurally related compound to styrene, as a selective pressure to evolve an *E. coli* BW25113 population. Styrene tolerant mutants were then screened and isolated from the population under styrene selection. The successful generation of styrene tolerant mutants using this two-stage approach contributes to the field of using ALE to evolve chemical resistance. An *E. coli* BW25113 mutant, mEtBCH 1, was isolated that could grow at a concentration of styrene and ethylbenzene at 4 mmol L^{-1} and 10 mmol L^{-1} respectively. Genome resequencing variant analysis identified 42 mutations in the *E. coli* BW25112 mEtBCH 1 mutant, with the more notable mutations including *acrR*, *acrB*, *rob* and *phoQ* suggesting a potential role of the AcrAB-TolC system in styrene tolerance. The aim to generate a mutant that could grow above the reported toxicity threshold concentration (2.9 mmol L^{-1}) was fulfilled. The implications of this result to the field

of styrene bio-production field could provide the basis for further study into microbial styrene tolerance. Such work could involve further transcriptomic and proteomic analysis to further characterise the mutant generated.

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Appendices

Appendix A

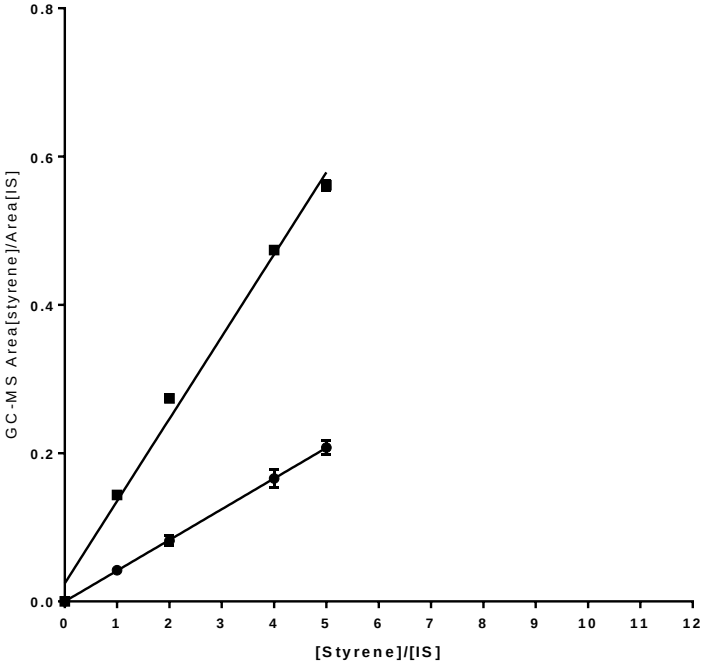


Figure 12.1 Styrene standard curve in hexane solvent + 10mM *tert*-butyl benzene

Appendix B

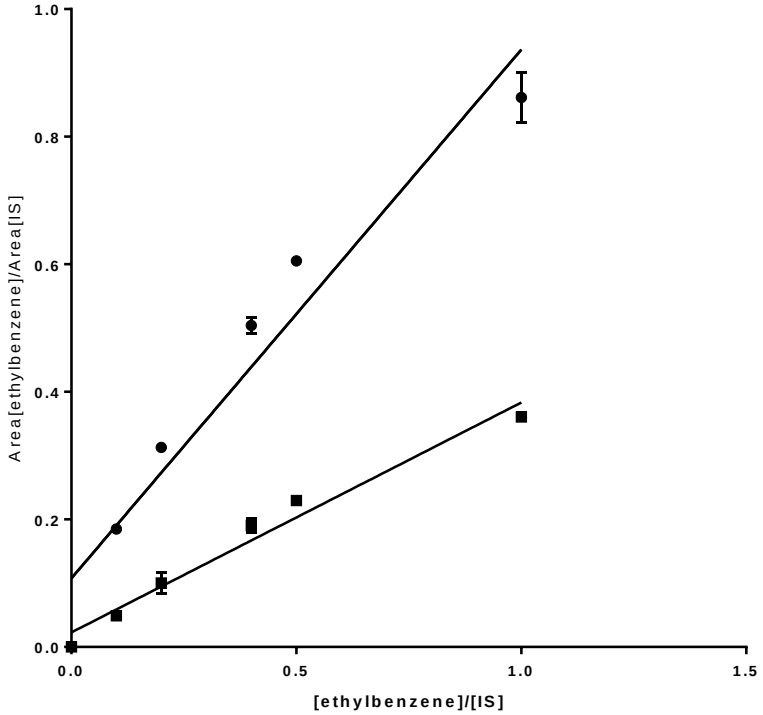


Figure 12.2 Ethylbenzene standard curve in hexane solvent + 10mM *tert*-butyl benzene

