Exploring the toxicity of styrene bio-production & the tolerance of other related organic solvents in *E. coli* by expressing different efflux pump systems

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

November 2018

Declaration

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Hana’a Muheisen

Acknowledgements

In the Name of Allah, the Most Beneficent, the Most Merciful. All the praises and thanks be to Allah.

First, I would like to thank my supervisor Prof. Gill Stephens for her constant support, suggestions and encouragement throughout this project. I would also like to thank my supervisor Prof. Ian Kerr for the support, encouragement, direction and the knowledge he has shared.

My gratitude and thanks to Dr Stephen Hall for his help and knowledge; especially in the bioreactor setup. In addition, I thank my research group; Andy, Luca, Charlotte, Russel, Laura, Valentine, Patricio, Amy and Rachael for their valuable discussion, help, and cooperation in this project.

I gratefully acknowledge the financial support by University of Nottingham/Islamic Development Bank (IDB).

I am sincerely grateful to my beloved parents, my family and friends; in the UK and in Jordan; for their love, endless support, and encouragement.

To Aziz,

my amazing husband,

whose sacrificial care for me and our children

made it possible for me to complete this work

and to my lovely children,

Mohammad, Adam and Talia

Abstract

The toxicity of styrene is the most important obstacle facing its production biologically in *E. coli* using the tools of synthetic biology. One potential solution to counter this toxicity involves manipulating a membrane transporter in *E. coli* to export the chemical outside the cell membrane.

In this project, SrpABC and AcrABTolC were used as candidate solvent pumps to investigate styrene resistance in *E. coli*. The solvent resistant pump, SrpABC, is a membrane protein that transports several organic solvents such as styrene and toluene out of the cell in *Pseudomonas putida* S12. Another important efflux system originally found in *E. coli* is the AcrABTolC system which expels a wide range of substrates from antibiotics to chemical solvents.

SrpABC genes were expressed in *E. coli* MG1655 (DE3), which was modified as part of this project by the incorporation of the λDE3 lysogen into a parental *E. coli* MG1655. Confirmation has been obtained regarding the overexpression of recombinant SrpABC in that strain by conducting RT–PCR analysis and an improvement of the detection of SrpA protein expression by western blotting has also been done. Subsequently, the other efflux system, AcrABTolC, has been overexpressed in *E. coli* BW25113 and confirmed by SDS PAGE and western blot techniques.

Tests of tolerance towards styrene and other organic solvents were conducted in the exponential phase of liquid cultures. The results showed that overexpressed SrpABC in *E. coli* confers more tolerance towards styrene than the AcrAB pump in *E. coli* C43 (DE3) Δ*acr*AB. Besides, the cells were tolerant towards cyclohexane,

cyclohexene, 1, 3-cyclohexadiene and ethylcyclohexane at concentrations 25, 10, 10 and 100 mM, respectively.

The effect of the SrpABC was tested on the bio-production of styrene proceeded in the same strain. Genes encoding PAL2 and FDC1 were cloned into pACYCDuet-1 and pETDuet-1 plasmids which were compatible with the efflux pump systems. The strain bearing the SrpABC pump (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC) produced 8.05 mM styrene using glucose (12 g /L) and L-phenylalanine (2 g / L) in a bioreactor, 30 % higher than the strain without the pump (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc); 6.2 mM. It was concluded that the SrpABC pump could improve styrene production.

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Abbreviations

Acr	Acridine
Amp	Ampicillin
bp	base pair
C	Carbenicillin
Cm	Chloramphenicol
Cti	cis-trans isomerase
DW	Distilled water
$EtBr_2$	ethidium bromide
FDC1	Ferulic acid decarboxylase
h	hour
IPTG	Isopropyl β-d-1-thiogalactopyranoside
kb	kilo base pair
kDa	kilo dalton
LB	Luria bertani
MCS	Multiple cloning site
Min	Minute
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PAL2	Phenylalanine ammonia lyase
PBS	Phosphate buffered saline
pHCA	p-hydroxycinnamic acid
RND	Resistance-Nodulation-Division
RT	Room Temperature
s	second
SDS	sodium dodecyl sulphate
Srp	Solvent resistant pump
TAL	tyrosine ammonia-lyase
TEMED	tetramethylethylenediamine
TUFA	*trans*-unsaturated fatty acids
USD	US dollar

1 Introduction

Styrene is one of the most important chemicals produced throughout the world, as it is a monomer of many commercially significant products, such as polystyrene and styrene-butadiene rubber, used in the rubber and plastics industry (James and Castor, 2000). The annual overall consumption of styrene is approximately 25 million metric tonnes and the approximate worth of the market is 30 billion USD (https://libweb.anglia.ac.uk/referencing/harvard.htm). Since the conventional production of styrene is dependent on oil, renewable and alternative sources must be found to meet future global demand in a low-cost manner to counter the increasing oil costs.

Few studies have explored the production of styrene or one of its derivatives from a biological standpoint. McKenna and Nielsen (2011) demonstrated that the bio-production of styrene could be achieved by the conversion of phenylalanine to styrene *via trans*-cinnamic acid in *E. coli* NST74 (McKenna and Nielsen, 2011). The co-expression of the PAL2 and FDC1 enzymes in *E. coli* NST74 produced titres of up to 260 mg/L, which is below the appropriate level for industrial production (McKenna and Nielsen, 2011). Unfortunately, a crucial limitation of their study was the inability to mitigate the toxicity of bio-produced styrene. After styrene accumulated in the medium to concentrations of approximately 260 mg/L, the growth and further styrene production ceased. Indeed, it is very important to overcome the toxicity of products in the cell to facilitate the production of both styrene and other chemicals. There are several possible ways to mitigate the toxicity of chemicals. One important biological solution may be to use efflux pumps to improve the bio-production of styrene (Dunlop *et al*., 2011). The aim of this project was to test the hypothesis that efflux pumps can be used to overcome styrene toxicity. This method was chosen because efflux pumps have been

previously tested as a solution to the toxicity problems of other chemicals (Chen *et al*., 2013 ; Dunlop *et al*., 2011).

The SrpABC transporter is an efflux pump that can export organic solvents, including styrene and toluene, in *Pseudomonas putida* S12 (Kieboom *et al*., 1998). The SrpABC pump has been chosen in this study for two reasons. The most important reason is that this efflux pump transports solely organic solvents such as styrene (Kieboom *et al*., 1998). In addition, no prior research has investigated its effect on *E. coli* with the bio-production of styrene. Therefore, this is the first study to use the SrpABC efflux pump in the toxicity tolerance of styrene and related solvents in *E. coli*.

The AcrABTolC transporter found in *E. coli* is also an efflux pump with a wide range of substrates including organic solvents and antibiotics. AcrAB overexpression confers slight resistance to exogenously added styrene in *E. coli* (Mingardon *et al.,* 2015), but the effect of the pump on endogenously produced styrene has not been studied yet. Therefore, in this project, the SrpABC efflux pump from *Pseudomonas putida* S12 was used and compared with a recombinant AcrAB pump to explore their effectiveness in the improvement of toxicity tolerance towards styrene and other organic solvents in *E. coli*.

2 Literature review

2.1 Styrene

Styrene, also known as ethylbenzene, vinylbenzene, or phenylethene, is an organic compound with a chemical formula (C_8H_8) (Fig 2.1). It is a derivative of benzene with no colour and a sweet smell. Styrene is named after the Styrax trees (*Styrax platanifolius*) from which sap (a type of benzoin resin) styrene can be extracted (James and Castor, 2000). Due to the excellent properties of polymers manufactured from styrene, including the high strength, low weight, flexibility and the low-cost, it is used to produce various industrial products from plastic containers to vehicle parts (Bond, 1989).

Figure 2.1 Structure of styrene.

Currently, all obtainable styrene is produced from the world's decreasing petroleum resources by the dehydrogenation of petroleum-derived ethylbenzene (Fig 2.2) (McKenna and Nielson, 2011).

ΔH (600 °C) = 124.9 kJ/mol

Figure 2.2 Dehydrogenation of ethylbenzene to styrene.

The worldwide consumption of styrene is approximately 25 million metric tonnes every year with a market of close to 30 billion USD (https://libweb.anglia.ac.uk/referencing/harvard.htm). By 2040, production levels of oil will be decreased to approximately 20% of what we consume currently and the world's population will double, thereby increasing the demand for production of commodity chemicals such as styrene from renewable resources (https://libweb.anglia.ac.uk/referencing/harvard.htm).

2.2 Styrene bio-production

Besides its petrochemical origins, styrene can be produced naturally in small amounts. Several species of Styrax trees produce <0.55% dry weight styrene from L-phenylalanine (Xavier *et al.*, 2005). In addition, the yeast *Penicillium camemberti* has been described as being able to produce low levels of styrene from L-phenylalanine (Pagot *et al*., 2007). Nevertheless, the concentrations of naturally occurring styrene are too low to match the global demands of producing styrene. One possible solution is the use of synthetic biology to design a pathway to produce styrene from sugars as an alternative to petroleum resources. McKenna and Nielson (2011) have demonstrated a biological approach by the design of a pathway that converts phenylalanine to styrene *via* *trans*-cinnamic acid (Fig 2.3).

Figure 2.3 Enzymatic pathways to produce styrene biologically.

They accomplished this by a two-step heterologous pathway, using phenylalanine ammonia lyase 2 (PAL2) from *Arabidopsis thaliana* and ferulic acid decarboxylase 1 (FDC1) from *Saccharomyces cerevisiae.* L-phenylalanine was produced endogenously from glucose and converted to the intermediate *trans*-cinnamate then decarboxylated to form styrene (McKenna and Nielsen, 2011). They have used the L-phenylalanine-overproducing strain, *E. coli* NST74, co-transformed with pSpal2At and pTfdc1Sc plasmids, expressing Pal2 and Fdc1, respectively. PAL enzyme pathway was the rate-limiting step for the styrene pathway in *S. cerevisiae*. However, their study encountered the problem of inability to mitigate the toxicity of bio-produced styrene.

2.2.1 Enzymes involved in the biosynthesis of styrene

2.2.1.1 Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) belongs to the ammonia lyase family, which cleaves carbon-nitrogen bonds and converts L-phenylalanine to ammonia and *trans*-cinnamic acid (Evans *et al*., 1987). PAL is widely found in plants in addition to some yeast and fungi species. It plays an essential function in the link between primary and secondary metabolism in plants by driving the deamination of L-phenylalanine to form *trans*-cinnamic acid. This is a crucial step in the general phenylpropanoid pathway (K Hahlbrock and Scheel, 1989) (Fig 2.4), which represents the source of metabolites (such as *p*-coumaroyl-CoA) in plants, being required for the biosynthesis of lignin and helping in the formation of many other important compounds such as flavonoids, coumarins, and lignins (Reichert *et al*., 2009).

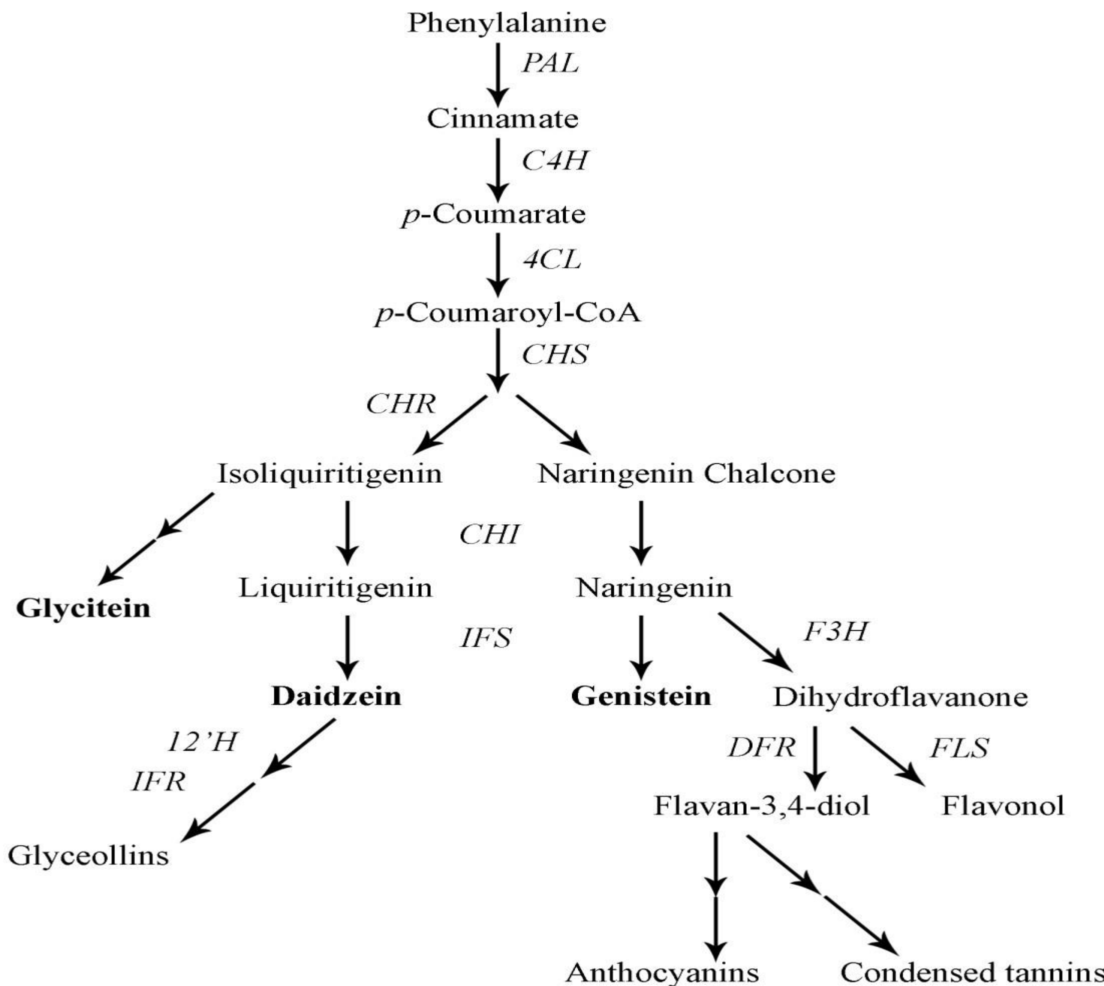


Figure 2.4 Phenylpropanoid pathway Phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxilase (C4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone reductase (CHR), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), flavonol synthase (FLS), isoflavone hydroxylase (12'H), isoflavone synthase (IFS), and isoflavone reductase (IFR). (Vogt, 2010).

Moreover, PAL is an inducible enzyme, and the incorporation of L-phenylalanine in the fermentation medium has been indicated as significantly inducing PAL activity (MacDonald and D'Cunha, 2007). L-Phenylalanine, the substrate of PAL, is produced by the shikimate pathway branches in bacteria, fungi, algae, and plants (Fig 2.5). The enzymes involved in the shikimate pathway are DAHP synthase, chorismate synthase, chorismate mutase, prephenic acid dehydratase 3-dehydroquinate synthase, shikimate dehydrogenase, shikimate kinase and EPSP synthase (Tzin and Galili, 2010).

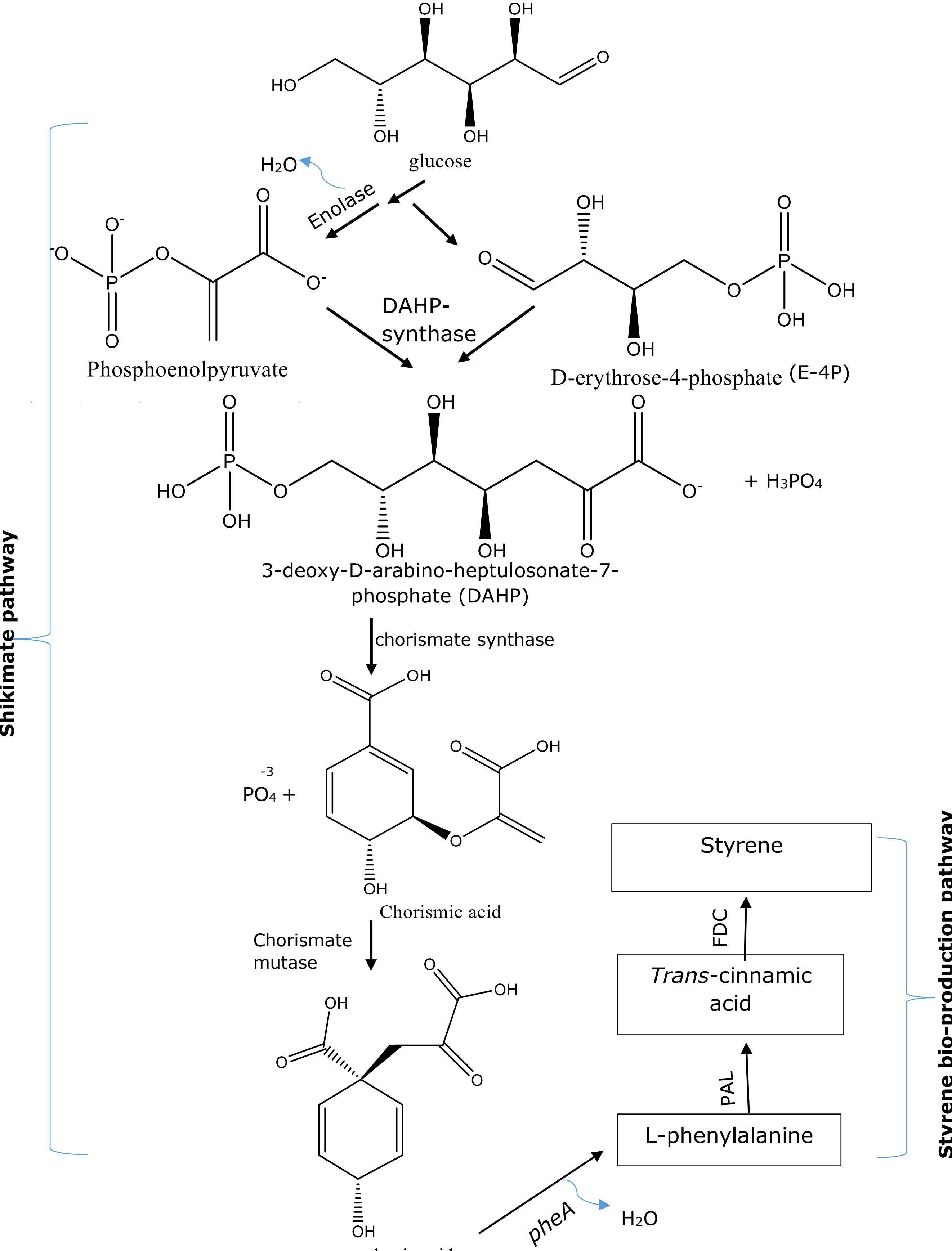


Figure 2.5 Shikimate pathway branches from glucose to L-phenylalanine. Chorismate prephenate dehydratase (*pheA*), 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP).

Some PAL enzymes, in addition to their ability to convert L-phenylalanine to *t*CA, can also use L-tyrosine as a substrate. In such reactions the enzyme is nominated tyrosine ammonia-lyase (TAL) and the reaction product is *p*-hydroxycinnamic acid (*p*HCA) (Rosler *et al*., 1997, Koukol and Conn, 1961).

The three-dimensional structure of PAL has been characterised using X-ray crystallography (Calabrese *et al*., 2004). PAL is composed of four identical subunits that are primarily alpha-helical, with two units of monomers forming a single active site. Identifying its structure allows understanding of the mechanisms that involve PAL-catalysed reactions. Catalysis in PAL may be controlled by the dipole moments of seven different alpha helices associated with the active site (Fig 2.7). The cofactor 3, 5-dihydro-5-methyldiene-4H-imidazol-4-one (MIO) is involved in the reaction and sits atop the positive pole of three polar helices in the active site (Calabrese *et al*., 2004).

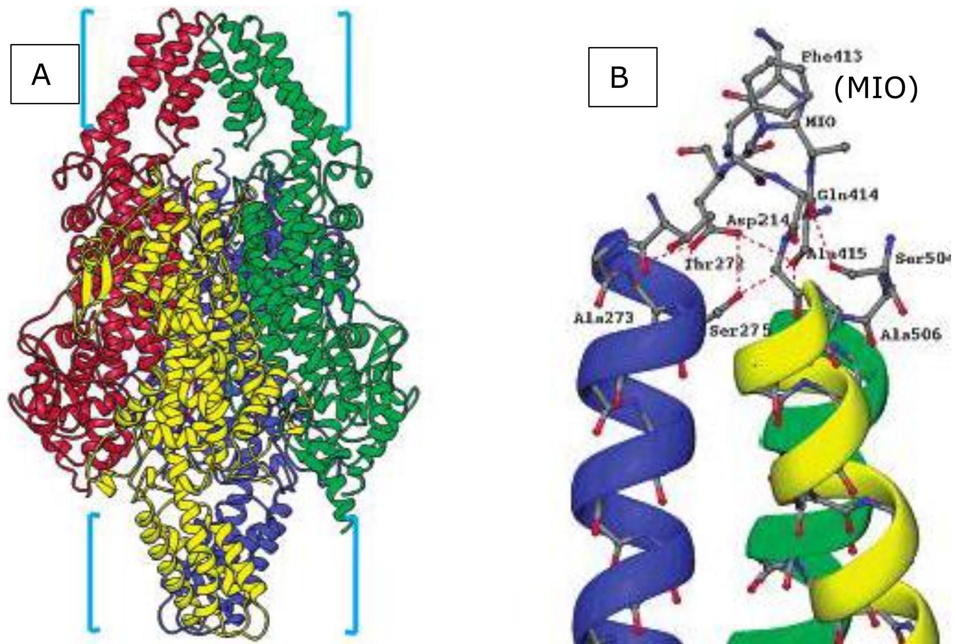


Figure 2.6 Crystal structure of PAL. A. Quaternary structure of PAL illustrates the four monomers of PAL; the brackets represent the residues that form a fan shape. B. PAL tertiary structure which demonstrate the three core helices that lead to the active site and cooperate with MIO-cofactor to increase the electrophilicity of the active site. MIO is reported to attack the aromatic ring of L-phenylalanine, which activates the C-H bond and leads to cleavage of the bond (Calabrese *et al*., 2004).

2.2.1.2 Aromatic acid decarboxylases

Ferulic acid, phenylacrylic acid, and phenolic acid decarboxylases are found widely in fungi, yeast and bacterial species (Bhuiya *et al.*, 2015). They catalyse the decarboxylation of many aromatic acids including cinnamic, *p*-coumaric and ferulic acids (Clausen *et al.*, 1994).

Ferulic acid decarboxylase (FDC) catalyses the decarboxylation of ferulic acid to 4-hydroxy-3-methoxystyrene (Huang *et al*., 1994) (Fig 2.7).

FDC1 $+CO_2$

Ferulic acid 4-hydroxy-3-methoxystyrene

Figure 2.7 Conversion of ferulic acid to hydroxy-methoxy styrene by FDC1.

FDC is found in several strains of yeast that decontaminate antimicrobial compounds that are normally used as food preservatives and flavours, such as cinnamic acid and sorbic acid (Mukai et al., 2010a), by decarboxylation to yield CO_2 as well as products such as styrene.

A number of decarboxylase enzymes working on their substrate ferulic acid were found in different microorganisms such as *P. fluorescens* (Huang *et al.*, 1994)(Huang *et al.* 1994), *B. pumilus* (Zago *et al*., 1995), *C. guilliermondii* (Huang *et al*., 2012), *Enterobacter sp*. Px6-4 (Gu *et al*., 2011), and *B. subtilis* (Cavin *et al.,* 1998) and have been recognised and characterised.