Appendix C

Calculation of specific growth rate

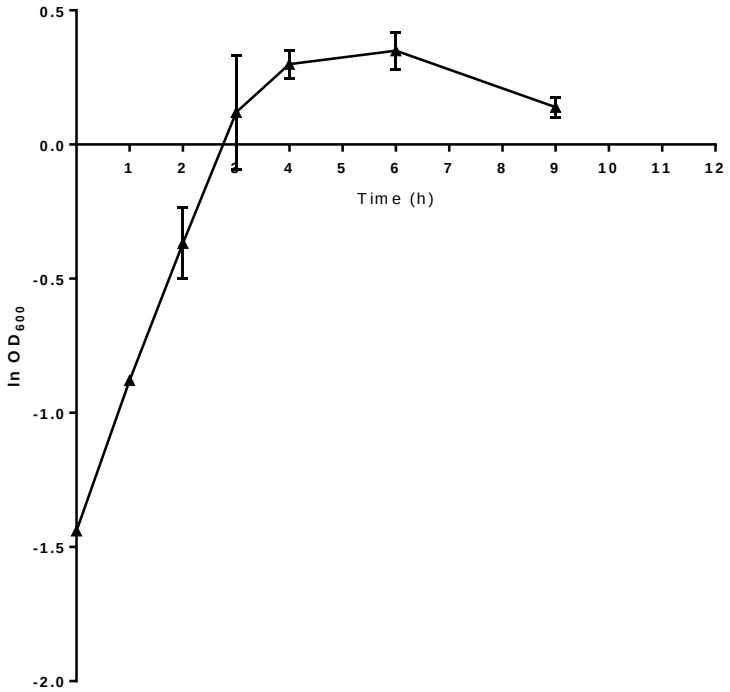


Figure 12.3 ln(OD_{600}) against time *E. coli* BW25113 parent strain at 0 mmol L^{-1} styrene

The specific growth rate, μ, was calculated from the growth OD_{600} measurements of the *E. coli* BW25113 strain in batch culture at 37 °C 250 RPM using at least 4 consecutive time points in exponential phase.

$$\mu = \frac{\Delta \ln OD_{600}}{\Delta t}$$

Appendix D

Calculation of bioreactor volume change

Volume of medium going into reactor / working volume of the reactor/day (v/v d^{-1})

Dilution rate = 0.1 $^{h-1}$

Volume of medium going into reactor in 1 day

= 5.7 mL h^{-1} x 24 h

= 136.8 mL

Therefore, volume of medium going into reactor / working volume of the reactor/day = (v/v d^{-1})