In addition, Lee *et al*. (2015) have provided evidence that using random and site-directed mutagenesis can enhance the bio-catalytic activity of FDCs for producing styrene derivatives from plant-originated phenolic acids, such as ferulic acid (Lee *et al.*, 2015).

Phenylacrylic acid decarboxylase (PAD) is a flavin mononucleotide (FMN)-containing protein that is widely found in microorganisms and fungi (Clausen *et al.*, 1994). It has been shown that cinnamic, *p*-coumaric (Fig 2.8) and ferulic acids are substrates for *S. cerevisiae* PAD (Clausen *et al.*, 1994). In *A. niger,* the decarboxylation activity is encoded by the gene *pad*A1 (Stratford *et al.*, 2012).

Figure 2.8 Decarboxylation of *p*-coumaric acid by PAD1. PAD1 undertakes the decarboxylation of *p*-coumaric to *p*-hydroxystyrene. FMN as a coenzyme for a number of oxidative enzymes, serving as an electron carrier by being by being alternately oxidized (FMN) and reduced (FMNH2).

PAD is also involved in the decarboxylation of sorbic and cinnamic acids into 1, 3-pentadiene and styrene, respectively (Fig 2.9) in germinating spores of *Aspergillus niger* (Plumridge *et al*., 2010).

O
COOH
OH
PAD
Sorbic acid
1, 3-pentadiene
COOH
O
PAD
OH
trans-cinnamic
Styrene

Figure 2.9 Decarboxylation reactions of the PAD1 enzyme. PAD1 decarboxylases sorbic acid and cinnamic acid to 1, 3-pentadiene and styrene, respectively (Plumridge *et al*., 2010).

Previous studies suggested that activity of both PAD1 and PadA1 also requires the expression of additional enzymes naturally encoded by adjacent genes. These enzymes are FDC1 and OhbA1 in *S. cerevisiae* and *A. niger*, respectively (Mukai et al., 2010b ; Plumridge *et al*., 2010). OhbA1 refers to a putative 3-octaprenyl-4-hydroxybenzoic acid decarboxylase. Initially it was believed that PAD1 and PadA1 were the main catalysts involved in sorbic and cinnamic acid decarboxylation and that FDC1 and OhbA1 were just co-catalysts (Mukai *et al*., 2010; Plumridge *et al*., 2007).

Recently, it has been shown that PAD1 utilises FMN as a substrate, but does not work as a decarboxylase. Instead, PAD1 catalyses the production of a unique,

diffusible cofactor required for decarboxylase activity of FDC1. Co-expression of PAD1 and FDC1 in *S. cerevisiae* results in FDC1 with high cofactor occupancy and an ability to decarboxylate a variety of phenylacrylic acids (Lin *et al*., 2015). Moreover, Richard *et al*., (2015) have reported the expression of the PAD1 and the FDC1 of *S. cerevisiae* in *E. coli*. The efficient activity of cinnamic acid decarboxylation in *E. coli* depends only on FDC1 over-expression and is not dependent on the co-expression of PAD1. The expression of the *S. cerevisiae* PAD1 in *E. coli* is not responsible for cinnamic acid decarboxylation activity, however, the expression of FDC1 alone resulted in activity suggesting that the PAD1 is not required for activity in *E. coli* (Richard *et al*., 2015).

2.2.2 Previous studies on styrene bio-production

Previously, Sariaslani (2007) demonstrated the production of aromatic chemicals from glucose, such as *p*-hydroxycinnamic acid (*p*HCA) and 4-hydroxystyrene (*p*HS) which are less toxic than styrene. The production of *p*HS from glucose depended on the overexpression of two enzymes, PAL to deaminate tyrosine to *p*HCA and a decarboxylase enzyme to convert *p*HCA to *p*HS (Fig 2.8). Decarboxylation of *p*HCA to *p*HS was achieved by overexpression of the *p*HCA decarboxylase (*pdc*) gene of *Lactobacillus plantarum* in the same *E. coli* strain under the control of T7 *lac* promoter (Ben-Bassat *et al*., 2003). Both *p*HCA and *p*HS are toxic to *E. coli*, and bacterial growth was abolished at 10 and 0.5 g/L concentrations, respectively. To mitigate this problem, Sariaslani (2007) decoupled the formation of the toxic product (*p*HS) from the first enzymatic step. The route consisted of a fermentation step that converts glucose to tyrosine using a tyrosine overproducing *E. coli* strain followed by another reaction that deaminates tyrosine to *p*HCA using *E*. *coli* cells overexpressing the PAL gene (Fig 2.10). The final step was a chemical decarboxylation of *p*HCA to produce *p*HS.

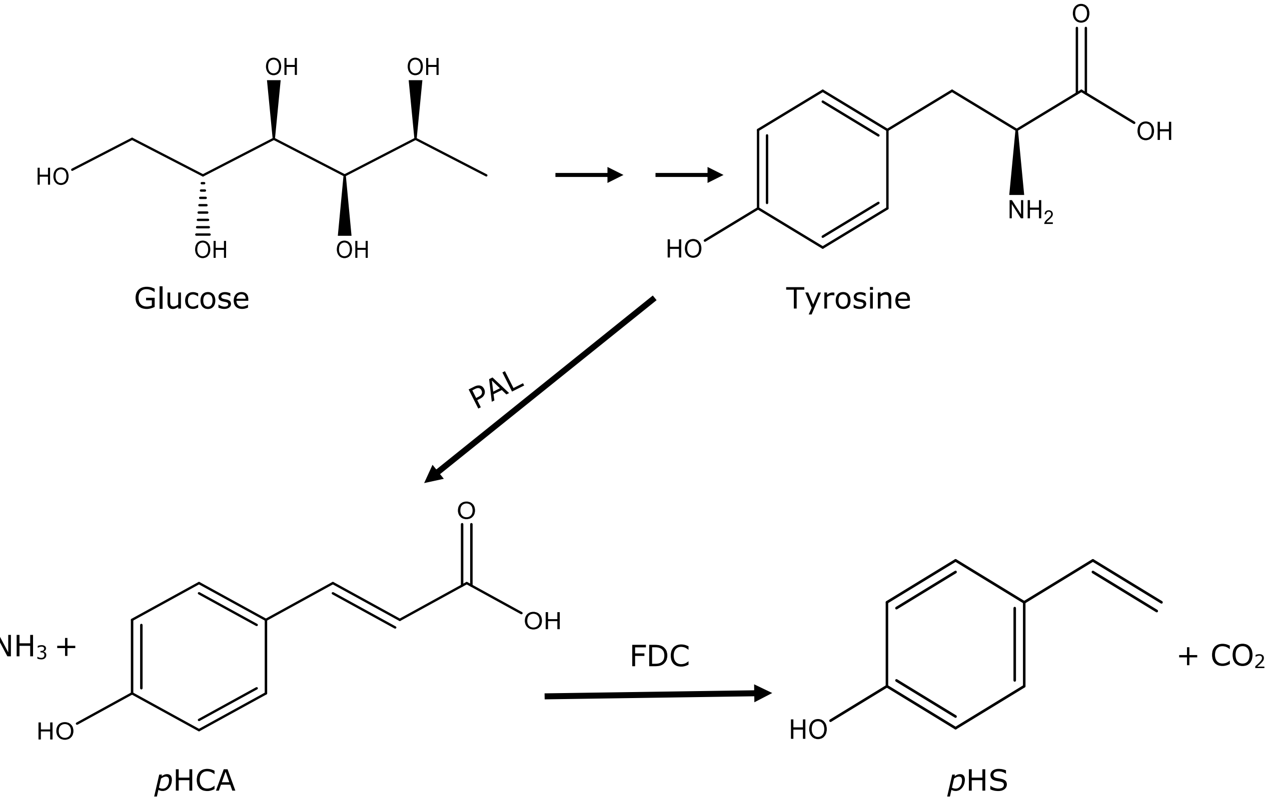


Figure 2.10 Enzymatic steps for the production of *p*HS from glucose. Production of *p*HS from glucose depended on the overexpression of two enzymes, PAL to deaminate tyrosine to *p*HCA and a decarboxylase enzyme to convert *p*HCA to *p*HS (Sariaslani, 2007).

Recently, styrene biosynthesis optimisation, such as codon and plasmid optimisation, enzyme screening, and *in situ* fermentation, was performed in *E. coli* BL21 (DE3) (Liu *et al*., 2018). The screening of isoenzymes of the enzyme PAL was studied from *Arabidopsis thaliana* (AtPAL2), *Artemisia annual* (AaPAL), *Petroselinum crispum* (PcPAL), and *Fagopyrum tataricum* (FtPAL). After codon optimisation, AtPAL2 was the most effective, and the recombinant strain resulted in styrene production of 55 mg/L (Liu *et al*., 2018).

In addition, Liu *et al*., (2018) have performed the plasmid optimisation, which improved styrene production to 103 mg/L. Besides, two upstream shikimate pathway genes, *aroF* and *pheA*, were overexpressed in the engineered strain, which was able to produce styrene up to 210 mg/L. Afterwards, they have

demonstrated that the co-expression of *tktA* and *ppsA* genes, responsible for the production of DAHP in the shikimate pathway, increased styrene production to 275 mg/L. Moreover, Liu *et al*., (2018) have used isopropyl myristate solvent as an *in situ* toxic product removal, styrene was produced of about 350 mg/L after 48 h of shake-flask fermentation, demonstrating a 636% improvement, compared with that produced in the original strain (Liu *et al*., 2018).

Styrene is toxic to microorganisms since it partitions preferentially in the cytoplasmic membrane. This effect is intricately related to the logarithm of the partition coefficient in *n*-octanol and water (log P_{ow}) (Ramos *et al*., 2002). Chemicals that have a log P_{ow} lower than 4.0, such as styrene (log P_{ow} 2.95), benzene (log P_{ow} 2.13), octanol (log P_{ow} 2.92), toluene (log P_{ow} 2.69), and xylenes (log P_{ow} 3.12–3.2), are very toxic for microorganisms (Ramos *et al*., 2002). Styrene can accumulate in the hydrophobic core of the membranes in microorganisms increasing the membrane fluidity and reducing the integrity of the membrane, leading to dissipation of ionic and pH gradients and loss of metabolites, proteins, and lipids (Salter and Kell, 1995). Ultimately, the leakage of cellular components leads to cell lysis and death (Weber *et al*., 1993). Therefore, the limiting obstacle in the bio-production is styrene toxicity (Liu *et al*., 2018). Possible toxicity solutions are discussed in the following section.

2.3 Chemical & biological solutions to counter styrene toxicity

There are several strategies to circumvent the effects of toxicity. One option is the use of *in situ* product removal (ISPR) is to employ an applicable partitioning method to eliminate the toxic product as it is produced from the culture, thus eliminating its possible accumulation to toxic inhibitory titres (McKenna *et al.*,

2014) such as gas stripping and solvent extraction. In solvent extraction, solvents such as *n*-dodecane, *n*-hexadecane, and BEHP (bis 2-ethyl hexyl phthalate) that are biocompatible and show high equilibrium partitioning of styrene over water (greater than 5) were examined in shake culture of *E. coli* NST74 to measure their ability to enhance the production of styrene (McKenna *et al*., 2015). Styrene production was enhanced in the presence of both *n*-dodecane and *n*-hexadecane, by 200% and 170%, respectively. However, when using BEHP, styrene production increased to 836 ± 64 mg/L (McKenna *et al*., 2015).

Gas stripping is the physical separation process during which one or more components are removed from a liquid stream by a vapour stream (https://libweb.anglia.ac.uk/referencing/harvard.htm). McKenna *et al*., (2015) demonstrated that gas stripping was effective for the prevention of styrene accumulation in the aqueous phase for up to 29 h with an average concentration of 68 ± 15 mg/L being maintained (McKenna *et al*., 2015). Subsequently, they have ceased the gas stripping to allow styrene to accumulate until all glucose was consumed then started gas stripping again to determine the cumulative volumetric styrene production, which reached a maximum of 561 ± 15 mg/L.

Although both processes were effective at reducing the toxic effects accompanying styrene production and accumulation in cultures, solvent extraction provided the maximum overall increase in production. Besides, McKenna *et al*., (2015) highlighted that there were limiting factors that exist for each method; solvent extraction is restricted by the necessity for other possible inhibitory solvents and additional downstream separation units (e.g., distillation) to maximise the purification of the product and extract the solvent. In the case of

gas stripping, at low aeration rates, the continuous air supply excessively diluted the styrene content leaving off-gas in the bioreactor, therefore at the industrial scale, this activity would be translated into increased energy requirements and reduced efficiency associated with capturing the product vapours for example *via* condensation (McKenna *et al*., 2014).

There are several possible biological mechanisms leading to solvent-tolerance in Gram-negative bacteria, including alterations in the membrane fatty acids and phospholipids (Heipieper *et al*., 2001) and formation of vesicles loaded with toxic compounds (Weber *et al*., 1994). Another mechanism a microorganism can use when exposed to toxic solvents is using efflux pumps to directly remove the toxic compounds from the cell. Some bacterial strains can use more than one mechanism to adapt to the exposure towards toxic solvents (Table 2.1) (Ramos *et al*., 2002).

Table 2.1 *Pseudomonas* solvent-tolerant strains and their tolerance mechanisms (Ramos *et al*., 2002).

Strain	*cis*-to-*trans* isomerization	Efflux pumps	Vesicle formation
DOT T1E	+	+	+
S12	+	+	n.d.
G73	-	+	n.d.
MTB6	+	+	n.d.

+ indicates that the character has been found in the strain.
− indicates that the character has not been found in the strain.
n.d.: not determined.

2.3.2.1 *Cis-trans* isomerization of unsaturated fatty acids

Cis-trans isomerization of unsaturated fatty acids facilitates the esterified fatty acids to adopt a denser packing in the membrane, which abolishes the fluidising effect of hydrophobic organic compounds (Kieboom *et al*., 1998) (Fig 2.11). In a similar way, the membrane is made more rigid by increasing the

abundance of saturated fatty acids relative to the unsaturated counterparts (Weber *et al*., 1993).

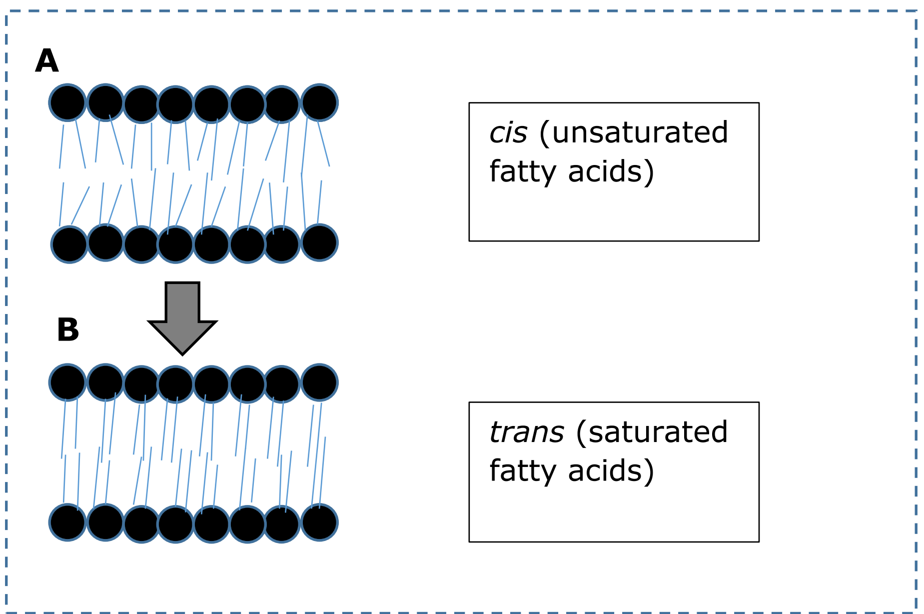


Figure 2.11 *Cis-trans* isomerization of unsaturated fatty acids in bacteria. *Cis-trans* isomerization of unsaturated fatty acids enables the esterified fatty acids to adopt a denser packing in the membrane (B). In a similar manner, the membrane is made more rigid by increasing the abundance of saturated fatty acids relative to the unsaturated counterparts (Ramos *et al.,* 2015).

Pseudomonas and *Vibrio* strains use this mechanism as an immediate response to exposure of organic compounds, including methanol, ethanol, butanol, phenol, hexanol, *p*-cresol, 4-chlorophenol, 3-nitrotoluene, toluene, octanol, and 2, 4-dichlorophenol (Heipieper *et al*., 2003). The *trans*-isomers are synthesised directly from the *cis*-isomers, with no shift in the position of the double bond, immediately after exposure (<1 min) (Heipieper *et al*., 2003). Similarly, an increase in *trans*-unsaturated fatty acids and the degree of fatty acid saturation was detected with *P. putida* when exposed to toluene, phenol, and *o*-xylene (Heipieper *et al.*, 2001, Heipieper *et al*., 1994). In the case of ethanol exposure, an increase in *trans*-unsaturated fatty acids occurred, but there was a decrease in the degree of overall fatty acid saturation (Heipieper and de Bont, 1994). The

arrangement increases in *trans*-unsaturated fatty acid in response to the exposure to various organic chemicals is rather consistent, whereas the shift in the degree of saturation is inconsistent/unclear and necessitates further investigation (Heipieper *et al.*, 1994); (Weber *et al.,* 1994).

Even though the effective *cis-trans*-isomerization adaptation mechanism is not a characteristic in Gram-negative bacteria, it apparently confers high capability of mainly *Pseudomonas* and *Vibrio* species overriding and displaying a great stability in various difficult environmental circumstances. One study has confirmed this idea by the increased tolerance towards octanoic acid of an engineered *E*. *coli* strain expressing the *cis-trans* isomerase (Cti) enzyme from *P. aeruginosa,* which synthesises *trans*-unsaturated fatty acids (TUFA) in *E. coli* membranes (Tan *et al*., 2016). The expression of Cti enhanced the adaptability of the *E. coli* strain to styrene toxicity. The engineered strain had significantly enhanced tolerance to styrene (17%) and toluene (20%). This improved tolerance of styrene has facilitated increased styrene production in the engineered strain more than the control strain that only expressed the styrene production pathway and did not produce TUFA. In addition, the study indicated that when L-phenylalanine was added to the minimal medium, the TUFA-producing strain produced 10.4% more styrene than the control strain. These results have demonstrated the effectiveness of TUFA production in increasing both tolerance and production of biorenewable chemicals (Tan *et al*., 2016).

2.3.2.2 Release of membrane vesicles

The second mechanism that can mitigate solvent tolerance is the formation of vesicles loaded with toxic compounds (Weber *et al.*, 1994). The membrane vesicles (MVs) of size 20-300 nm are primarily made up of components of the

periplasmic and the outer membrane (McBroom and Kuehn, 2007). Through pinching off and expanding the outer membrane that is made up of some periplasmic contents, the MVs are released to the outside environment from the outer membrane of Gram-negative bacteria (Mashburn-Warren *et al*., 2008). Based on the work of Kobayashi (2000), an observation was made that the MVs could be used to remove the poisonous chemical from the membrane (Kobayashi *et al*., 2000). This observation was made after the volume of toluene in the membrane of the wild-type *P. putida IH-2000* was reduced to approximately half the amount that had been collected inside the toluene-sensitive strain after the MVs were released. The MVs that were released from *P. putida* IH-2000 had 0.625 mol toluene/ mol lipid after 0.5 h, but after 2 hours the amount reduced to 0.16 mol toluene/ mol lipid due to toluene exposure (Kobayashi *et al*., 2000). Consequently, the production of MVs that have toxic components helps in a fast reaction to toxic chemicals, which enables the cell to avoid accumulation of the compound in the membrane. Moreover, through modification of the membrane composition and surface properties, it helps the cell to adapt (Manning and Kuehn, 2011). However, a huge amount of membrane components are lost from the cells and as a result, this increases the load for the biosynthesis of the material shed off from the periplasm and the outer membrane (Schwechheimer and Kuehn, 2015).

In bacteria having both adaptation mechanisms, most likely, they occur at the same time in a stress situation because of their passive activation. This phenomenon of an associated increase in surface hydrophobicity as well as for the *trans/cis* ratio was already observed for bacteria in stress situations (Löffler et al., 2010); (Naether *et al*., 2013).

2.3.2.3 Efflux pumps

Efflux pumps are membrane transporters that recognise and export toxic compounds from the cell using the proton or sodium motive force or ATP hydrolysis in the case of ABC transporters. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms. Some examples of efflux pumps and their substrates are found in Table (2.2).

Table 2.2 Summary of efflux pumps & their substrates.

	Bacterial strains	**Efflux pump**	**Substrate range**	**Reference**
1	*Pseudomonas putida* S12	SrpABC	Styrene, toluene, hexane & octanol	(Kieboom *et al*., 1998a)
2	*P. putida* DOT T1E	TtgABC	Ampicillin, carbenicillin, tetracycline, nalidixic acid, chloramphenicol, styrene, isopropylbenzene, *p*-xylene & toluene	(Rojas *et al*., 2001)
3	*P. putida* DOT T1E	TtgDEF	Toluene, styrene	(Rojas *et al*., 2001)
4	*P. putida* DOT T1E	TtgGHI	Antibiotics & solvents	(Rojas *et al*., 2001)
5	*P. aeruginosa*	MexAB-OprM	β-lactams, β-lactam inhibitors, tetracycline, chloramphenicol, novobiocin, macrolides, fluoroquinolones, crystal violet, ethidium bromide, acriflavin, SDS, thiolactomycin, acylated homoserine lactones, aromatic hydrocarbons, cerulenin	(Keith *et al.*, 1996)
6	*P. aeruginosa*	MexCD-OprJ	β-lactams, fluoroquinolones, tetracycline, chloramphenicol, novobiocin, macrolides, crystal	(Keith *et al*., 1996)

			violet, ethidium bromide, acriflavin, SDS, aromatic hydrocarbons, triclosan	
7	*P. aeruginosa*	MexEF-OprN	fluoroquinolones, tetracycline, chloramphenicol, aromatic hydrocarbons, trimethoprim, triclosan	(Keith *et al.*, 1996)
8	*P. putida* KT2442	MepABC	Toluene, styrene, *ρ*-xylene, and antibiotics	*(Fukumori et al.*, 1998)

Efflux proteins have been classified into six different families: (1) major facilitator superfamily (MFS); (2) ATP-binding cassette (ABC) superfamily; (3) small multidrug resistance (SMR) family; (4) resistance-nodulation-division (RND) superfamily; (5) multidrug and toxic compound extrusion family (Nikaido and Takatsuka, 2009) and (6) proteobacterial antimicrobial compound efflux (PACE) (Hassan *et al*., 2018).

Recent advances in DNA technology and the advent of the genomic era have led to the identification of numerous new members of the above pump families, and the ubiquitous nature of efflux pumps is remarkable. Transporters that efflux multiple substrates, including antibiotics, have not evolved in response to the stresses of the antibiotic era (Nikaido and Takatsuka, 2009). All studied bacterial genomes contain several different efflux pumps, which indicate their ancestral origins. It has been estimated that ~5%–10% of all bacterial genes are involved in transport and a large proportion of these genes encode efflux pumps (Nikaido and Takatsuka, 2009).

The best-studied efflux pumps are those from the RND family that play an important role in the intrinsic resistance towards antibiotics in Gram-negative bacteria (Wong *et al*., 2014).

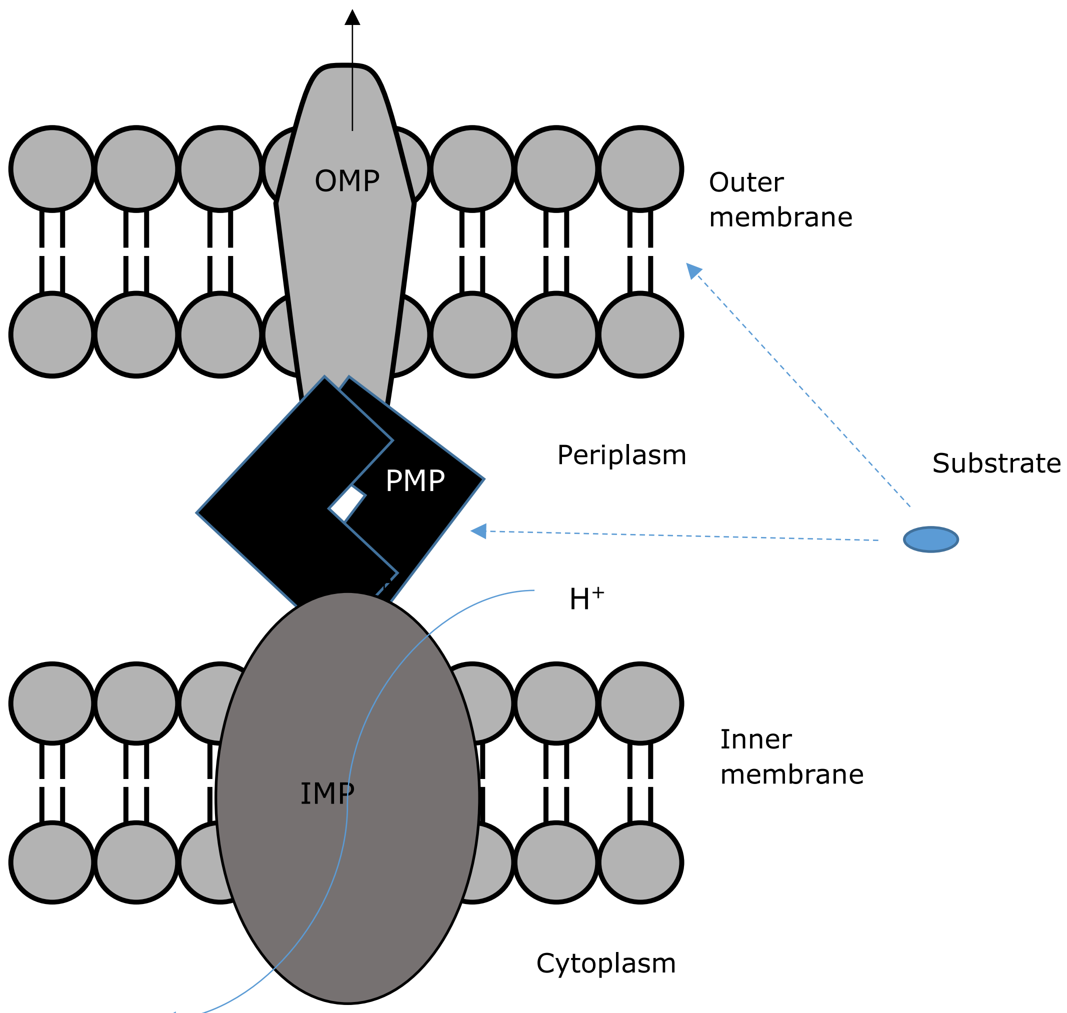


Figure 2.12 RND pump structure. A demonstration of the tripartite efflux pump AcrAB-TolC, which consists of an outer membrane protein TolC, an inner membrane protein (IMP) AcrB, and a periplasmic membrane protein (PMP) AcrA. The system utilises its energy from a proton motive force to expel substrates.

RND pumps are composed of three components (Fig 2.12): the inner membrane protein (IMP), which is responsible for the substrate recognition; a periplasmic membrane fusion protein (MFP) anchored in the cytoplasmic membrane; and an outer membrane protein (OMP) that forms a channel (Nikaido and Takatsuka, 2009).

Solvent export pumps that could promote resistance in *E. coli* were used in this project, as described below.

SrpABC membrane protein

An energy-dependent active efflux pump is found in the membrane of the Gram-negative *Pseudomonas putida* S12 (Kieboom *et al*., 1998). This organism can withstand toxic concentrations of toluene and other organic solvents such as styrene (1% v/v) by at least two mechanisms: First, *cis*- to *trans*-isomerization of unsaturated fatty acids of the membrane (Weber *et al*., 1993). Second, *Pseudomonas putida* S12 uses the SrpABC efflux pump that belongs to the RND family of transporters. SrpABC consists of an inner membrane transporter (SrpB), an outer membrane channel (SrpC), and a periplasmic linker protein (SrpA). It was first discovered by Kieboom *et al*. (1998) who employed transposon mutagenesis to generate a library of genetic mutations in *P. putida* before screening the library for solvent tolerance (Kieboom *et al*., 1998c).These mutations caused a loss of solvent toxicity tolerance of the strain by the inability of the mutated colonies to grow in the presence of the toxic solvents, such as toluene, propyl-benzene, hexane, cyclohexane, ethylbenzene, *p*-xylene, and di-methyl-phthalate (Kieboom *et al*., 1998a). The genes (*srp*ABC) for this solvent efflux system were subsequently cloned, sequenced, and shown to impart the solvent-resistant phenotype to solvent-sensitive *Psudomonas putida* strains (Kieboom *et al*., 1998d, Kieboom *et al*., 1998b).

Moreover, Kieboom *et al*. (1998) revealed that the *srp* promoter represented by the expression of β-galactosidase could be induced by several toxic organic solvents, such as toluene, benzene, styrene, *p*-xylene, ethylbenzene, and propyl-benzene. Induction by toluene resulted in an increase in gene expression of 15 to 17-doublings, *srp*-*lac*Z expression was observed after 30 minutes after addition of the solvent (Kieboom *et al*., 1998a). The aromaticity and the charge

are not required for organic solvents to act as inducers. In addition, the *srp*ABC operon is not induced by an environmental stress such as pH and NaCl or heavy metals (Kieboom *et al*., 1998).

Sequence analysis shows that the efflux pump, SrpABC, from *P. putida S12* is highly homologous to other known solvent/antibiotic transporters, including MepABC, TtgABC (Kieboom and de Bont, 2001), and MexAB/OprM in *P. aeruginosa* (Kieboom *et al*., 1998). For example, SrpABC is homologous to MexAB/OprM in *P. aeruginosa* and SrpA, SrpB, and SrpC are 57.8%, 64.4%, and 58.5% identical to MexA, MexB, and OprM, respectively (Ramos *et al*., 2002).

Bui le *et al*. (2015) were the first to introduce the *srp*ABC gene into different strains of *E. coli.* They used it to improve butanol production tolerance in *E. coli* by engineering *E. coli* MG1655 (DE3) strain under the control of the *lac* promoter and investigated the effect of the newly engineered strain on butanol tolerance. Subsequently, they concluded that the cloned MG1655 (DE3) strain expanded the tolerance limit of *n*-butanol (Bui le *et al*., 2015).

Further, *srp*ABC genes from *P. putida* S12 were also cloned and inserted into a solvent sensitive *E. coli* strain by Garikipati and Peeples (2015) to transfer solvent tolerance. Recombinant *E. coli* strains carrying either plasmid pJG001 or pJG002, containing *srp*ABC genes in low-copy-number or high-copy number, respectively, grew in the presence of a saturated concentration of toluene. Both strains were more tolerant to 1% (v/v) of toxic solvents (decanol and hexane), reaching similar cell density as the control where no solvent was added (Janardhan Garikipati and Peeples, 2015).

AcrAB-TolC efflux system

AcrAB-TolC is a multidrug efflux system found in Gram-negative bacteria, such as *E. coli* and *Salmonella typhimurium.* The pump has been reported to have a wide range of substrates including antibiotics and organic solvents. In *E. coli*, the pump expels chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid, and β-lactam antibiotics (Piddock, 2006). In addition, the pump has shown an ability to expel other organic solvents, such as heptane and hexane (Takatsuka *et al*., 2010).

AcrAB-TolC comprises of an outer membrane channel TolC, an inner membrane protein AcrB, and a periplasmic protein AcrA. Previous electron microscopy studies of the AcrAB-TolC pump have revealed the overall shape of the pump and the relative arrangement of its components (Du *et al*., 2014) (Fig 2.13).

The AcrABTolC efflux pump has a stoichiometric ratio of 3:3:6:3 for AcrB:AcrZ:AcrA: TolC within the fully assembled pump. TolC has a transmembrane β-barrel and a periplasmic α-helical domain, whereas AcrA has a long α-helical hairpin, a lipoyl domain, a small β-barrel, and a membrane-proximal domain. AcrB has a periplasmic docking, porter and transmembrane domains, and the proton pathway for the antiport system is located in the transmembrane domain (Seeger *et al*., 2009). AcrZ is an α-helical protein that binds to the transmembrane domain of AcrB and is thought to affect the selectivity of AcrB for certain substrates (Hobbs *et al.,* 2012).

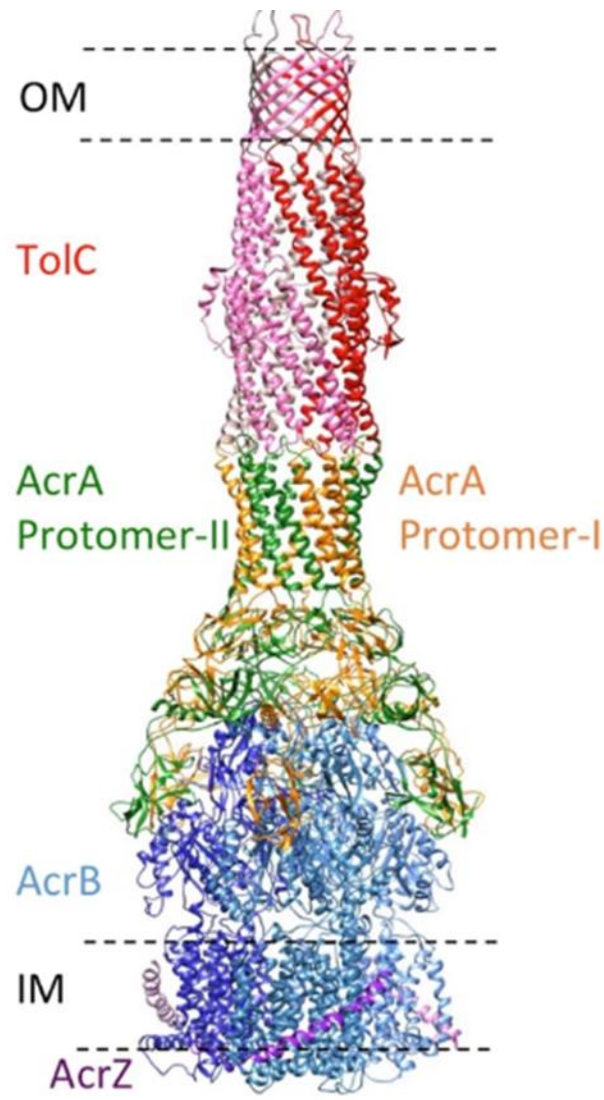


Figure 2.13 Asymmetric crystal structure of the multidrug efflux pump AcrABZ-TolC. The four subunits components of the pump are coloured; starting from the outer membrane component: TolC (red), AcrA (orange and green), AcrB (blue), and AcrZ (purple) that are located in the inner membrane (Wang *et al*., 2017).

Many studies have been conducted regarding the structure of this pump and how the efflux pump subunits interact with each other. Du *et al*. (2014) have observed in their model that there is no direct interaction between TolC and AcrB in the assembly, in contradiction to previous models. Instead, the two proteins are bridged in the periplasm entirely through AcrA. They have also shown that the assembly comprises an AcrB trimer, an AcrA hexamer and a TolC trimer (Du *et al.*, 2014).

The entry of chemicals into AcrB is still not clear (Du *et al*., 2015), but there is an evidence for substrate entry from the periplasm (Murakami *et al.*, 2006) or from the outer leaflet of the inner membrane (Seeger *et al*., 2006). Nevertheless, the efflux mechanism is relatively well understood. During the transport cycle in the porter domain, the three AcrB subunits have different conformational states, namely the access, binding, and extrusion states (i.e., one will be in the access state, one in the binding state, and the other in the extrusion state) (Murakami *et al.,* 2006). In the access state, the drug-binding pocket is expanded, the chemical enters the subunit, and a proton from the periplasm binds to the subunit. In the binding state, the chemical binds to the hydrophobic drug-binding pocket in the porter domain. Finally, in the extrusion phase, conformational changes lead to a reduction in the size of the drug-binding pocket, the chemical is released into the top funnel towards TolC, and the proton is released to the cytoplasm (Seeger *et al*., 2006). Thus, each subunit will transition from the access to the binding state and then to the extrusion state, sequentially.

Ruiz and Levy have found that the inactivation or inhibition of AcrABTolC activates the *acr*AB promoter. The activation was mediated by three loci known to regulate *acr*AB: *acr*R, *sox*RS and *mar*A (Ruiz and Levy, 2014). Expression of *sox*S and *mar*A was increased in the absence of the AcrAB-TolC pump. The role of AcrR encoded by *acr*R is a repressor of the *acr*AB operon (Ruiz and Levy, 2014). Overexpression of *acr*AB by the mutation in *acr*R gives the strain the ability to grow in the presence of cyclohexane (White *et al*., 1997).

Recently, Mingardon *et al*. (2015) demonstrated that *E. coli* cells harbouring the AcrAB-TolC efflux pump had increased growth in the presence of up to 120 mg/L of exogenously added styrene and displayed enhanced 1-hexene production relative to the *acr*B deletion mutant. The previously mentioned results suggest that manipulating membrane transporters may facilitate increased styrene production (Mingardon *et al*., 2015).

2.3.2.3.1 Overexpression of membrane proteins

During this project it was necessary to express membrane proteins in *E. coli.* Many studies have reported the difficulty of their overexpression and the ambiguity of the optimal system for overproduction. For example, Miroux and Walker (1996) investigated the expression of seven membrane proteins in *E. coli.* After the induction of the expression, most of *E. coli* BL21 (DE3) cells died due to the toxicity associated with membrane protein expression, i.e. *via* damage to the integrity of the cell membrane (Miroux and Walker, 1996). Further research identified several mutated strains of BL21 (DE3) which had improved membrane expression compared to *E. coli* BL21 (DE3). These strains contain genetic mutations phenotypically selected after induction with IPTG during the expression of the mitochondrial oxoglutarate carrier protein (OGCP) in the BL21 (DE3) bacterial host. The first mutant isolated, named C41 (DE3), showed OGCP protein levels to be strongly increased despite a tenfold reduction of corresponding mRNA levels (Miroux and Walker, 1996). The mutation in C41(DE3) is most likely the replacement of the *lac*UV5 promoter located upstream of the T7 RNA polymerase by the genomic wild-type copy of the *lac* promoter (Wagner *et al*., 2008) that reduces the level of T7 RNA polymerase activity, thereby preventing cell death associated with overexpression of many recombinant toxic proteins. A second

round of selection was conducted expressing *unc*F, the *E. coli* b-subunit of the F1Fo ATP synthase, in C41 (DE3). A second mutant C43 (DE3) was isolated. It carries at least one additional mutation that provides a greater level of tolerance to toxic proteins. Although the mutation in the C43 (DE3) genome remains unknown, a delay in the transcription of the *unc*F gene (60 min) was observed, allowing membrane synthesis and proper folding of the b-subunit (Miroux and Walker, 1996).

The C41(DE3) and C43(DE3) strains have subsequently been used to produce proteins (plant membrane protein) that were either expressed poorly in BL21(DE3) or for which the expression plasmids could not be transformed into BL21(DE3) (Voet-van-Vormizeele and Groth, 2003). Therefore, in these examples, strains C41 (DE3) and C43 (DE3) were generally superior to BL21 (DE3) as a host for protein overexpression.

Project approach

The toxicity of styrene could be solved biologically by the aforementioned techniques. In conclusion, the formation of OMVs is an abundant mechanism in Gram-negative bacteria and can be increased in certain situations (Schwechheimer and Kuehn, 2015). However, the regulation of the vesiculation mechanism remains a challenging research field.

The research including *cis-trans* isomerization and styrene production in *E. coli* is recent (Tan *et al*., 2016) and the regulation of OMVs formation remains a challenging research field. Dunlop *et al.* 2011, highlighted the importance of efflux pumps in solvent tolerance in bacteria (Dunlop *et al.*, 2011). Efflux pumps were

used as a promising option to overcome the toxicity of styrene. Based on this finding, it is anticipated that work undertaken on efflux pump, SrpABC, in addition to the AcrABTolC system will improve the production of styrene. Therefore, in this study, AcrAB and SrpABC candidate systems were used for styrene tolerance investigation. Moreover, resistance/tolerance to a range of other organic solvents was also been studied with the same efflux systems.

3 Aims and objectives

This project aims for the improvement of styrene toxicity tolerance in *E. coli* by the identification of styrene efflux pumps and their incorporation into *E. coli* strains capable of bio-production of styrene. To achieve this, they were several objectives:

1- Identify candidate efflux pumps for styrene:

The SrpABC transporter can export organic solvents, including styrene and toluene, in *Pseudomonas putida* S12 (Kieboom *et al*., 1998). The SrpABC pump has been chosen in this study because it transports solely organic solvents such as styrene (Kieboom *et al*., 1998). The AcrABTolC transporter with a wide range of substrates including organic solvents and antibiotics. AcrAB overexpression confers slight resistance to exogenously added styrene in *E. coli* (Mingardon *et al.,* 2015).

2- Overexpression of candidate efflux pumps in *E. coli*:

By identification of the appropriate culture media and conditions conducive to membrane protein expression. In addition, multiple bacterial strains (including the so-called "Walker" strains (Miroux and Walker, 1996) were investigated to identify optimal conditions for pump protein expression and overproduction.

3- Screening the toxicity tolerance of styrene and other related organic solvents:

This work was carried out to identify and evaluate the toxic concentrations of styrene by examining toxicity tolerance assay of styrene in the transporter

transformed strains. This information is essential for styrene bio-production. Wider exploration of the substrate list for these efflux systems was carried out by examining toxicity tolerance assay of other chemicals in order to allow the opportunity for these chemicals to be synthesized endogenously in the bacterial systems in the future.

4- Identify the appropriate chemical solution for styrene production:

In this study, *n*-dodecane for styrene gas stripping was used in the bioreactor, *n*-dodecane was previously used to extract styrene and was effective solution for styrene production (McKenna *et al.*, 2015), by using *n*-dodecane with a different and modified method accompanied with our biological solution (SrpABC and AcrABTolC efflux pumps), in order to reduce the toxicity and improve styrene bio-production

5- Finally, the bio-production of styrene endogenously by the most tolerant strain:

The most important objective in this project was the ability to produce more styrene using our solutions, this was by expressing the styrene-synthesizing enzymes previously mentioned in McKenna and Nielson (2011) study with the transporter-cloned, styrene- tolerant strain.

4 Materials & methods

4.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich: yeast extract, peptone, glucose, agar, carbenicillin, chloramphenicol, ethanol (EtOH), isopropanol, isobutanol, glycerol, cyclohexene, cyclohexane, 1,3-cyclohexadiene, ethylcyclohexane, imidazole, isopropyl β-D-1-thiogalactopyranoside (IPTG), β-mercaptoethanol, acrylamide:N,N′-methylenebisacrylamide (37.5:1), ammonium chloride, sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), dimethyl sulfoxide (DMSO), ethylenediaminetetracetic acid (EDTA), Tween 80, Triton-X-100, Tris, bromocresol green, cresol red, magnesium sulphate ($MgSO_4$), HEPES, ammonium acetate, sodium acetate, hydrochloric acid (HCl), sodium chloride (NaCl), manganese chloride ($MnCl_2$), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium chloride (KCl), zinc chloride ($ZnCl_2$), potassium permanganate ($KMnO_4$), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), sodium carbonate ($NaHCO_3$), *trans*-cinnamic acid, Ethidium bromide ($EtBr_2$), agarose was purchased from Fisher and *n*-dodecane (Acros®). The purity of all chemicals was >99%.

4.2 Reagents

Tris/glycine/SDS Premixed Electrophoresis Buffer, TAE buffer and Laemmli Sample Buffer were purchased from Bio-Rad. Instant blue stain solution from Expedeon®. DNA loading dye, Gene Ruler Kb DNA Ladder were purchased from Thermofisher scientific. Gibson Assembly Master Mix (2X) reagent was purchased from New England BioLabs. Ethidium bromide buffer and TE buffer were purchased from Qiagen. BugBuster ReagentTM and nucleotides were purchased from Merck-Millipore. SOC medium and complete EDTA-Free Protease Inhibitor were purchased from Novagen and Roche, respectively.

4.3 Enzymes

RNase A, lysozyme from chicken egg white and pyruvate kinase/lactic dehydrogenase (PK/LDH) solution were purchased from Sigma-Aldrich. KOD DNA polymerase and rLysozyme™ solutions were purchased from Novagen. Phusion® High-Fidelity DNA polymerase, T4 DNA ligase and the restriction enzymes were purchased from New England BioLabs. Benzonase® nuclease was purchased Merck-Millipore. Reverse Transcriptase was purchased from ThermoFisher scientific.

4.4 Kits

GenElute PCR Clean-up Kit, GenElute Gel Extraction Kit, GenElute MiniPrep and MidiPrep kits were purchased from Sigma-Aldrich. TransformAid™ bacterial transformation Kit was purchased from ThermoFisher scientific and the λDE3 lysogenization kit from Novagen®.

4.5 Genes and plasmids

*srp*ABC gene (5742 base pair) (Kieboom *et al*., 1998) accession no. AF029405.1 was synthesized and purchased from Biomatik© in *p*sk (+) vector with ampicillin resistance. PT pal-fdc plasmid harbouring *pal2* and *fdc1* genes was purchased from Addgene©(McKenna *et al.*, 2013). The vectors pETDuet-1 and pACYCDuet-1 (low copy plasmid) were purchased from Novagen© (Table 4.1).

Table 4.1 List of plasmids were used in this study

Plasmids	**Source**
pETDuet-1	Novagen©
pACYCDuet-1	Novagen©
pETDuet_MCS1_SrpABC	This study
pACYCDuet_MCS2_SrpABC	This study
PT pal fdc	addgene©
pACYCDuet_pal fdc	This study

pETDuet_AcrAB	This study
pBAD AcrAB TolC	Ingenza©

4.6 Strains

E. coli DH5α, *E. coli* BL21 (DE3) competent cells were kindly supplied by Dr Ian Kerr's laboratory. Competent *E. coli* C41 (DE3) and C43 (DE3) strains were purchased from Lucigen ©. *E. coli* BW25113 and MG1655 strains were available in our lab.

4.7 *In silico* methods

All gene sequences were searched and found in the NCBI website with their GenBank accession numbers. The ClustalW website was used to find the alignments between specific genes and the phylogenetic similarities between them. The NetPlasmid tool was used to draw plasmids with cloning designs. NetPrimer was used to design and choose the suitable primers for sequencing and PCR amplification. All sequencing data were analysed by Chromas, Emboss-water, BLAST and NEBcutter websites.