= 136.8 mL/57 mL/1 day

= 2.4 v/ v d^{-1}

Appendix E

Determination of glucose limitation

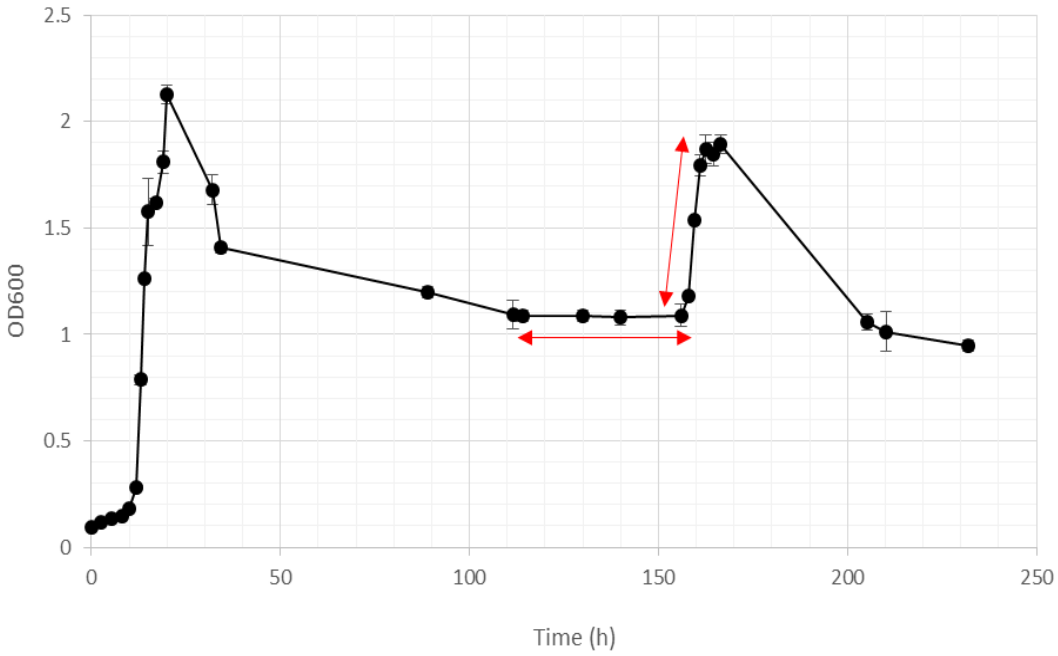


Figure 12.4 Determination of chemostat glucose limitation at 1 g L^{-1}. This was tested by spiking the glucose concentration up to 2 g L-1. A proportionate rise in OD600 was observed confirming the system was glucose limited.

Appendix F Calculation of the solubility limit of styrene in water at 37 °C

The (+) styrene saturated atmosphere aqueous phase concentration was calculated using Henry's Law equation and calculated styrene K_H and $V\rho_{gas}$ values.

$$[C]_{aq} = K_H \bullet V\rho_{gas}$$

K_H -Henry's Law constant for styrene was obtained from the NIST chemistry webbook standard reference data entry 69 which stated it to be:

K_H (25°C) = 0.38.

Temperature dependent constant (K)

(K) = ln (kH))/d(1/T) = 4800

K_H (T) = $\mathbf{k°_H}$ exp(*d***(ln(k_H))**/*d***(1/T)** ((1/T) - 1/(298.15 K))

K_H (37°C) $= 0.24 \frac{mol}{Kg \bullet bar}$

$V_{\rho gas}$ – saturated vapour pressure value for styrene at 37°C calculated as a function of temperature from (Perry, 1954).

$V\rho_{gas}$ = 0.0144 bar

Application of these values to Henry's Law

$[C]_{aq} = K_H \bullet V\rho_{gas}$

$[C]_{aq} = 0.24 \frac{mol}{Kg \bullet bar} \bullet 0.0144$ bar

$[C]_{aq} = 3.46 \times 10^{-3} \frac{mol}{L}$

$= 3.46$ mmol L^{-1}

Appendix G

PIPS Reflective Statement Template

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

This reflective statement describes a professional internship I completed at Hanna IP in Belfast NI. Hanna IP is an intellectual property consultancy firm with a large portfolio of clients in the UK and Ireland. The primary service offered by Hanna IP is the acquisition of patent protection for their client's developing technology, products, and processes. Hanna IP works on several different client projects at a time, meaning that the nature of the work varies day to day. This was made clear at the start of the internship, it was therefore agreed that the continual "project" was for me to gain a better understanding of the profession, with the measurable outcomes being to contribute effectively to the firm and share the findings of my professional development with Hanna IP.

Hanna IP gave me the opportunity to get involved with client projects that would give me a good insight into the profession. In the first couple of days I was shown how to complete a pre-filing patentability search report. These reports are carried out prior to a patent being drafted, to ensure that the idea is patentable against previously published documents (i.e. other patents, peer reviewed papers, articles). My task was to read through the report and the cited documents and formulate an opinion as to whether the invention was patentable. I was also exposed to a wide variety of work that a trainee patent attorney would be given such as examination reports; amending claims; drafting correspondence to clients and the Intellectual Property Office.

The main project outcomes achieved during my time at Hanna IP was that by the end of the internship, I was able to contribute effectively to the firm. From the experience gained, I drafted important documents on behalf of the firm which is evidence that I truly gained an understanding of the work patent attorney's do.

There were also several skills which I developed during my time at Hanna IP. I learnt how to understand and explain complex matters clearly and break down scientific/technical matters to give clear advice. I also learnt that I enjoy being in a workplace environment that involves a client facing role and a good balance of independent and team-based work. This is something I would not have known if I did not do a professional internship of this nature. My internship had a positive influence on my career plans as I now plan to embark on a career in ideas and innovation.