4.8 Growth of strains

E. coli strains were grown on Luria Bertani (LB) medium, composed of peptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L). The medium was dissolved in distilled water (dH_2O) and then autoclaved for 15 min. For the preparation of agar plates, agar (15 g/L) was also added before autoclaving. The agar media was then cooled to ~50 °C and poured manually into Petri dishes. Carbenicillin (50 mg/mL) (instead of ampicillin) and chloramphenicol (34 mg/mL) stock solutions that were prepared in dH_2O and ethanol respectively, were filter sterilised and added separately as required. For the tolerance toxicity assays, the recombinant strains harbouring the efflux pump proteins were grown in sterilised M9 minimal media which was prepared from 1x M9 solution (Sigma Aldrich®), 1 M $MgSO_4$ (2 mL), 2%

(w/v) casamino acids solution (50 mL), 1M $CaCl_2$ (100 µL), 0.5% (w/v) thiamine solution (100 µL) and 20% (v/v) glycerol solution (50 mL) or glucose (20 % v/v) as a carbon source as required. All these ingredients were added to DW to form a one-litre solution.

4.8.1 Liquid media chemical- tolerance assay

E. coli MG1655 (DE3) transformed with plasmids encoding the SrpABC system and *E. coli* BW25113 transformed with plasmids encoding the AcrABTolC efflux system were assayed for their tolerance to different concentrations of different solvents: styrene, cyclohexane, cyclohexene, 1,3- cyclohexadiene, ethylcyclohexane and toluene separately. The assay was as follows:

Overnight cultures of control strains (transformed with empty-plasmid) and the strains transformed with the efflux pumps were incubated in 50 mL M9 minimal media broth in an orbital incubator at 200 rpm for 16 h at 37 °C. All subsequent culture was performed in QuickFit© flasks (250 mL) which are compatible with Suba-Seal™ rubber septa to provide the chemical resistance required. Cultures were diluted down 100-fold and grown to an OD_{600} of 0.5 at which point the inducer (IPTG for pET plasmids or L-arabinose for pBAD plasmids, 0.5 mM or 0.002% w/v respectively) was added. Subsequently, 30 minutes after induction, the solvent was added by syringe to the required final concentrations; 0, 2.5, 4 and 5 mM styrene, 100, 50, 25 and 10 mM of cyclohexane, cyclohexene, 1, 3-cyclohexadiene, ethylcyclohexane and sterile suba seals were inserted. For toluene tolerance, 0 and 6.2 mM concentrations were used. OD_{600} readings of the cultures were measured in triplicates per hour by Shimadzu UV-spectrophotometer.

4.8.2 Solid tolerance assay

Antibiotic tolerance was examined on LB agar plates as follows

4.8.2.1 Ciprofloxacin MIC assay

Antibiotic agar plates (20 mL) were prepared at concentrations from 0 - 100 ng/mL ciprofloxacin using freshly made antibiotic stocks (Table 4.2). M9 minimal media broth (5 mL) was inoculated with a colony from a freshly inoculated plate (<1 week) and incubated for 16 h at 37 °C.

This culture was diluted by a factor of 1/100 the next day in 5 mL M9 minimal media and incubated until OD_{600} reached 0.5 then protein expression was induced by the addition of IPTG (0.5 mM) for 30 minutes. Three spots (1 µL) of diluted inoculums; OD_{600} = 1, 0.1 and 0.01 were pipetted onto each antibiotic plate including the antibiotic-free control and this was incubated at 37°C for 16 hours. The plates were inspected for growth and the Minimum Inhibitory Concentration (MIC) was defined as the antibiotic concentration at which there is zero growth on the plate (Wiegand *et al*., 2008).

Table 4.2 Ciprofloxacin antibiotic stocks and relevant concentrations in LB agar plates

Concentration (ng/mL)	Stock (mg/mL)	Volume added to 20 mL agar (µL)
0	N/A*	N/A
5	0.01	10
10	0.01	20
15	0.01	30
20	0.01	40
50	0.01	100

*N/A = non- applicable

4.9 Preparation of chemically competent cells

For the preparation of chemically competent cells, a transformation buffer $MnCl_2$ (55 mM), $CaCl_2$ (15 mM), KCl (250 mM), PIPES (pH 6.7, 10 mM) was prepared and sterilised by filtration through 0.22 µm or 0.45 µm filters.

In a sterile process, a single colony from a plate of the strain of interest was selected and inoculated into 10 mLs of LB-broth in a 50 mL tube and grown for 6-8 hours at 37°C. Afterwards, 200 mL of LB-broth in three large flasks (1 L) were inoculated with the starter culture (1, 2 or 4 mL) and grown at 20-22 °C with vigorous shaking (200-220 rpm) overnight. The ODs were monitored the following morning until an OD_{600} for one of the cultures was between 0.45 to 0.65. The flask was put on ice for 15 min. The culture was dispensed into four Falcon tubes (50 mL) and centrifuged in a refrigerated centrifuge (5 °C) at 3500 g *x* for 12-15 min. On the ice, the pellets were re-suspended in chilled transformation buffer (20 mL each). The bacteria then were re-centrifuged at 3500 g *x* at 4°C for 10-15 min and re-suspended in the transformation buffer (10 mL) with the addition of DMSO (1.5 mL) to protect the frozen bacteria from ice crystals. The bacteria were incubated on ice for 10-15 min. Finally, aliquots (800 µL) in chilled, sterile micro-centrifuge tubes were kept at -80°C.

Competent cells prepared with the TransformAid® bacterial transformation kit (ThermoScientific™) were also used in other transformations

4.10 Molecular biology

4.10.1 Gene synthesis

The sequence of the genes encoding the SrpABC transporter from *Pseudomonas putida* S12 was modified by the addition of two sets of different restriction enzymes at 3′ and 5′ ends to enable cloning (Appendix 8.1). The gene was then

synthesised externally (Biomatik©) and inserted in *p*SK (+) plasmid. The synthesis of the gene with *E*coRI, *N*deI restriction enzymes at 5′ end and the *N*otI, *X*hoI restriction enzymes at 3′ end allowed for subsequent sub-cloning with either of the two multiple cloning sites (MCS) of pETDuet-1 and pACYCDuet-1 vectors. After its delivery, it was reconstituted in Tris-EDTA (TE) buffer (40 µL) in a final concentration equals 100 ng/ µL in order to be prepared for the cloning into these two vectors: pETDuet-1 and pACYCDuet-1.

4.10.2 Cloning of the *srp*ABC gene

Four different plasmids were constructed with the insertion of the transporter gene (*srp*ABC) into two multiple cloning sites (MCS1, MCS2) of pETDuet-1 and pACYCDuet-1 vectors separately; pETDuet_MCS1_srpABC, pETDuet_MCS2_srpABC, pACYCDuet_MCS1_srpABC, and pACYCDuet_MCS2_srpABC (maps in Appendix 8.2) using the following steps:

4.10.2.1 Restriction digests

Restriction enzymes (or restriction endonucleases) are enzymes that cut double-stranded DNA by making two breaks. Purified *p*SK (+)SrpABC DNA was typically digested in a total reaction volume of 20 µL, containing 5-10 U/microliter of enzyme per µg of DNA and 2 µL of 10X reaction buffer (supplied with the enzyme). Both the insert (*srp*ABC genes) and the vectors (pETDuet-1, pACYCDuet-1) were cut with the same restriction enzymes to create compatible single-stranded extensions to facilitate ligation.

There were two double digests; *E*coRI, *N*otI restriction enzymes were used to clone into MCS_1, whereas *X*hoI and *N*deI restriction enzymes were used to clone into MCS_2. The enzyme buffer was selected for the optimal condition using NEB Custom Digest tool. The digests reactions were performed at 37 °C for 1 h. The

products of DNA digests were resolved on agarose gel electrophoresis (as described later) and the desired bands of the previous constructs (insert or vector) was excised with a scalpel and purified by NucleoSpin© Gel & PCR Clean-up kit (Macherey-Magel).

4.10.2.2 Ligation

The purified DNA samples were resolved again on agarose gel electrophoresis for the confirmation of the band size. The NanoDrop2000© spectrophotometer was used to measure the concentrations of DNA samples and to estimate the insert/vector ratio for the ligation reactions. The DNA purity and quality was determined by the A260/A280 ratio; only the samples with a A260/A280 ratio of > 1.8 were used. The ligation reactions were prepared with a 3:1 insert-to-vector ratio and T4 DNA ligase (BioLabs©) was added. These reactions were performed overnight at 16˚C.

4.10.3 DNA sequencing

For the sequencing of the putative recombinant plasmids with the *srp*ABC genes, forward and reverse primers were designed (Table 4.3) using NetPrimer website and the alignments were done using BLAST website. The GC % was 40 to 60% and C- dimer and hairpin structures were avoided. This was done because these structures can self-bound, reducing the ability and the availability of the primer to bind to the DNA sequence.

Table 4.3 Synthetic primers sequences for *srp*ABC gene sequencing.

Primers	Sequence (5′ □□3′)	Tm* °C	GC %
SrpF1	CTATGAAACGCTGTTGAAGAC	59.0	42.8
SrpF2	GGATCATTACCGAAGGTGTTC	62.1	7.6
SrpF3	CCAACTCGCTCAGGCGTCCAG	73.5	66.6

SrpF4	CCTTCTTTGGTGGCTCCGCTGGC	76.9	65.2
SrpF5	CTCCAGGCAGTTCGTCCAAAC	68.0	57.1
SrpF6	GCTGTATGAGAAGGAGGG	57.2	55.5
SrpF7	GACTGCCTACGAGTTGGAC	59.9	57.8
SrpF8	GGAACTTACGGCAAACAGC	62.2	52.6
SrpR1	CTGCGTGACGGTGGCGAGCG	78.8	75
SrpR2	GATGTTTGGATATTGGC	53.2	41.1
SrpR3	CTTGCGGTTGGCGAGTCC	69.4	66.6
SrpR4	CCTCAGGGGAGATTCGC	63.5	64.7
SrpR5	GACTTCACTATTGATTTG	46.7	33.3

*Tm: Melting temperature

After the synthesis of the primers by Source BioScience (Nottingham), they were diluted (100 ng/μL) and sent with our constructed pETDuet_MCS1_srpABC, pETDuet_MCS2_srpABC, pACYCDuet_MCS1_srpABC, and pACYCDuet_MCS2_srpABC plasmids (100 ng/μL) to the DNA Sequencing facility at School of Life Sciences/ University of Nottingham. DNA Chromatograms from the sequencing data were analysed using Chromas Lite along with sequence data that was aligned with the predicted sequences using the BLAST local alignment tool.

4.10.4 Cloning of the *acr*AB gene

To clone the *acr*AB gene into the pETDuet_1 plasmid, *p*BAD_acrABTolC and pETDuet_1 plasmids were cut by *X*hoI and *B*glII restriction enzymes; this had the effect of removing the *tol*C gene piece from AcrABTolC plasmid. Afterwards, the resulted sticky ends were ligated by T4 DNA ligase and transformed into *E. coli* DH5α by plating transformed cells on LB solid agar containing carbenicillin (50

mg/mL) and growing at 37 °C overnight. Subsequently, the plasmid was purified from an overnight culture using the GenElute MiniPrep kit.

The plasmid then was sequenced using the following primers.

Table 4.4 Synthetic primers sequences for *acr*AB gene sequencing.

Primers	Sequence (5'⟶3')	Tm °C	GC %
AcrABF1	CAGCGAGTCAGTGAGCG	56	64
AcrABF2	GTCACTGATGCCTCCG	52.7	62
AcrABF3	CGCTCACTGCCCGCTTTCCAG	63.4	66
AcrABF4	CGGTCAGGCGACTG	56	73
AcrABF5	GCCTAATTTCTTTATCG	41.8	36
AcrABF6	GCAGAAGCAGCGAAGC	54.6	60
AcrABF7	GTGGTGGTTCGCCGCCG	63	66
AcrABF8	GTCAGAAGTAAGTTG	49.5	60

*Tm: Melting Temperature

The *acr*AB gene was also cloned into the *p*ACYCDuet-1 plasmid using *X*hoI and *B*glII restriction enzymes. Subsequently, the plasmid was purified from an overnight culture using the GenElute MiniPrep kit. The plasmid was also then sequenced using the previous primers.

4.10.5 Cloning of *pal fdc* genes

4.10.5.1 Gibson's assembly

Other plasmid-insert fragments without sticky ends have been ligated using Gibson Assembly® Master Mix such as the cloning of *p*ACYCDuet_pal-fdc and pETDuet_pal-fdc plasmids for the production of styrene. For the assembly of

pACYCDuet_pal-fdc plasmid, insert and plasmid fragments amplification was performed using the following primers (Table 4.5) using the NEBuilder® tool.

Table 4.5 Synthetic primers for the PCR amplification of pACYCDuet_pal-fdc plasmid

primers	Sequence	Anneals	** F/R	Ta *
pal fdc	TATAGTGAGTCGTATTAATTTCCTAATG	pACYCDuet-1	R	62.6°C
pACYCDuet-1	aattaatacgactcactataCCATGGGCGGGAGGTAAC	Pal fdc	F	62.7°C
pACYCDuet-1	gacttaagcattatgcggccCGCCAAAACAGCCAAGCTTTTATTTATATC	Pal fdc	R	62.7°C
pal fdc	GGCCGCATAATGCTTAAG	pACYCDuet-1	F	62.6°C

*Ta (recommended annealing temperature for PCR), ** F: forward, R: reverse

The highlighted sequences represent the complementary ends of each plasmid fragments

In details, styrene-producing encoding genes, including *pal* and *fdc1* were amplified *via* PCR (Q5 High-fidelity DNA polymerase) using genomic DNA templates derived from PT pal-fdc plasmid (McKenna *et al.*, 2013). The *E. coli* expression vector pACYCDuet-1 vector was similarly amplified by PCR, then the amplified DNA fragments were purified from the agarose gel. Subsequently, the purified fragments were assembled in a reaction using the Gibson assembly master mix reagent (New England BioLabs®) and incubated at 50 °C for 20 to 60 minutes followed by the transformation into chemically competent *E. coli* DH5α. The selection was then achieved by plating transformed cells on LB solid agar containing chloramphenicol (34 mg/L) and growing at 37 °C overnight.

Table 4.6 Gibson's assembly ligation reaction volumes

	Recommended Amount of Fragments Used for Assembly	
	2-3 Fragment Assembly	Positive Control
Recommended DNA Ratio	Vector :insert = 1:2	
Total Amount of Fragments	0.03–0.2 pmols X μL	10 μL
Assembly Master Mix (2X)	10 μL	10 μL
Deionized H_2O	10-X μL	0
Total Volume	20 μL	20 μL

The genes *pal-fdc* were also cloned into the pETDuet-1 plasmid using Gibson Assembly technique and amplified by the following primers (Table 4.7).

Table 4.7 Synthetic primers for the PCR amplification of pETDuet-pal_fdc plasmid

Primers	Sequence	Anneals	F/R	Ta*
pal_fdc	TATAGTGAGTCGTATTAATTTCGCGGGATCGAG	pETDuet-1	R	64.3°C
pETDuet-1	aattaatacgactcactataCCATGGGCGGGAGGTAAC	pal_fdc	F	69.2°C
pETDuet-1	gttatccgctcacaattcccGATGCCTGGCAGTTCCCTAC	pal_fdc	R	69.2°C
pal_fdc	GGGAATTGTGAGCGGATAAC	pETDuet-1	F	64.3°C

*Ta: annealing temperature

The assembled plasmids pETDuet_pal-fdc and pACYCDuet_pal-fdc were verified by gene sequencing using the following primers (Table 4.8).

Afterwards, the plasmid was transformed with DH5α competent cells. After confirmation of the correct transformant, the plasmid pACYDuet_pal-fdc was sub-cloned into *E. coli* MG1655 (DE3) and the protein expression was confirmed by SDS PAGE

Table 4.8 Synthetic primers sequences for *pal fdc* genes sequencing.

Primers	Sequence (5'□3')	Tm °C	GC %
palfdcF1	GACAGGACTATAAAGATAC	43.6	47.0
palfdcF2	CCAGTCGGGAAACC	52.8	66.7
palfdcF3	GACGCCGCTTCGTTCTACCATC	60.2	59.1
palfdcF4	GCATTACAAACAGAACTC	45.9	38.9
palfdcF5	GACATTTGGAGGAGAATC	47.4	44.4
palfdcF6	GCATATCACTGGTCTGG	49.6	52.9
palfdcF7	GAATTGTGAGCGGATAAC	48.2	44.4
palfdcF8	GGCAAGAATGTGAATAAAG	46.5	47.1

4.10.6 Cell transformation (Heat shock transformation)

E. coli competent cells (100 µL) were thawed on ice, then the required vector (up to 1 µL) was added to the competent cells and incubated on ice for 30 min. After that, the mixture was transferred to a 42 °C water bath for 90 s, then moved to the ice for 2-3 min.

A volume (900 µL) of sterilised LB broth (without antibiotic) was added to the mixture and incubated for 1 hour in 37 °C shaker incubator. Aseptically, 100 µL of the cells were plated on LB agar plates supplemented with carbenicillin (50 mg/mL) overnight at 37 °C incubator. The remaining (900 µL) cells were pelleted by brief centrifugation (10,000 g X for 1 min) and then spread on LB agar plates with the same antibiotic.

Co-transformation of two plasmids was also used as the previously mentioned steps of standard transformation, but the concentration of both plasmids was diluted to 2 – 20 ng/μL.

4.10.7 Plasmid purification & long-term storage

After transformation, 1 to 4 colonies of the growing transformed bacterial cells were inoculated into sterilised LB broth (5 mL) containing carbenicillin (final concentration 0.1 g/L) and incubated overnight (up to 16 h) in 37 °C shaker incubator. For plasmid purification, grown cultures were centrifuged at 4000 rpm for 10 min, the bacterial pellets were used for DNA purification by QIAprep Miniprep kit or Midiprep (QIAGEN) as required. For long-term storage, glycerol stocks were prepared by adding 500 μL of sterilised glycerol (30% v/v) to 500 μL of grown cultures and kept at -80 °C.

4.11 Analytical methods

4.11.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation, visualization of DNA and for the estimation of DNA concentration. The gel was made by dissolving agarose (1% w/v) in 10x TPE buffer (108 g Tris base was dissolved in 900 mL dH_2O, 15.5 mL of 85% phosphoric acid and 40 mL of 0.5 M EDTA, pH 8.0). Subsequently, after microwaving the solution for 1 to 2 min, ethidium bromide ($EtBr_2$, 5 μL stock/100 mL gel) was added to the solution; then the mixture was poured in pre-assembled electrophoresis system following which the comb was inserted immediately. After the gel was set and solidified, the samples were loaded after the addition of 6x DNA loading dye (0.3% w/v) bromophenol blue, 0.3% (w/v) xylene cyanol FF, and 30% (v/v) glycerol (MELFORD). In addition, 1 kb DNA ruler (Thermo-Fisher Scientific®) was loaded. Electrophoresis was run using TPE

buffer at 70 to 90 volts (V) for approximately 50 to 90 min. The DNA bands were seen under 315 nm ultraviolet trans-laminator and compared to the ladder. The photos of the agarose gels were taken by an uGenius3 Gel documentation system (Syngene).

4.11.2 Strain λDE3 phage-lysogenisation

This method is designed for site-specific integration of λDE3 prophage into an *E. coli* host chromosome, so the lysogenised host can be used to express target genes cloned in T7 expression vectors tightly. The lysogenisation protocol was used as follows:

E. coli MG1655 wild-type strain was grown in LB broth supplemented with maltose (0.2% w/v) and $MgSO_4$ (10 mM) at 37 °C to an OD_{600} of 0.5. Stock phages (as provided with the kit), titres of 10^8 pfu λDE3, 10^8 pfu of the helper phage, and 10^8 pfu of the selection phage were mixed with 1–10 µL host cells. The host/phage mixture was incubated at 37 °C for 20 min to allow the phage to adsorb to the host cells. Then the mixture was plated evenly onto an LB plate and incubated at 37 °C overnight (Fig 4.1).

Most surviving colonies from the previous steps should be λDE3 lysogens. These lysogens can be evaluated by their ability to support the growth of the T7 tester phage. The T7 tester phage is a T7 phage deletion mutant that cannot grow unless provided by T7 RNA polymerase from the host cells. It makes very large plaques on λDE3 lysogens in the presence of IPTG.

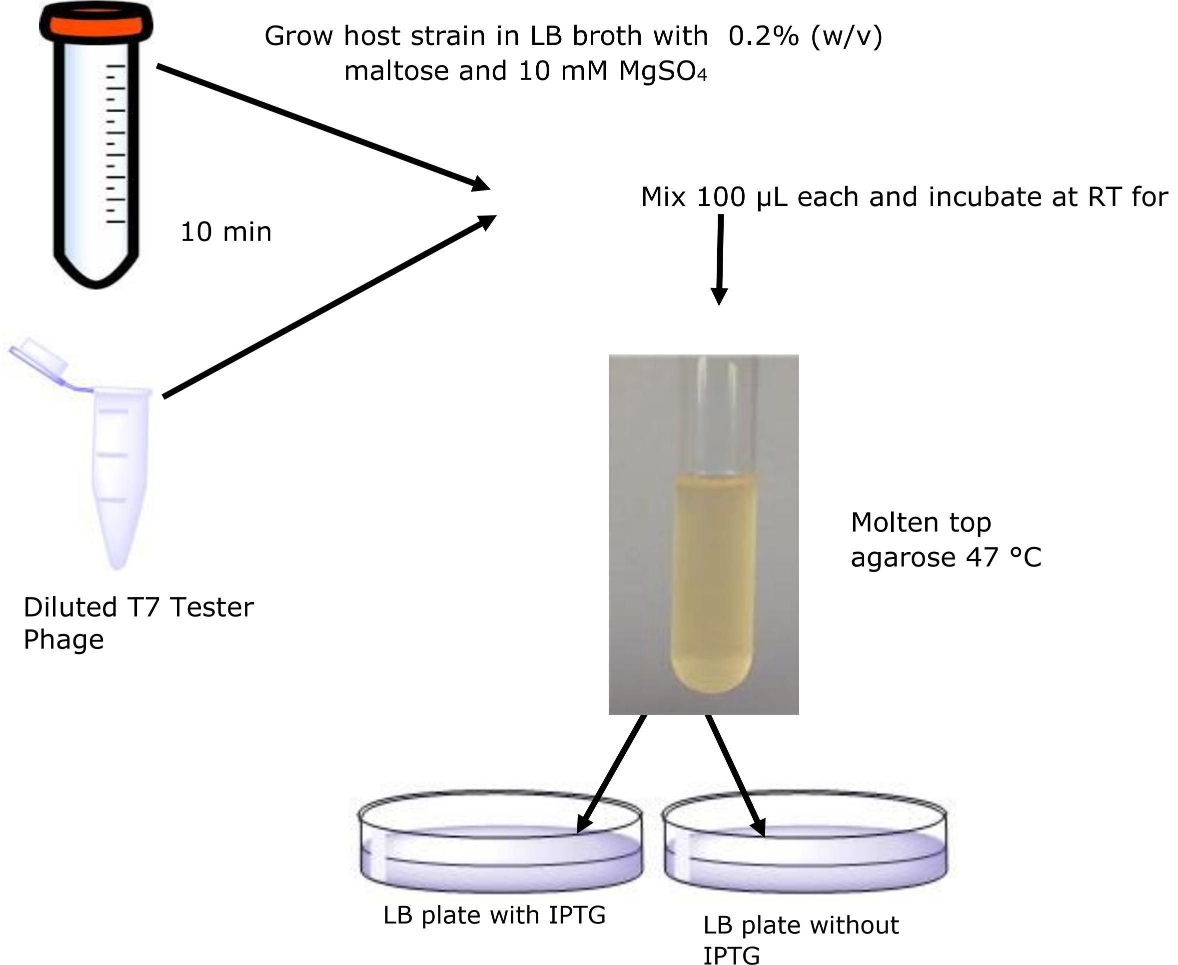


Figure 4.1 λDE3 phage lysogenisation of *E. coli* MG1655 strain. In order to express *srp*ABC genes tightly, *E. coli* MG1655 (DE3) strain was constructed using the λDE3 lysogenisation kit (Merck Millipore®).

For the verification of λDE3, the following procedure was used:

From the previous step, 50 to 200 candidate lysogens (the surviving colonies) were plated onto an LB agar plate and incubated at 37 °C overnight. From those colonies, one colony was inoculated onto LB broth supplemented with 10 mM $MgSO_4$ and maltose (0.2% w/v) to an OD_{600} of 0.5. T7 Tester phage was diluted to a titer of 1-2*10^3 pfu/mL using 1X Phage dilution buffer. A volume of host cells (100 µL) was mixed with the diluted phage (100 µL) in duplicate tubes.

The host/phage mixture was incubated at room temperature for 10 min to allow the phage to adsorb to the host cells. Molten top agarose (3 mL) was added to each tube containing the host/phage mixture. Then the contents of one duplicate

were poured onto an LB plate and the other duplicate onto LB plate supplemented with 0.4 mM IPTG to evaluate the induction of T7 RNA polymerase. The plates were set until the agarose layer was hardened and then incubated at 37 °C incubator for 2 to 4 hours. As expected the colony showed a large plaque on the IPTG plate confirming that this colony was MG1655.lambdaDE3. This strain was then frozen at -80 for long-term storage and competent cells made (section 4.9) for protein expression.

4.11.3 Protein expression

Most cloning steps were performed in *E. coli* DH5α cells that help in maintaining the stability of the recombinant vector because the *end*A1 gene in *E. coli* DH5α is mutated so that the intracellular endonuclease it encodes is inactive, and thus, no degradation of plasmid DNA would occur during any preparation. After confirmation of successful cloning, the vectors were transferred to different *E. coli* strains to investigate the protein expression.

4.11.3.1 IPTG/L-arabinose induction of proteins

A single colony was taken from freshly transformed cells and inoculated in LB/M9 broth (20 mL) supplemented with carbenicillin (50 mg/mL) and incubated at 37 °C shaker incubator (200 rpm) overnight (up to 16 h). The next day a volume of the culture (0.5 mL) was diluted (1:100) in fresh LB/M9 broth supplemented with carbenicillin and incubated in 37 °C shaker incubator until it became turbid and the OD_{600} reached 0.4 to 0.6.

A volume (4 mL) of the previous culture was taken without any induction as a non-induced culture; the remainder was induced by the addition of 0.4 mM IPTG or 0.002 to 0.2 % (w/v) L-arabinose (final concentration) for pET -or pBAD-plasmids respectively. The cultures were incubated at 37 °C in a shaker incubator

(200 rpm) for 2 to 24 h. Samples (1.5 mL) were taken at time intervals for analysis.

4.11.3.2 Membrane protein preparation protocol

Alternatively, cultures (50 mL) were induced and grown as above to determine the expression of proteins in the bacterial membrane. After induction, samples were centrifuged at 4000 rpm for 10 min at 4 °C. The bacterial cell pellet was re-suspended in an excess of ice-cold PBS and re-centrifuged to remove any growth media. The supernatant was removed and the tubes were weighed to know the mass of cells. The cell pellet was re-suspended in a suitable volume of membrane isolation buffer 4 (MIB4, 10 mM Tris (pH 7.4), 250 mM sucrose) containing protease inhibitors and transferred to a chilled tube (1.5 mL). The samples were sonicated on ice (40% power, 3 bursts of 10 s).

The cell lysates were centrifuged in JA-20 rotor at 3000 g for 15 min at 4 °C to pellet out any unbroken cells and large debris. The first supernatant (S1) was retained and the first pellet (P1) was re-suspended in the same volume as the original re-suspension. The supernatant was loaded into pre-weighed 1.5 mL ultracentrifuge tubes (TLA100) for approximately 75% full and was centrifuged at 100,000 g for 1 h at 4 °C. The second supernatant (S2) was retained and the pellet (P2) was re-suspended in a suitable volume of MIB4 supplemented with protease inhibitors by shearing initially through a broad needle (19 G) 3-4 times and then through a narrow-gauge needle (25 G) at least 20 times.

All produced fractions were analysed by a modified Lowry protein assay before proceeding to the SDS-PAGE.

4.11.4 Protein assay

4.11.4.1 Protein assay (Lowry protein assay (BioRad®))

To determine protein concentration, duplicate standard curves were prepared containing Bovine Serum Albumin (BSA) (0-10 μg to 5 μL water). The standard protein (BSA) and unknown protein samples (5 μL) were pipetted in duplicates in the wells of polypropylene 96-well plate.

Subsequently, using a repeating pipette, 20 μL of Reagent A (an alkaline copper tartrate solution), and then 200 μL of Reagent B (a dilute Folin Reagent) was added onto each well and allowed to react for 15 min at room temperature. The absorbance at 650-750 nm was then measured by placing the plate into a microplate spectrophotometer reader (DYNEX Technologies®). The results were then analysed using an Excel spreadsheet specifically designed for this assay to determine protein concentrations and to estimate the volume of each protein sample that would be loaded onto SDS-PAGE gels.

4.11.5 SDS-PAGE

For the analysis of protein expression, sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis was performed (Laemmli, 1970). Protein samples (typically 20 μg) were denatured by incubation in protein-loading buffer (50 mM Tris base (pH 6.8), 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.005 % (v/v) bromophenol blue, 1.25 % (v/v) β-mercaptoethanol), by heating (75-95 °C for 5 min). Acrylamide gels were prepared using acrylamide (8 % w/v) resolving gels in separating buffer (375 mM Tris base, pH 8.8, 0.1 % w/v SDS) and 4 % acrylamide stacking gels in stacking gel buffer (125 mM Tris base, pH 6.8, 0.1 % w/v SDS). "Any kD" and "12%" Mini-PROTEAN TGX precast gels (Bio-Rad®) were sometimes used instead of preparing gels manually.

Samples were resolved alongside molecular weight markers (ColorPlusTM Pre-Stained Protein Ladder, NEB) in protein running buffer (50 mM Tris Base (pH 6.8), 0.192 M glycine, 0.1% (w/v) SDS). Electrophoresis was run at 30 mA per gel (constant current) or 160 V per gel (constant voltage) until the dye front was just eluted from the gel. The gels were then removed from glass plates and stained with Instant Blue stain for one hour at room temperature. The stain was then discarded and the gels were washed with dH_2O. Pictures of the gels were obtained using an uGenius3 Gel Documentation System (Syngene).

4.11.6 Western blotting

Two antibodies were used in this procedure; one being the monoclonal anti-His_6-HRP primary antibody (Thermo-fisher Scientific) which was used to target the His-tagged SrpA protein product of the expressed pETDuet_MCS1_SrpABC, and pACYCDuet_MCS2_SrpABC plasmids, and the His-tagged AcrB protein product of the expressed pBAD harbouring the AcrAB-TolC system. The second antibody used was the rabbit polyclonal Anti-strep tag II antibody HRP (LifeSpan Biosciences, Inc.) that targeted the Strep-tagged TolC protein product of pBAD_AcrAB-TolC in *E. coli* BW25113.

SDS-PAGE gels were transferred to a nitrocellulose membrane in transfer buffer (25 mM Tris base, 190 mM glycine, 20% v/v methanol) and electrophoresed at constant current 250 mA for 1 h at room temperature. Blots were incubated in blocking solution (5% w/v of non-fat milk in PBST, PBS containing 0.1% (v/v) Tween 20 (Thermo-Fisher Scientific)) for 1 h at room temperature. Subsequently, blots were incubated with the antibody in blocking solution (1: 5000 dilution) for 1 hour at room temperature or overnight at 4 °C. Blots were then washed four times every 5 min with PBS.

For the detection of the antibody on the nitrocellulose membranes, SuperSignal West Pico Chemiluminescent Substrate (Thermo-Fisher Scientific) was used as the enhanced chemiluminescent (ECL) HRP substrate. Blots were incubated in Working Solution (mixing equal amounts of Stable Peroxide Solution and Luminol/Enhancer Solution) for 1 min before they were drained, wrapped up in cling film and placed face up inside an X-ray developing cassette. Final western blots were either exposed to X-ray films for various intervals (30 s to 15 min) in a dark room or were imaged using a digital imaging system (Fujifilm LAS3000).

4.11.7 Liquid chromatography–mass spectrometry

Liquid chromatography–mass spectrometry (LCMS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). Liquid chromatography separates mixtures with multiple components, mass spectrometry provides a structural identity of the individual components with high molecular specificity and detection sensitivity. This technique can be used to analyse biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin. In this study, LCMS was used to confirm the protein expression of the SrpABC subunits in *E. coli*. Bacterial cultures were grown at 37^{0}C until OD became 0.5, then induced by IPTG (0.5 mM). An SDS gel was run for the culture samples. The gel with marked bands was sent to Mass Spectrometry Unit, School of Life Sciences, The University of Nottingham. The mass spectra were analysed used to determine the mass of the protein subunits.

4.11.8 Purification of total RNA from the bacterial lysate using the RNeasy® mini kit

To complement the protein expression data above, gene expression of the multidrug pump components was verified by RT-PCR. RNA was extracted from the bacterial cultures of MG1655 (DE3)-pETDuet_MCS1_SrpABC and MG1655 (DE3)-pACYCDuet_MCS2_SrpABC grown in M9 minimal medium by using RNeasy® protect bacteria mini kit (QIAGEN®; which contains reagents to prevent degradation of RNA transcripts) including enzymatic lysis and proteinase K digestion of bacteria followed by DNase digestion and RNA purification. The concentration of RNA samples was measured by Nanodrop 2000c UV/IV Spectrophotometer. Extracted RNA samples were reverse transcribed to cDNAs by the reverse transcriptase (Thermofisher®) (Figure 4.2).

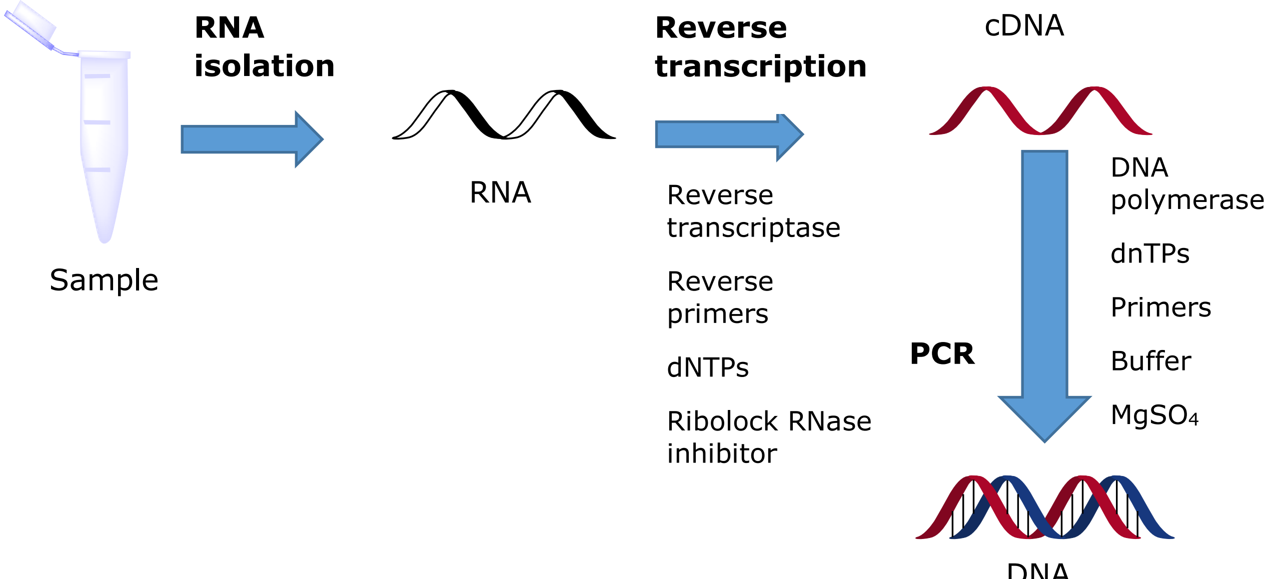


Figure 4.2 Reverse transcription process. cDNAs were produced by the reverse transcription reaction that includes reverse transcriptase enzyme which reverses transcribed RNA to cDNA, reverse primers, dNTPs and Ribolock RNase inhibitor that inhibits the degradation of RNA samples in the reaction.

The reverse transcription reaction was performed in nuclease-free tubes as in Table (4.9):

Table 4.9 Reverse transcription reaction

Component	Volume	Final Concentration
5X Reaction Buffer	4 µL	1x
Template RNA	3 µL	1.5 mM
dNTPs (2 mM each)	2 µL	1 mM
gene-specific primer	-	15-20 pmol
Thermo Scientific™ RiboLock RNase Inhibitor (#EO0381)	0.5 µL	
RevertAid Reverse Transcriptase	1 µL	
PCR Grade Water	X µL	
Total Volume	20 µL	

The reaction was incubated for 60 min at 42 °C. Subsequently, the reaction was terminated by heating at 70 °C for 10 min. The reverse transcription reaction products were used in PCR for amplification and verification.

4.11.9 Polymerase chain reactions

Polymerase chain reaction (PCR) was performed to amplify the genes of interest. The reactions (50 µL) contained genomic cDNA resulted from the reverse transcription at different concentrations, primers (0.4 mM), nucleotides (dNTP, 0.3 mM), Novagen buffer for KOD polymerase and KOD DNA polymerase (2.5 U). When required, DMSO and $MgCl_2$ were added at different concentrations. Previously designed forward and reverse primers (Janardhan Garikipati and Peeples, 2015) were used in this study to allow the extension of the desired fragments in the SrpA, SrpB, and SrpC gene regions (Table 4.10).

Table 4.10 *srp*ABC gene-specific primers for RT-PCR (Janardhan Garikipati and Peeples, 2015)

Gene Amplified	Fragment size	Primer & Position	DNA Sequence
***srp*A**	1016 bp	(SrpAF)Forward primer at 331	bp 5′ – CCT TAA CTG CGC TGA TGC TA – 3′
		(SrpAR)Reverse primer at 1347	bp 5′ – CAC GTT GAA CAC CTT CGG TA – 3′
***srp*B**	824 bp	(SrpBF)Forward primer at 1992	bp 5′ – GCC TTA TGC CAT GCG TAT CT – 3′
		(SrpBR)Reverse primer at 2816	bp 5′ – GGT ACG AAT ACA GCC GAC AG – 3′
***srp*C**	897 bp	(SrpCF)Forward primer at 4995	bp 5′ – TGG TGA CTG CCT ACG AGT TG – 3′
		(SrpCR)Reverse primer at 5892	bp 5′ – CGT TGA GCA TCC AGC ACT GT – 3′

The PCR reactions were run as follows Table (4.11).

Table 4.11 RT-PCR reactions using KOD Hot Start DNA Polymerase

Component	Volume	Final Concentration
10x Buffer for KOD Hot Start DNA polymerase	5 µL	1x
25 mM $MgSO_4$	3 µL	1.5 mM
dNTPs (2 mM each)	5 µL	0.2 mM
PCR Grade Water	X µL	
Sense (5′) Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3′) Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	0.02 U/µL
Template DNA	X µL	
Total Volume	50 µL	

All PCR products were verified by agarose gel electrophoresis.

4.11.10 High pressure liquid chromatography (HPLC)

HPLC is an analytical technique for the separation and quantification of substances in a mixture. HPLC analysis was carried out using Agilent© 1260 Infinity II HPLC series system equipped with an autosampler.

Samples were prepared by removing 1 mL of culture from a shake flask culture and pelleting the cells at 11,000 x g for 2 min. The supernatant (0.75 mL) was then transferred to a glass HPLC vial and sealed with a Teflon-lined cap.

For the quantification of glucose, Rezex ion-exclusion column was used. The mobile phase was 100% H_2SO_4 (0.005 M, 0.7 mL/min) and the components were separated using a Rezex ion-exclusion column at 60 °C. Detection was performed at 215 nm using a G1362A VWD (Variable Wavelength Detector) and by a G1362A Refractive Index Detector at 35 °C. Calibration for each compound analysed was performed using authentic standards at a concentration range between 0.1 and 10 mM. Samples were filtered through 0.2 µm filters and filtrates (20 µL) injected into the apparatus using an auto-sampler. Data analysis was performed with ChemStation software.

For the separation of L-phenylalanine, *p*-coumaric acid, *t*CA, and styrene, diode array (UV/Vis) detector, and ZORBAX Eclipse XDB-C18 column (4.6mm x 150 mm 5 µm; Agilent©). Samples (5 µL) were injected for analysis at a total constant flow rate of 1.0 mL/min and a constant column temperature of 45°C. The column was eluted with 'solvent A' (consisting of double-distilled water) and 'solvent B' (consisting of methanol (99.8% grade) plus 0.1% trifluoroacetic acid (TFA)). The eluent began as a mixture of 90% solvent A and 10% solvent B before a linear gradient was applied over 8 min to then reach a mixture of 20% solvent A and 80% solvent B. This eluent composition was then held constant for 2 min before a second linear gradient was then applied over the course of 4 min to achieve a final mixture of 95% solvent A and 5% solvent B. The eluent was monitored at each of 215 nm for L-phenylalanine and 258 nm for *t*CA, and styrene. Under these conditions, L-phenylalanine, *t*CA, and styrene were eluted at 6.1, 17.5 and 23.7 min, respectively.

4.11.11 Gas chromatography–Mass Spectrometry

GC-MS is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. In this project, I used this method for the confirmation of the biosynthesis of styrene. Culture supernatant (1 mL) was added to hexane (1 mL) and vortexed for 20 min at maximum speed. The biphasic mixture was centrifuged for 1 min at 11,000 *x g* to settle. 750 µL of the hexane layer was removed for analysis by GC-MS.

Styrene was detected and quantified using an Agilent 5975 GC-MS System equipped with an Agilent capillary HP5-MS column (0.32 mm x 30 m x 0.5 µm), an Electron Impact ionisation source and a 5975C inert MSD with quadrupole mass analyser. For each compound, detection methods were optimised starting from a standard method already used in the lab for the detection of similar compounds. 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 in a concentration range of 1 – 7 mM for styrene.

For the detection of styrene, samples (1 µL) were injected using an auto-sampler a split ratio of 100:1. The inlet temperature was 250°C, the oven temperature was initially kept at 45°C for 5 min but, for styrene, it was increased to 300°C at 20°C/min. The final temperature was held for 10 min.

4.12 Bioreactor fermentations

All bioreactor fermentations were performed using a 1 L aqueous working volume in a 3 L New Brunswick BioFlo®/CelliGen® 115 (eppendorf®) bioreactor. *BioCommand* software was used for process control and data acquisition. Agitation

was performed using two Rushton-style blade turbine impellers, each with a diameter, height, and spacing of 6.3, 1.2, and 7 cm, respectively. Aeration was performed with the use of a sparger positioned just below the base of the agitator shaft. Dissolved oxygen (DO) levels were measured using DO probe whereas pH was measured using a pH probe. Control of pH was performed *via* the automated addition of ammonium hydroxide (30%) and sulphuric acid (2 M) solutions, as appropriate. The temperature was controlled using an electric heating blanket that controls the heating and the cooling of the fermentation chamber.

For culture fermentation, 5x salt solution composed of $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4 and NaH_2PO_4. Trace Elements (TE) solution was prepared of: $CaCl_2.{}_2H_2O$ (0.5 g/L), $FeCl_3$ (10.03 g/L), $ZnSO_4.7H_2O$ (0.18 g/L), $CuSO_4.5H_2O$ (0.16 g/L), $MnSO_4.H_2O$ (0.15 g/L), $CoCl.6H_2O$ (0.18 g/L) and $Na_2EDTA.2H_2O$ (22.3 g/L). Glucose (pre-culture) 10 g/L, glucose (batch phase) 12 g/L. Glucose feed solution (D-glucose 325 g, 30 mL Thiamine (0.225 g/L solution), TE solution (6 mL) and yeast extract (6 g) and dH_2O was added to 500 mL. All solutions were sterilised by autoclaving at 121 °C for 15 min. Thiamine solution was sterilised by filtration through 0.21 μm filter.

Inoculum medium (50 mL) was prepared in a 250-mL sterile baffled flask composed of 5X salt solution (10 mL), 25% D-glucose solution (2 mL), 1M $MgSO_4$ solution (0.25 mL), TE solution (0.1 mL), 0.5 mL of Thiamine (0.225 g/L) with the addition of sterile deionised H_2O using the appropriate antibiotic. The next day, the pH probe was calibrated before adding the batch media to the fermenter.

Afterwards, the whole fermenter with the probes and attached tubes containing the batch medium was autoclaved and sterilised. Subsequently, the DO probe was calibrated with N_2 for zero and then with air until reach 100 min^{-1} to ensure that

the fermentation is O_2 enriched. The OD_{600} of the inoculum was checked before adding to the fermenter starting with OD_{600} equals 0.0075 and grown overnight. Next day, the glucose was checked for negativity before attaching the sterilised glucose feed (65% glucose solution (464 mL), thiamine (0.225 g/L solution) (30 mL), TE solution (6 mL)) as indicated by a sharp increase in DO and confirmed using glucose oxidase test strips. The flow rate was adjusted manually, the feed pump rate was 0.1 mL/min at the first hour from the start of feeding, 0.2 mL/min[1] during the second hour and 0.3 mL/min to the end to avoid the accumulation of excess glucose in the culture and to maintain a pseudo-exponential growth rate. Sampling was done each 2 hr. IPTG induction (1.5 mM) was performed when the OD_{600} of the culture reached 30 to 40 this happened concomitantly with L-phenylalanine (2 g/L) addition.

The air flow rate was 100 min^{-1} and the DO was maintained at 30 % of saturation by automatic control of the stirrer speed between 600–1200 r.p.m. Besides the dodecane trap (250 mL) (Fig 4.3) was fixed at the outlet gas of the bioreactor for stripping the produced styrene and to reduce the toxicity of styrene in the fermenter medium. When OD was about 30, styrene production was induced by the addition of sterilised IPTG (1.5 mM) and L-phenylalanine (2 g/L) directly to the bioreactor.

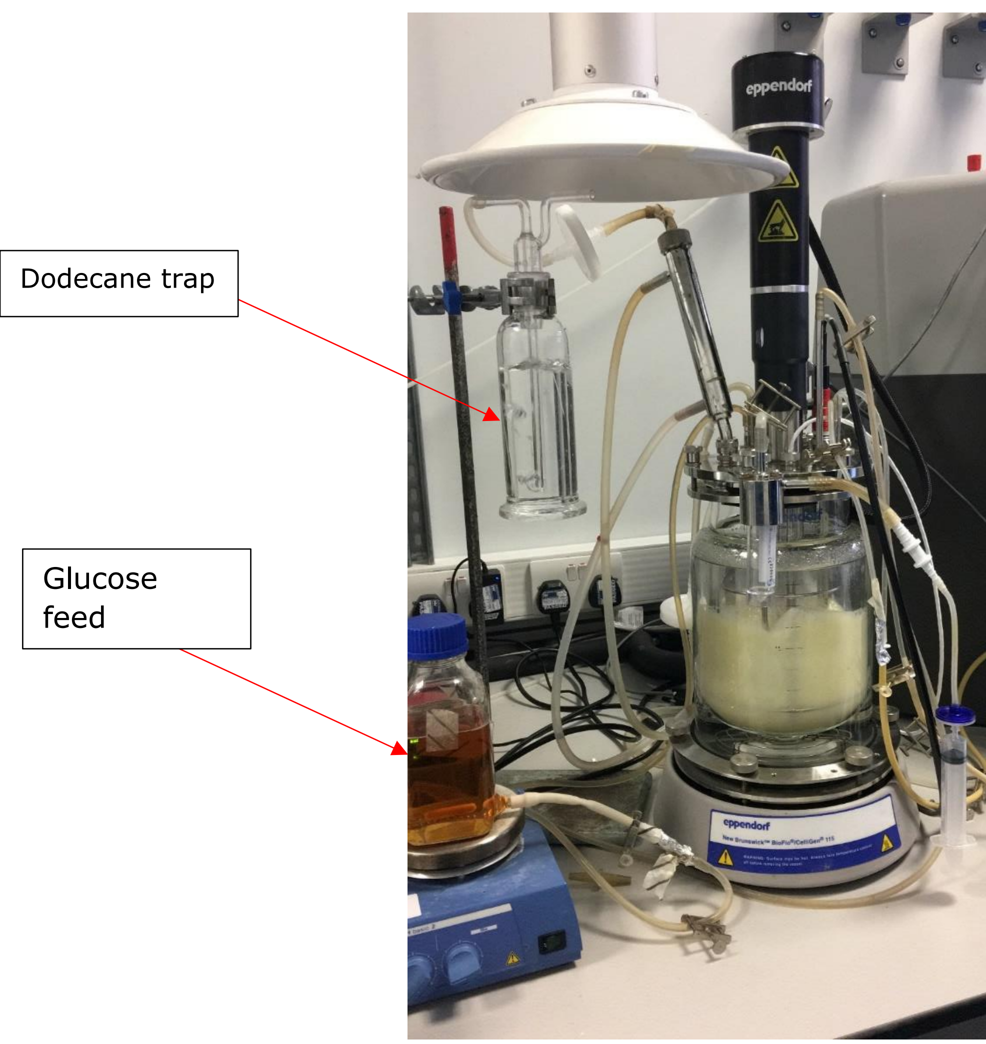


Figure 4.3 Bioreactor demonstration. Bioreactor fermenter for styrene production with dodecane trap to catch all produced styrene

4.13 Light Microscopy

The light microscope was used to check the viability and morphology of bacteria. One drop (100 µL) of bacterial culture with added styrene was put on slide and covered and checked under light microscope. Immersion oil (one drop) was added by Pasteur pipette to the slide cover when using 100x magnification lens to increase the resolving power of the microscope.

Results

5 SrpABC efflux pump

5.1 Protein expression

The aim of this procedure was to express the SrpABC protein in *E. coli*. The *srp*ABC genes from *Pseudomonas putida* S12 (accession no. AF029405.1) (Kieboom *et al*., 1998) were inserted into pETDuet-1 and pACYCDuet-1 plasmids. In details, *srp*ABC genes were inserted into the restriction sites of the pETDuet-1 plasmid using *E*coRI, *N*otI and *X*hoI, *N*deI restriction enzymes in MCS1 and MCS2 respectively resulting in pETDuet_MCS1_srpABC, pETDuet_MCS2_srpABC constructs. The pACYCDuet_MCS1_srpABC and pACYCDuet_MCS2_srpABC constructs produced from the cloning of the *srp*ABC genes into the pACYCDuet-1 plasmid by using the same restriction enzymes. DNA sequencing showed that only pETDuet_MCS1_srpABC and pACYCDuet_MCS2_srpABC plasmids (Fig 5.1) had the correct sequence.

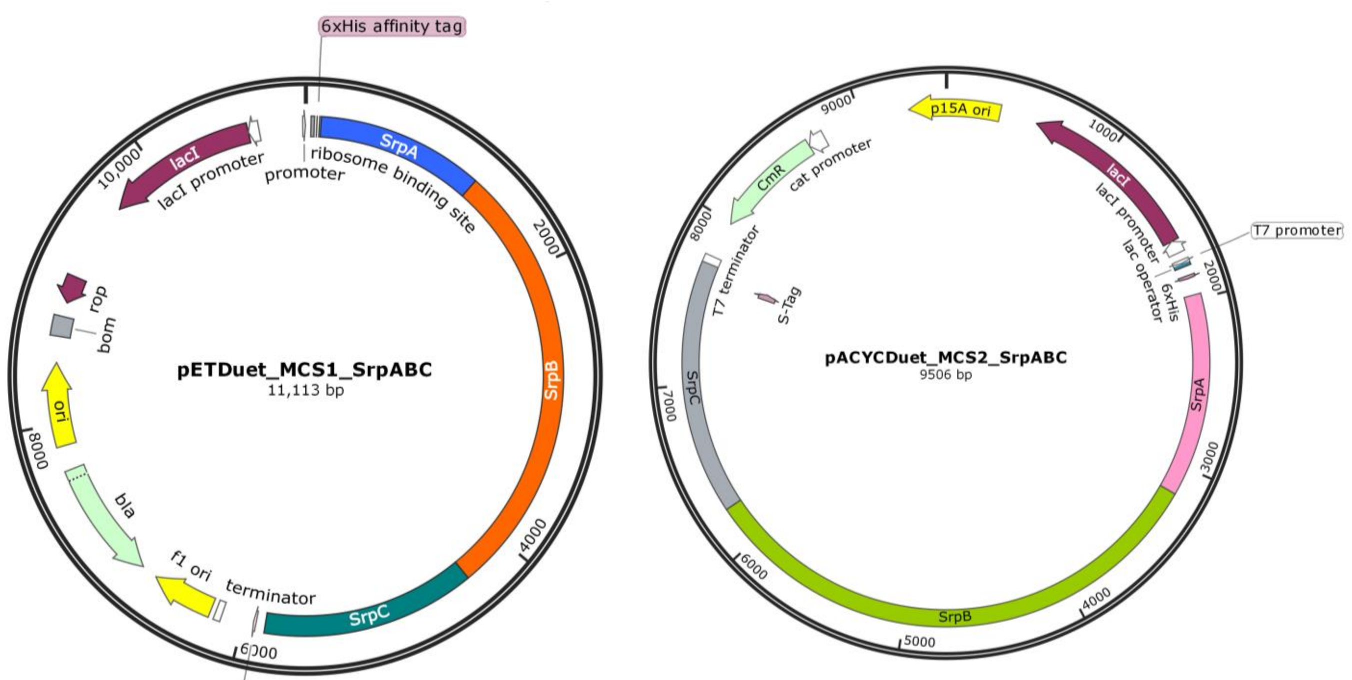


Figure 5.1 *p*ETDuet_MCS1_SrpABC and pACYCDuet_MCS2_srpABC maps. Expected plasmid maps for pETDuet-1_MCS1_SrpABC and pACYCDuet_MCS2_srpABC (drawn by SnapGene®). *lac*I is the *lac* repressor which inhibits the expression of genes coding for proteins, SrpA has 6xHis affinity tag. P15A: origin of replication for pACYCDuet-1 plasmid that have 10 copy number of replication.

The confirmation of protein production of these plasmids was investigated in different *E. coli* strains. First, protein production was investigated in *E. coli* BL21 (DE3). T7 expression hosts such as DE3 strains e.g. *E. coli* BL21 (DE3) have a chromosomal copy of the phage T7 RNA polymerase gene (Studier and Moffatt, 1986). When an inducer such as IPTG is added to the culture, T7 RNA polymerase is produced and transcribes the gene of interest, followed by translation of the desired protein by the endogenous protein translation machinery. The SDS PAGE gels of several trials of SrpABC protein expression in LB or M9 medium at 30 and 37°C in *E. coli* BL21 (DE3) did not show positive confirmation by Western blotting with anti-His tag antibody of the His-tagged SrpA. In addition, the same negative result was obtained by Liquid chromatography mass spectrometry (LCMS) for the corresponding mass of the SrpABC subunits. This method was used to confirm protein production of SrpABC subunits.

A second trial was performed in this project to express the *srp*ABC gene using *E. coli* C41 (DE3) and *E. coli* C43 (DE3). The C41(DE3) and C43(DE3) strains have been used to produce proteins (frequently, membrane proteins) that were either produced poorly in BL21(DE3) or for which the expression plasmids could not be transformed into BL21(DE3) (Miroux and Walker, 1996). Therefore, strains C41 (DE3) and C43 (DE3) are generally superior to BL21 (DE3) as a host for membrane protein overexpression. The mutation in C41 (DE3) is most likely the replacement of the *lac*UV5 promoter located upstream of the T7 RNA polymerase by the genomic wild-type copy of the *lac* promoter (Wagner *et al*., 2008). A second mutant C43 (DE3) has been isolated (Miroux and Walker, 1996).

Although the mutation in the C43(DE3) genome remains unknown, a delay in the transcription of the *unc*F gene (encoding the beta-subunit of *E. coli* ATPase) was

observed, allowing membrane synthesis and proper folding of the β-subunit (Miroux and Walker, 1996). These mutations will reduce the level of T7 RNA polymerase activity preventing cell death associated with overexpression of many recombinant toxic proteins.

Therefore, the pETDuet_MCS1_srpABC and pACYCDuet_MCS2_srpABC plasmids were transformed into C41 (DE3) and C43 (DE3) strains and protein production was analysed *via* SDS PAGE. However, there was no evidence that SrpABC was produced in the C41 and C43 strains. This confirms other recent studies about SrpABC protein expression in *E. coli*. The overexpression of SrpABC was problematic (Bui le *et al.*, 2015) because they were not able to detect expression by SDS-PAGE, probably because of the toxicity caused by the overproduction of the transmembrane protein, SrpB. Alternatively, when they performed Reverse Transcriptase-PCR (RT-PCR), they were able to verify the presence of *srpABC* transcripts when they were cloned in pACYCDuet-1. Therefore, *srpABC* could be expressed in the *E. coli* MG1655 strain under the control of the T7/*lac* promoter (Bui le *et al.*, 2015). As the required strain MG1655 (DE3) is not commercially available, I have generated this using a lysogenisation kit designed for site-specific integration of λDE3 prophage into the *E. coli* host chromosome. The host strain was mixed with the phage mixture to allow the phage to adsorb to the host cells (methods pg. 47) and grown onto plates. Most surviving colonies should be λDE3 lysogens. The constructed strain was verified by growing the λDE3 phage-infected bacteria on IPTG containing LB agar. Subsequently, the successful strain colonies were able to form plaques in the agar after the addition of a T7 tester phage that has a DE3 mutation and cannot grow unless provided by T7 RNA polymerase from the host cells in the presence of IPTG, this indicates the activity

of λDE3-phage (Fig 5.2). The culture of the successful colonies of the constructed strain was then frozen at -80 for long-term storage and competent cells made.

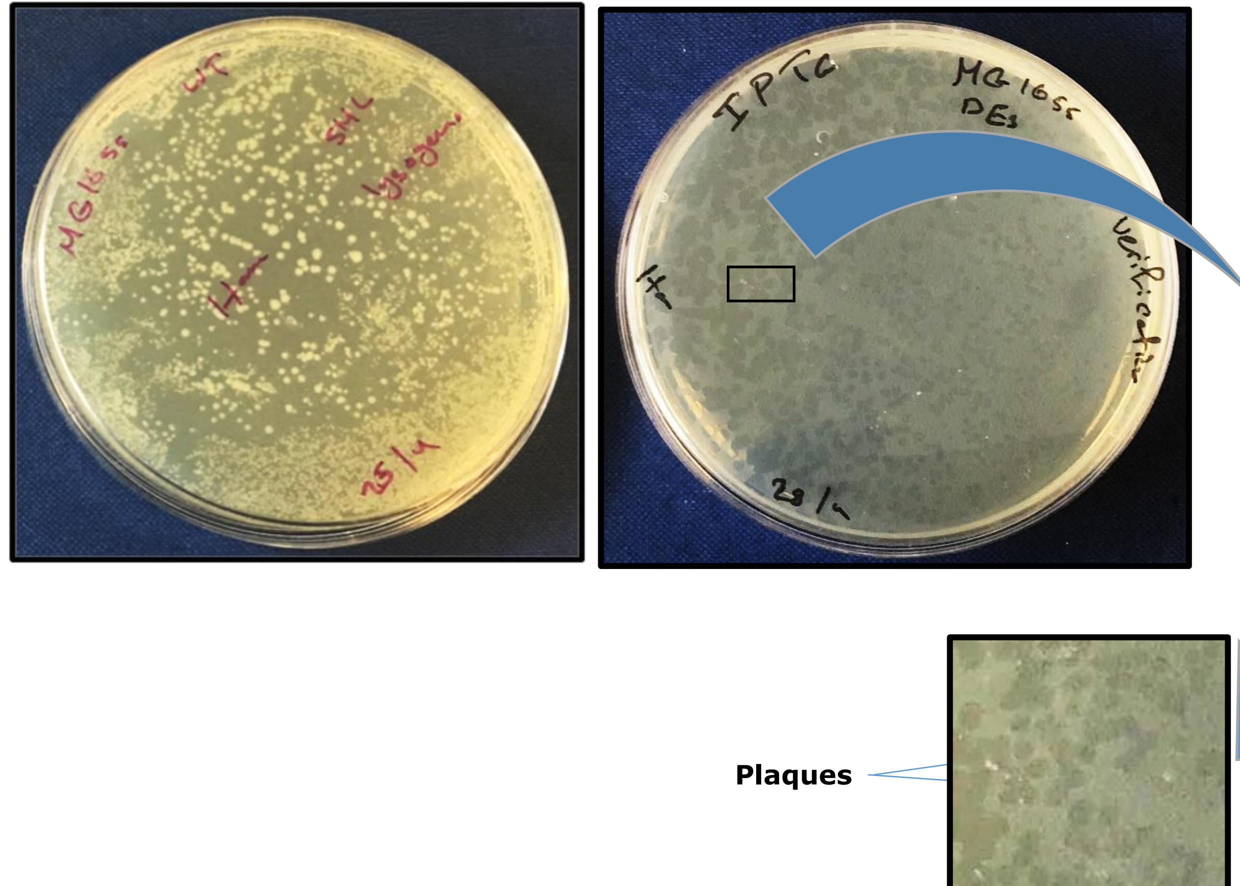
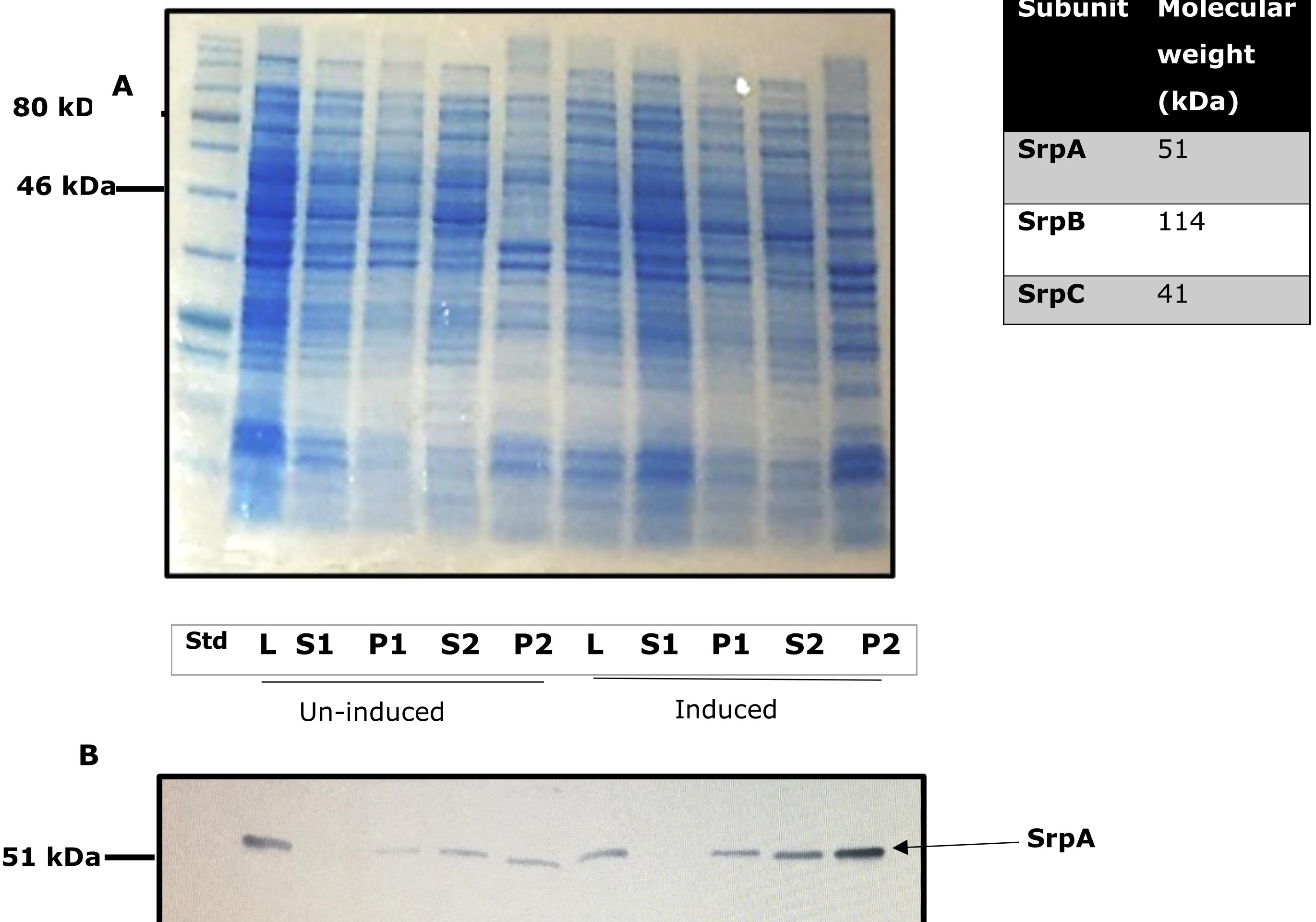


Figure 5.2 λDE3 phage lysogenisation of *E. coli* MG1655 strain. MG1655 (DE3) strain was constructed using the λDE3 lysogenisation kit (Merck Millipore®). The λDE3 phage integrated into the strain chromosome and the colonies of MG1655 strain harbouring the λDE3 phage were verified by growing on IPTG-supplemented LB agar plates. Only the lysogenized colonies can help T7 tester phage to form plaques on the plate indicating expression of T7 RNA polymerase and obtaining the desired strain harbouring λDE3 phage MG1655 (DE3).

Subsequently, the strain was transformed with pETDuet_MCS1_srpABC and pACYCDuet_MCS2_srpABC plasmids. For protein production analysis, cultures of the resulting strains were grown in M9 liquid medium (methods section (4.11.3.1, page 48) and samples were harvested at OD_{600} equal to 1 for both SDS PAGE and RT PCR analyses. The SDS PAGE gels of MG1655 (DE3) cells carrying SrpABC (pETDuet-1 or pACYCDuet-1 plasmids) indicated that production of SrpA (51 kDa),

SrpB (116 kDa) and SrpC (41 kDa) could not be detected against the background of total cell or membrane proteins (Figures 5.3(A), 5.4 (A)). However, production of the SrpA protein (which carried a C-terminal-His_6 tag) could be detected by western blotting of the same fractions (Figures 5.3 (B), 5.4 (B)), with much greater production in the induced fractions. Lower level production in the un-induced fractions presumably reflects the "leaky" T7 promoter.



Subunit	Molecular weight (kDa)
SrpA	51
SrpB	114
SrpC	41

Figure 5.3 Protein production expression in *E. coli* MG1655 (DE3)-pETDuet_MCS1_SrpABC. The cultures were grown in M9 minimal media induced by 0.4 mM IPTG at 37 °C. (A) SDS PAGE gel of the membrane preparation samples; the whole cell lysate (L), a low speed supernatant (S1), a low speed pellet (P1) which represents cellular debris, the second supernatant (S2) which represents the periplasmic and cytoplasmic protein fraction and the second pellet (P2) represents the membranous protein fraction along with the molecular weight markers were resolved in 8 % w/v polyacrylamide. (B) Western blot with anti-His tag antibody (1:5000) following which membrane incubated in chemiluminescent reagent and then exposed for 90 s to X-ray film to allow detection. 'L' represents lysate, 'P' represents pellet and 'S' represents

supernatant. 'Std' implies the standard. SrpB and SrpC were not seen in the SDS gel. SrpA about 51 kDa has been detected.

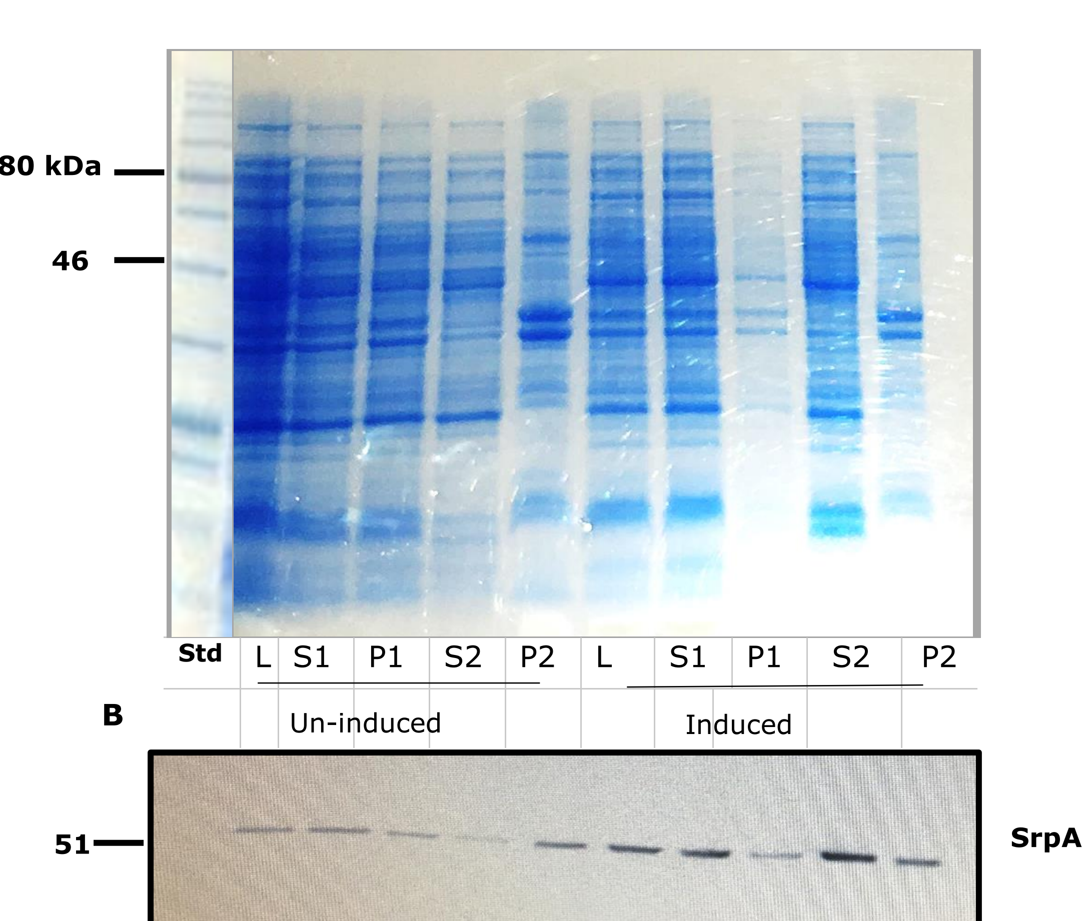


Figure 5.4 Protein production in *E. coli* MG1655 (DE3) pACYCDuet_MCS2_SrpABC. The cultures were grown in M9 minimal media induced by 0.4 mM IPTG at 37 °C. (A) SDS PAGE gel of the membrane preparation samples: L, S1, P1, S2 and P2 along with the molecular weight markers were resolved in 8 % w/v acrylamide. (B) Western blot with anti-His$_6$ tag antibody (1:5000) following which membrane incubated in chemiluminescent reagent and then exposed for 90 seconds to X-ray film to allow detection. 'L' represents lysate, 'P' represents pellet, and 'S' represents supernatant. SrpB and SrpC were not seen in the SDS gel. SrpA (51 kDa) has been detected.

Gene expression of *srp*B and *srp*C was confirmed by RT-PCR analysis of the RNA samples extracted by RNeasy protect cell kit from the same cultures of *E. coli* MG1655 (DE3) cells carrying SrpABC (pACYCDuet_MCS2_SrpABC and pACYDuet_MCS2_SrpABC) that were used for SDS analysis. Following RT-PCR (see section 4.9), all the DNA samples produced from the PCR were analysed by

agarose gel electrophoresis to identify the transcripts (methods section 4.11.8, page 54).

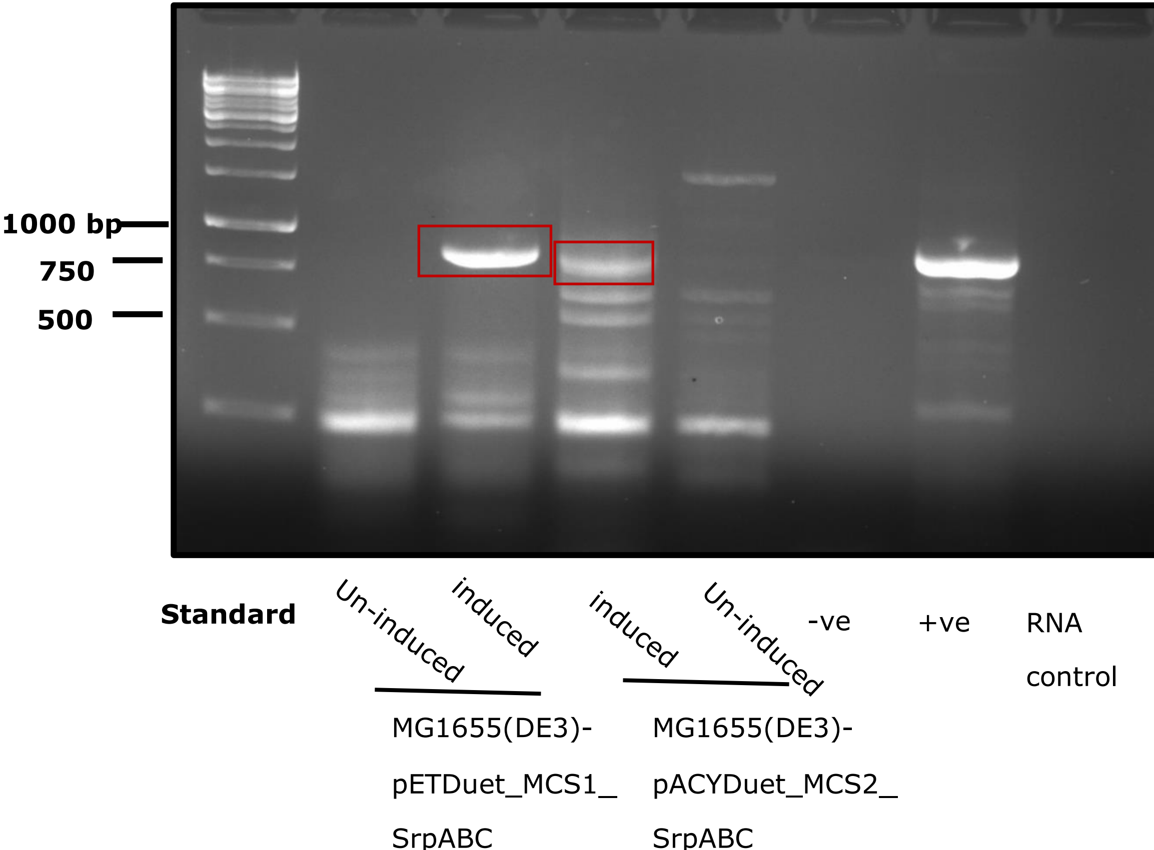


Figure 5.5 RT-PCR validation of *srp*B expression in *E. coli* MG1655 (DE3). Agarose gel of RT PCR products from *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC and pACYCduet_MCS2_SrpABC using the forward and the reverse primers of SrpB. Negative control: empty pUC 19 plasmid, positive control: pSK-SrpABC plasmid DNA template. RNA control is the RNA sample without reverse transcription.

*srp*B and *srp*C were detected from the samples harvested and prepared from *E. coli* MG1655 (DE3) cells carrying pETDuet_MCS1_SrpABC plasmid by matching the expected transcript length (824, 897 bp respectively) (Figures 5.5, 5.6).

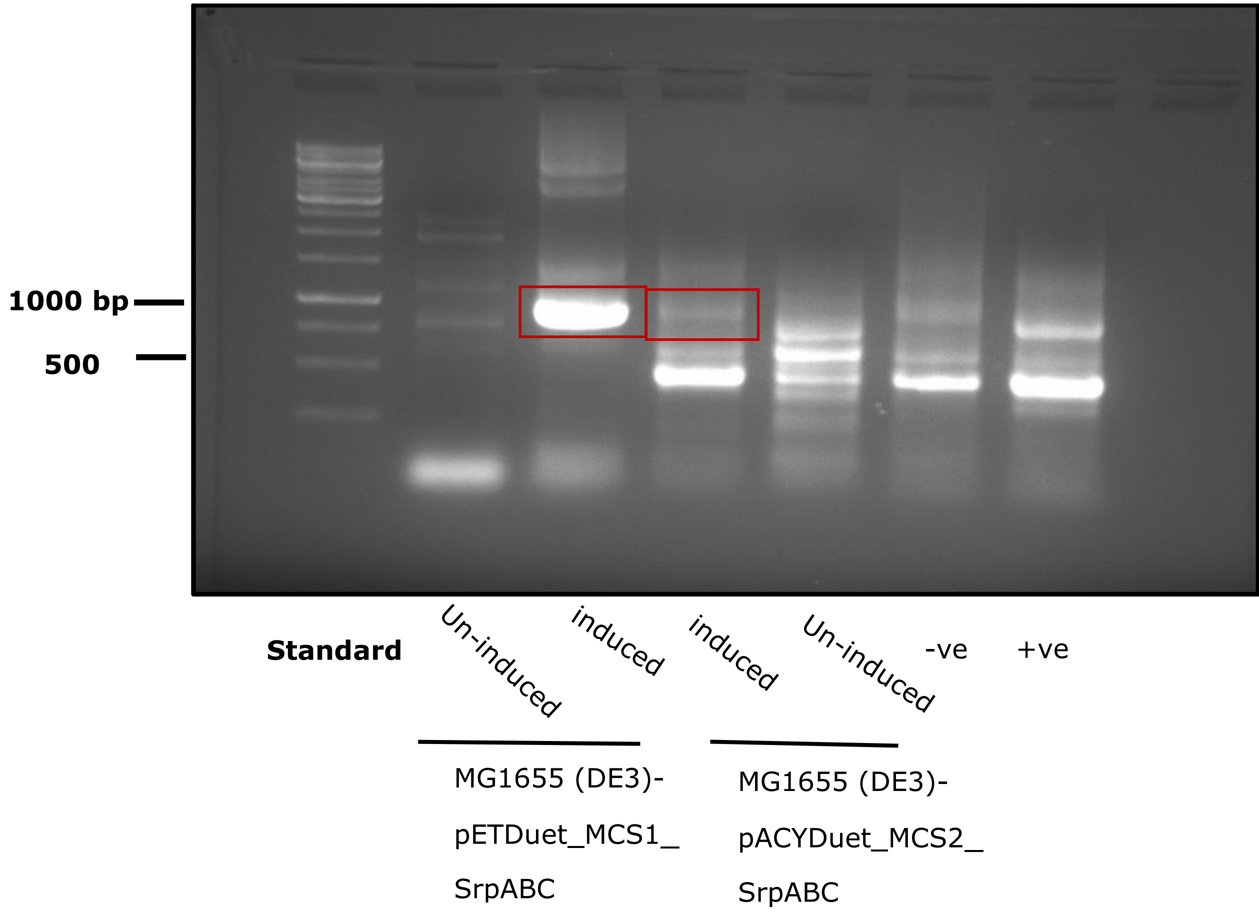


Figure 5.6 RT-PCR validation of *srp*C expression in *E. coli* MG1655 (DE3). Agarose gel of RT PCR products from *E. coli* MG1655 (DE3)-pETDuet_MCS1_SrpABC and pACYCduet_MCS2_SrpABC using the forward and the reverse primers of SrpC. Negative control: pUC 19, positive control: SrpABC gene in psk+ plasmid.

However, the gene expression of *srp*C *E. coli* MG1655 (DE3) cells carrying pACYCduet_MCS2_SrpABC samples was less than that from cells carrying pETDuet_MCS1_SrpABC, presumably because the expression vector pACYCduet-1 is a low copy plasmid.

In conclusion, the expression of the SrpABC pump genes was confirmed in *E. coli* MG1655 (DE3) cells carrying pETDuet_MCS1_SrpABC and pACYCduet_MCS2_SrpABC plasmids by the improved detection of SrpA protein expression using western blotting (this study), and SrpB and SrpC units by RT-PCR analysis (Janardhan Garikipati and Peeples, 2015).

5.2 Toluene toxicity tolerance in *E. coli* MG1655 (DE3) bearing SrpABC pump

The engineered strain transformed with SrpABC was grown in M9 medium with a saturated concentration of toluene (6.2 mM) to verify the pump activity. The cultures of the cells were grown in M9 minimal media at 37°C until the OD_{600} was 0.5, then *srp*ABC expression was induced by the addition of IPTG (0.4 mM). Toluene (6.2 mM) was added 30 min after IPTG induction. The negative control (empty pETDuet-1 plasmid) was also grown in the same conditions. ODs were monitored 4 hours after toluene addition (Fig 5.7).

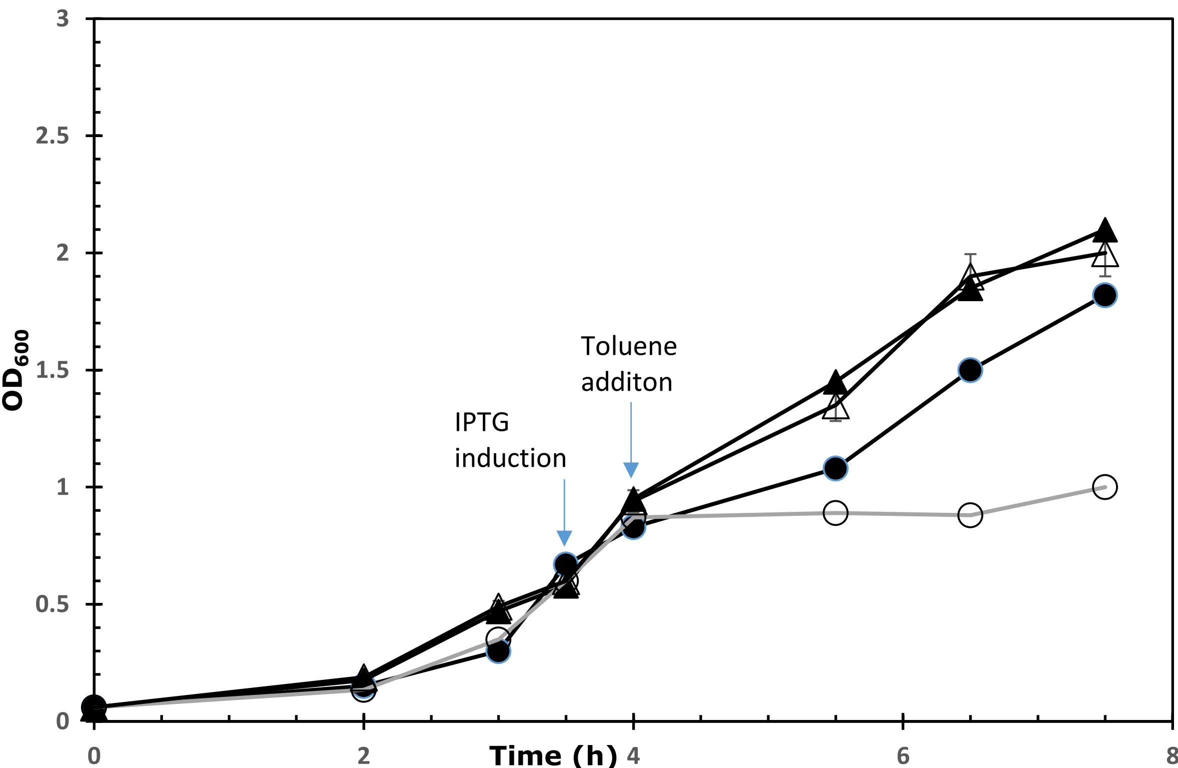


Figure 5.7 *E. coli* MG1655 (DE3)-pETDuet_SrpABC efflux pump activity assay. SrpABC efflux pump activity was assayed in *E. coli* MG1655 (DE3)-pETDuet_SrpABC-, cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.5 mM IPTG. Afterwards, toluene (6.2 mM) (Circles) was added 30 min after IPTG induction. The triangles represent no added toluene. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-ΔpETDuet-1) was also grown at the same conditions. ODs were monitored for 4 hours after toluene addition. Error bars represent one standard deviation from triplicate experiments.

The negative control *E. coli* strain with empty plasmid showed growth inhibition under toluene exposure, whereas *E. coli* cells transformed with *srp*ABC genes were more tolerant to toluene compared to the control strain. Similar results were obtained earlier when *srp*ABC genes were transferred into a solvent-sensitive *Pseudomonas putida* strain (Kieboom *et al*., 1998b).

The same assay was also performed with the same strain having SrpABC on the lower copy number plasmid pACYCDuet-1 (Fig 5.8).

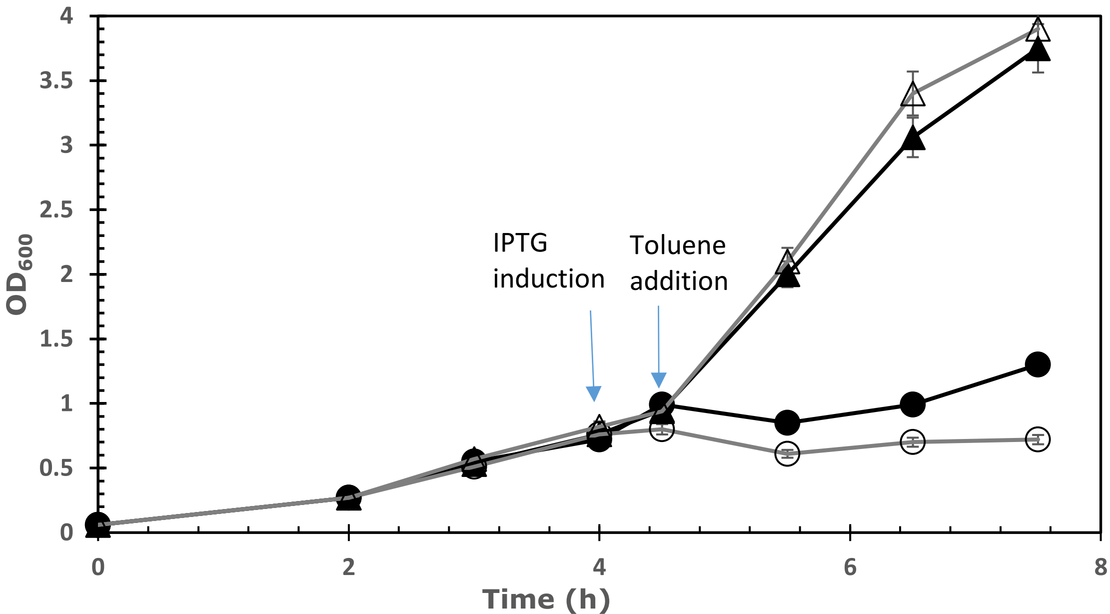


Figure 5.8 *E. coli* MG1655 (DE3)-pACYCDuet_SrpABC efflux pump activity assay. SrpABC efflux pump activity was assayed in *E. coli* MG1655 (DE3)-pACYCDuet_SrpABC, cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.5 mM IPTG. Afterwards, 0 (Triangles) and 6.2 mM toluene (Circles) was added 30 min after IPTG induction. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-Δ pACYCDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after toluene addition. Error bars represent one standard deviation from triplicate experiments.

The results using pACYCDuet_SrpABC plasmid were the same as when using a medium copy number plasmid (pETDuet-1), except one difference which was that a longer time that needed for the cells of MG1655 (DE3)-pACYCDuet_SrpABC to

recover after toluene addition. This was expected because of the low copy number of plasmid pACYCDuet-1.

5.3 Styrene toxicity tolerance in *E. coli* harbouring the SrpABC system

The aim of this experiment was to investigate the toxicity tolerance of styrene in *E. coli* MG1655 (DE3) harbouring SrpAB. Growing cultures of the cells in M9 minimal medium at 37°C until OD_{600} becomes 0.5, then inducing with 0.4 mM IPTG. Exogenously added styrene was added 30 min after IPTG induction. The negative control (empty pETDuet-1 plasmid) was also grown at the same conditions (Fig 5.9).

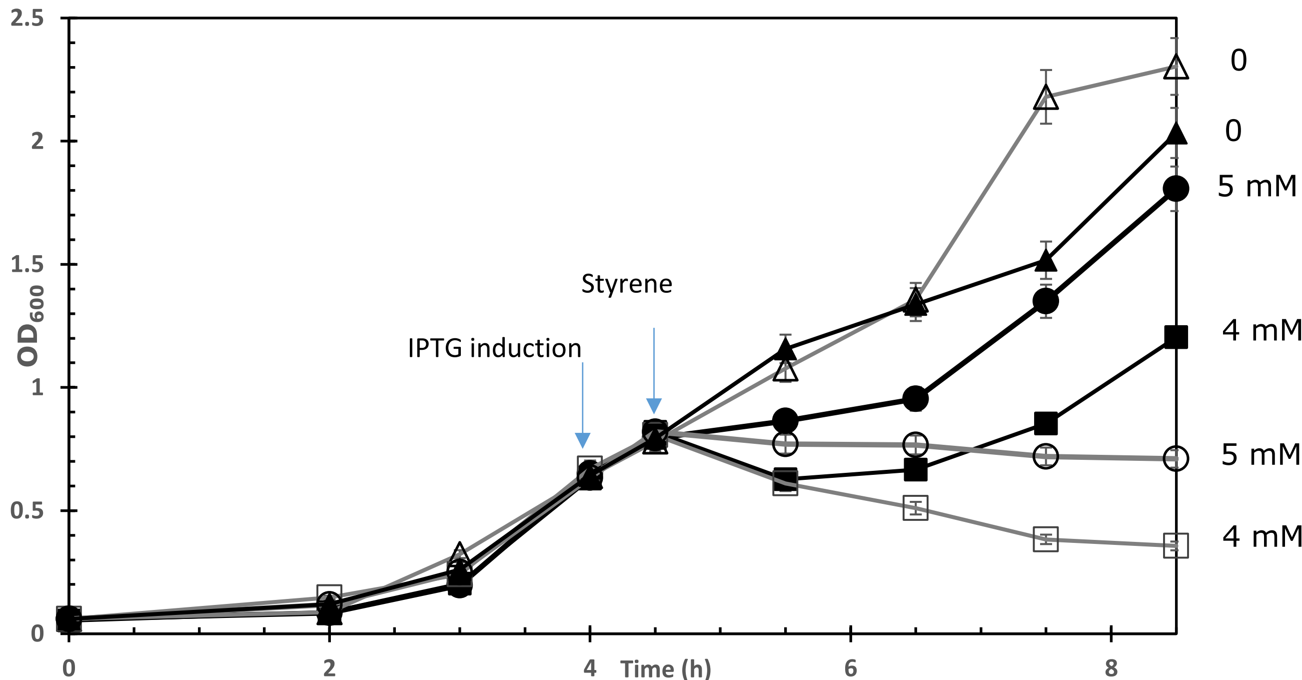


Figure 5.9 Toxicity tolerance of *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC cells to styrene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, styrene (0 (Triangles), 4 (Squares) and 5 mM (Circles)) was added 30 min after IPTG induction. The solid shapes represent the strain with the pump. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-ΔpETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after styrene addition. Error bars represent one standard deviation from triplicate experiments.

The strain bearing SrpABC pump-encoding genes was more tolerant of styrene at 2.5 mM than the control strain whereas when using 4 mM styrene, the growth of both strains was inhibited, but the growth of the pump bearing strain began to recover two hours post induction with IPTG.

In conclusion, exogenously added styrene at 4 mM concentration is toxic to the cells of the pump-harbouring strain and the control but the strain harbouring the pump could recover its growth after approximately 2 h from styrene addition.

Styrene toxicity was also investigated in *E. coli* MG1655 (DE3) harbouring SrpABC pump genes in a low copy plasmid (pACYCDuet-1). Styrene tolerance was examined in this strain in identical experiments. Negative control (empty pETDuet-1 plasmid) was also grown at the same conditions (Fig 5.10).

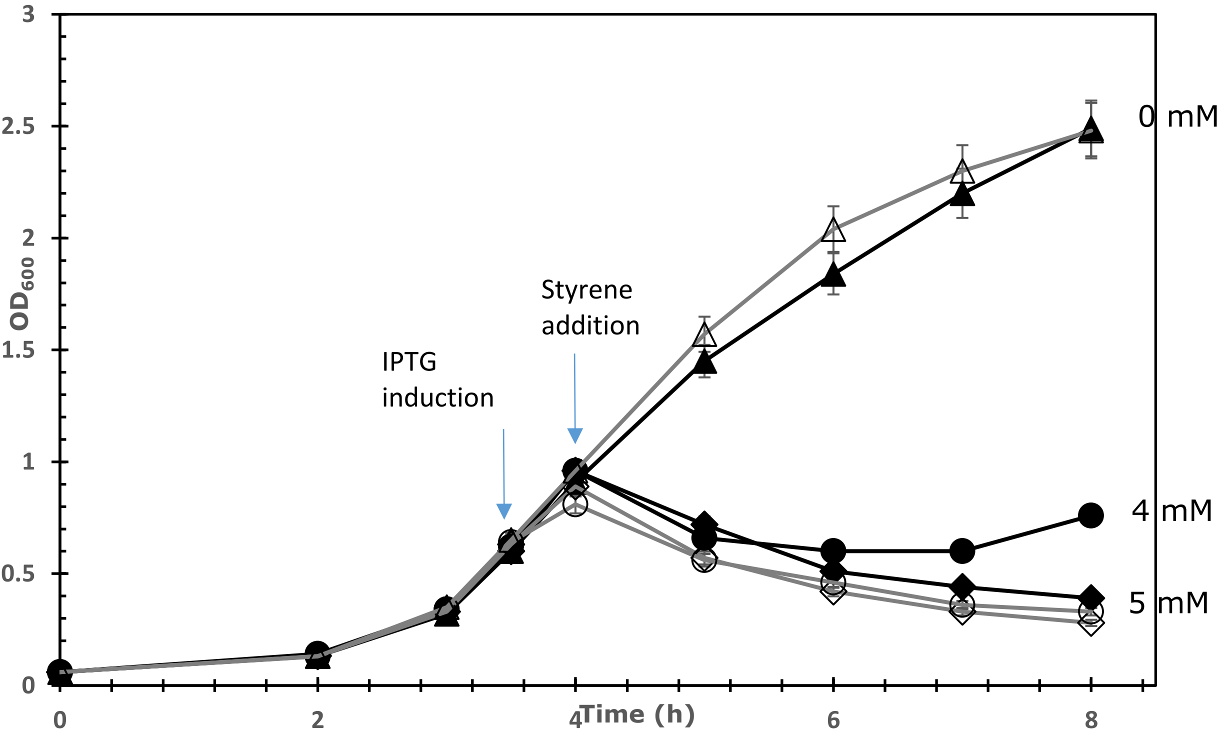


Figure 5.10 Toxicity tolerance of *E. coli* MG1655 (DE3)-pACYCDuet_MCS2_SrpABC cells to styrene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, styrene 0 (Triangles), 4 (Circles) and 5 mM (Diamonds) 30 min after IPTG induction. The solid shapes represent the strain with the pump. The open shapes represent the negative control (empty pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after styrene addition. Error bars represent one standard deviation from triplicate experiments.

Toxicity tolerance assay results have shown similar results to the previous experiment with the medium copy plasmid (pETDuet-1) which when using 4 mM styrene, the growth of both strains was inhibited but the growth in the SrpABC pump-bearing strain began to recover again and the recovery took a long time because of the low copy number plasmid (pACYCDuet-1) was used. In addition, when using 5 mM styrene neither strain could grow.

Samples from the cultures were taken and investigated under the light microscope to check the morphology of the cells. Some of the cells of bacterial cultures (*E. coli* MG1655 (DE3)-pETDuet_SrpABC) exposed to 5 mM styrene were non-motile and some were moving slowly indicating the toxicity (Fig 5.11). In addition, some longer filamentous cells were noticed but not in the cultures without added styrene. This is could be they are the dividing cells during the binary fission phase. Therefore, more analysis is needed in future research including the study of the morphology of the cells using the Electron microscope to gain more insights about the morphology of the cells and the presence of mutated bacterial cells.

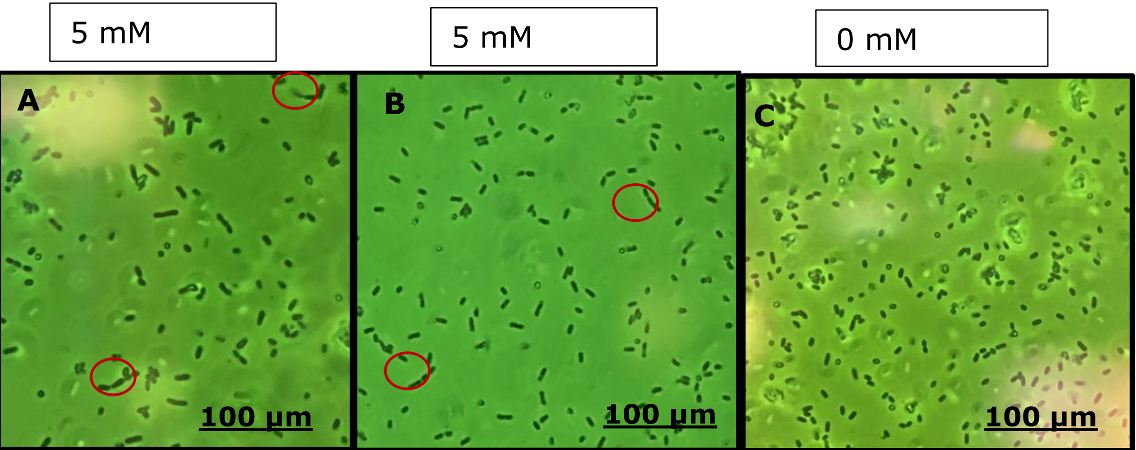


Figure 5.11 Morphology of *E. coli* MG1655 (DE3)-pETDuet_SrpABC cells under the light microscope. Bacterial cells were inspected after styrene (5 mM) addition from separate samples. The magnification was 100x. Red circles indicate the filamentous cells. A and B panels represent samples from cultures with styrene (5 mM). C panel represents sample from a culture without styrene addition.

In addition, I did the viability test of the cells, however technically it was difficult to apply the experiment after 1 min incubation with several dilutions, so I decided to determine the concentrations (0 to 5) and the dilutions up to 10^{-4} and add an extra time interval for 30 minutes was added to detect the accurate results. In addition, I measured the viable count in the control cultures (without styrene) and grow each concentration culture in triplicate (Tables 11.1, 11.2 Appendix). After a brief exposure to 2.5 mM styrene (1 minute) results in no toxicity with the number of viable cells unaffected. There was no significant difference in the mean number of the viable cell after one-minute incubation for the first three dilutions up to 10^{-4}. At higher concentrations there was an apparent 2-fold reduction in the number of viable cells. After longer exposure this effect was much more evident with considerable toxicity observed at both 2.5 and 5 mM styrene. Indeed, at 5 mM complete cell death was observed (Table 11.3 Appendix1). Therefore, I concluded that the concentration of styrene at 5 mM is toxic to *E. coli* MG1655 (DE3).

Besides, during exposure to styrene, the increased turbidity was noticed with higher concentrations of styrene in the experiment, possibly due to increased number of dead cells. Moreover, after 30 minutes' incubation with styrene, the solubility of styrene in the bacterial culture seems to be increased in comparison to the cultures with one-minute incubation, since some styrene droplets were seen at the surface of the culture. This due to the production of surfactants when the cells died that may have helped to increase the solubility of styrene and the homogeneity of the solution.

Conclusion

Styrene toxicity tolerance was investigated in *E. coli* MG1655 (DE3) using the efflux pump SrpABC. Three styrene concentrations were used; 5, 4 and 2.5 mM styrene. Styrene at concentrations 5 and 4 mM is toxic to *E. coli* cells but in the case of 4 mM styrene, some growth recovery was observed with the cells bearing SrpABC pump two hrs after styrene exposure.

Toxicity tolerance of other solvents was also investigated in the following section:

5.4 Toxicity tolerance of other related organic solvents

Having observed that SrpABC expression could confer resistance to styrene and toluene, confirming previous research findings (Kieboom *et al.*, 1998b), it was important to explore the wider substrate specificity of the SrpABC pump in our strains. Toxicity of other related organic solvents was undertaken to determine the substrate list of the newly *srpABC*-transformed bacterial cells. The ChemSpider tool was used to search for chemicals of interest as a target group for toxicity screening and tolerance in *E. coli*, the most important aspect is that the chosen chemicals are related to styrene structure (Table 5.1). Especially since searches of the literature did not reveal any studies including such chemicals with the SrpABC and AcrABTolC transporters in *E. coli*.

Table 5.1 List of the organic solvents that will be added to the substrate list of the pumps

	Name	Structure	CAS no.	Melting point °C	Physical state at RT
1.	Cyclohexane		110-82-7	6.47	Liquid
2.	Cyclohexene		110-83-8	−104	Liquid
3.	1,3-Cyclohexadiene (1,2-Dihydrobenzene)		592-57-4	N.D.	Liquid
4.	Ethylcyclohexane ≥99%		1678-91-7	−111	Solid

5.4.1 Cyclohexane toxicity tolerance

To determine cyclohexane tolerance in *E. coli*, cultures of *E. coli* MG1655 (DE3) harbouring SrpABC pump genes (pETDuet-1 plasmid) were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. After 30 min, cyclohexane was added. Negative control (empty pETDuet-1 plasmid) was also grown at the same conditions (Fig 5.12).

The strain harbouring the SrpABC pump was more tolerant to cyclohexane at 10 mM than the control strain (with the empty plasmid). In addition, 25 mM cyclohexane was toxic for both strains, but the strain bearing the pump could recover after 1.5 h from cyclohexane addition. Moreover, at 100 and 50 mM cyclohexane, the growth of both strains was inhibited concluding that all these concentrations are non-tolerable and killing the bacteria. Kieboom *et al.*, (1998)

have found that *P. putida S12* having the SrpABC pump can grow with 1% v/v (11.9 mM) cyclohexane concentration.

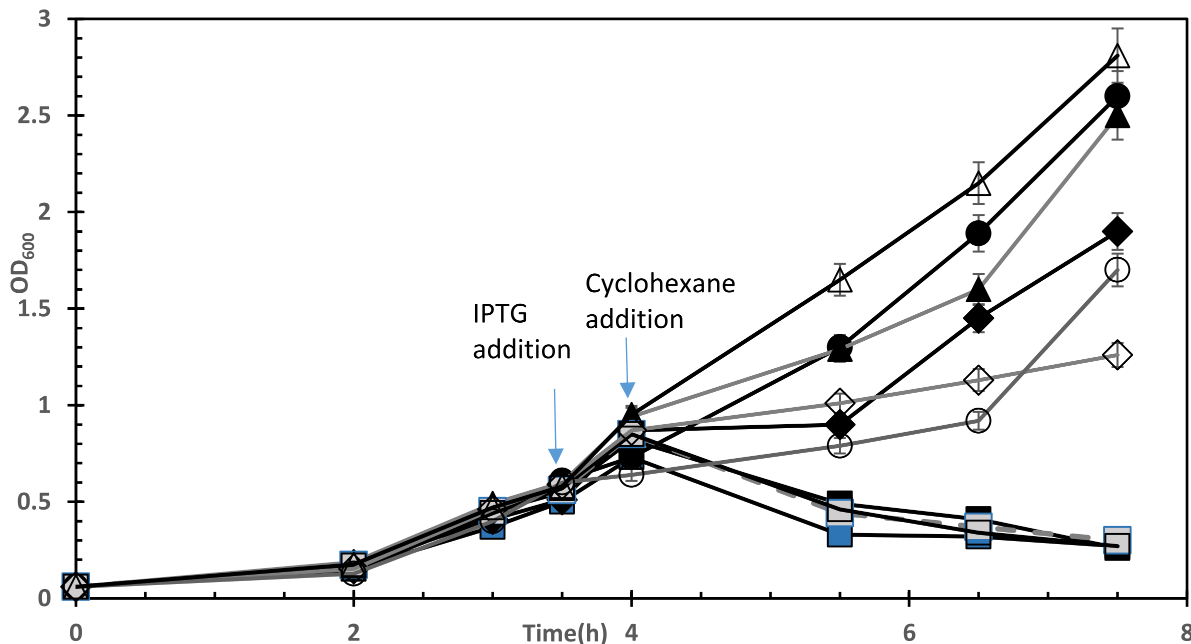


Figure 5.12 Toxicity tolerance of *E. coli* MG1655 (DE3)-pETDuet_MCS1_SrpABC cells to cyclohexane. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, cyclohexane (100 (Blue squares), 50 (Black squares), 25 (Diamonds) and 10 mM (Circles)) was added 30 min after IPTG induction. The triangles represent no added cyclohexane. The solid shapes represent the strain with the pump. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-Δ pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

Conclusion

SrpABC-bearing strain, *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC, could tolerate to up to 10 mM cyclohexane over the control strain. In addition, the strain harbouring the pump showed some recovery 2 hrs after exposure to 25 mM cyclohexane

5.4.2 Cyclohexene toxicity tolerance

To determine cyclohexene toxicity tolerance in *E. coli*, cultures of *E. coli* MG1655 (DE3) harbouring SrpABC pump genes (pETDuet-1 plasmid) were grown the identically as the previous experiments. Cyclohexene was added 30 min after IPTG induction (Fig 5.13).

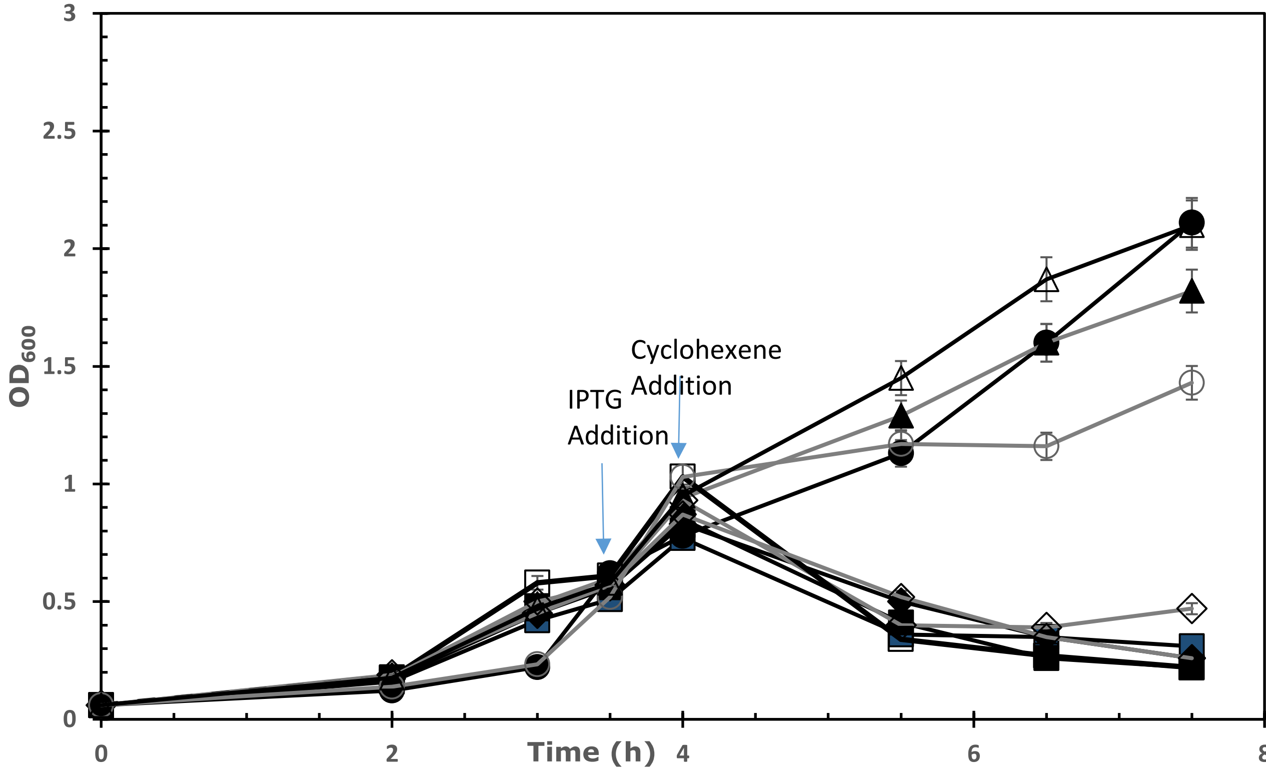


Figure 5.13 Toxicity tolerance of *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC cells to cyclohexene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, cyclohexene (100 (Blue squares), 50 (Black squares), 25 (Diamonds) and 10 mM (Circles)) was added 30 min after IPTG induction. The triangles represent no added cyclohexene. The solid shapes represent the strain with the pump. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-ΔpETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

The strain harbouring SrpABC pump was more tolerant to cyclohexene at 10 mM than the control strain. In addition, at 25, 50 and 100 mM cyclohexene, the growth of both strains (with- and without the pump) was inhibited indicating that all these concentrations are toxic and killing the bacteria.

Conclusion

SrpABC-bearing strain, *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC, could tolerate to up to 10 mM cyclohexene over the control strain.

5.4.3 1, 3-cyclohexadiene toxicity tolerance

To verify 1, 3-cyclohexadiene toxicity tolerance in *E. coli*, cultures of *E. coli* MG1655 (DE3) harbouring SrpABC pump genes (pETDuet-1 plasmid) were grown in M9 minimal media until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, 1, 3- cyclohexadiene was added after 30 min from IPTG induction. Negative control (empty pETDuet-1 plasmid) was also grown at the same condition (Fig 5.14).

The strain harbouring the SrpABC pump was more tolerant to 1, 3-cyclohexadiene at 5 and 10 mM than the control strain. In addition, 25 mM 1, 3-cyclohexadiene represents the toxic concentration in both strains as the growth was inhibited indicating that this concentration is non-tolerable and killing the bacteria.

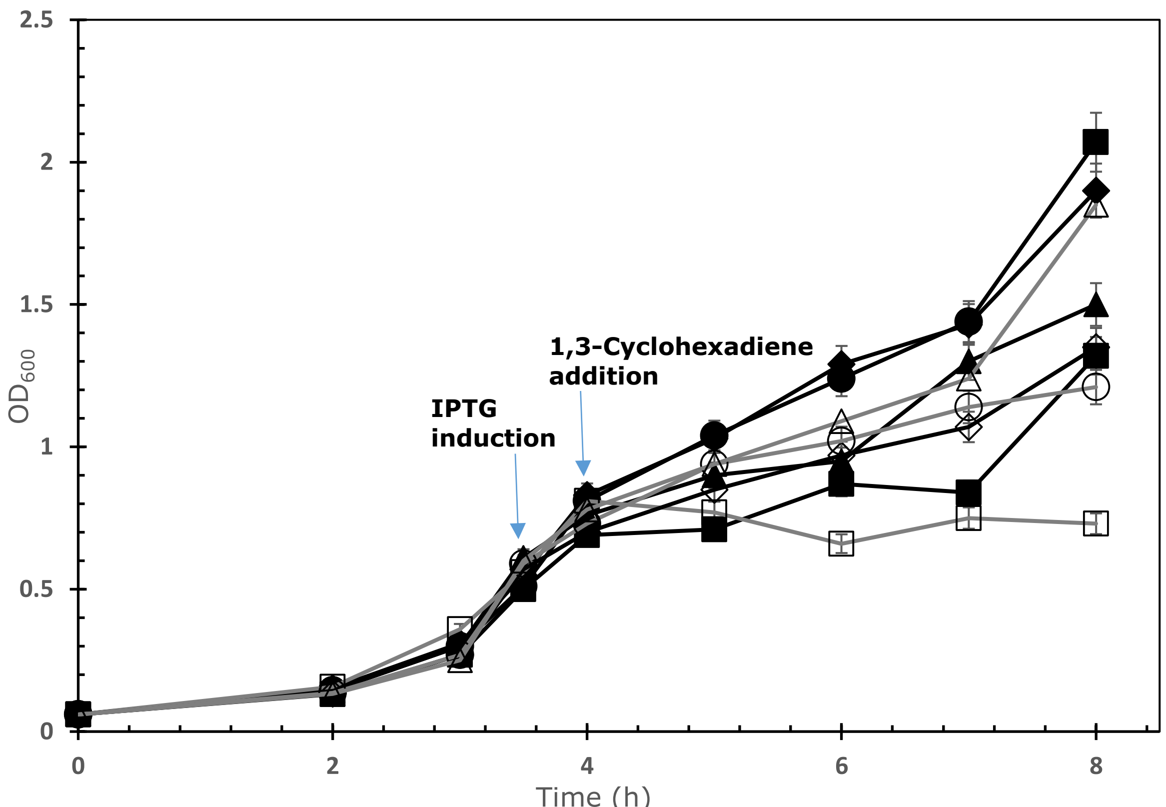


Figure 5.14 Toxicity tolerance of *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC cells to 1, 3-cyclohexadiene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, 1, 3-cyclohexadiene (25 (Squares), 10 (Diamonds) and 5 mM (Circles)) was added 30 min after IPTG induction. The triangles represent no added 1, 3-cyclohexadiene. The solid shapes represent the strain with the pump. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-Δ pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

<u>Conclusion</u>

E. coli MG1655 (DE3) pETDuet_MCS1_SrpABC was tolerant up to 10 mM 1, 3-cyclohexadiene over the control.

5.4.4 Ethylcyclohexane toxicity tolerance

To determine ethylcyclohexane toxicity tolerance in *E. coli*, cultures of *E. coli* MG1655 (DE3) harbouring SrpABC pump genes (pETDuet-1 plasmid) were grown identically as previous experiments. Ethylcyclohexane was added 30 min after IPTG induction (Fig 5.15).

The chemical was toxic over a short time period (up to 6 hours) in the control strain, then the control recovered and started to grow quickly. This is different from other experiments where the higher concentrations of chemical see no growth even at 8 hours. The results from the previous experiment have revealed that the strain harbouring SrpABC pump was more tolerant to ethylcyclohexane at all concentrations than the control strain. In addition, 50 and 100 mM ethylcyclohexane were toxic to the control strain.

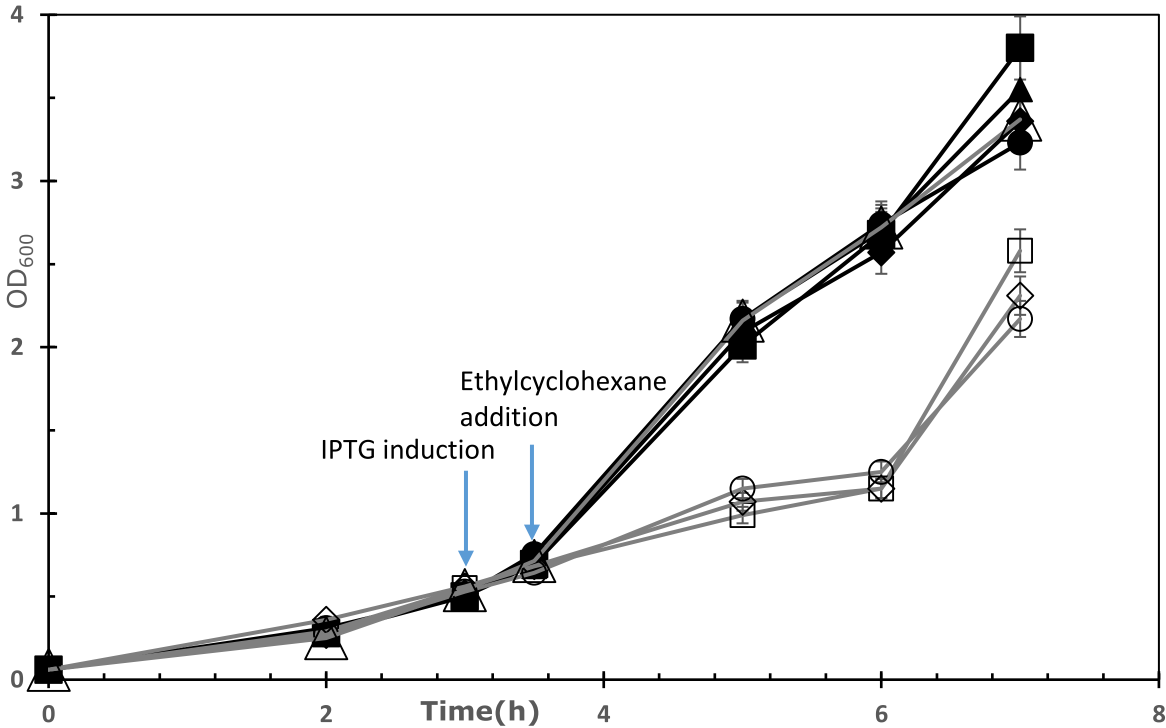


Figure 5.15 Toxicity tolerance of *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC cells to ethylcyclohexane. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, ethylcyclohexane (100 (squares), 50 (Diamonds) and 25 (Circles) mM) was added 30 min after IPTG induction. The triangles represent no added ethylcyclohexane. The solid shapes represent the strain with the pump. The open shapes represent the negative control (*E. coli* MG1655 (DE3)-Δ pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

General conclusion

Overexpression of the SrpABC pump was confirmed in *E. coli* MG1655 (DE3)-pETDuet_MCS1_SrpABC and *E. coli* MG1655 (DE3)-pACYCDuet_MCS1_SrpABC by RT-PCR of SrpB and SrpC transcripts, and by Western Blotting of the anti-Hi tagged SrpA antibody.

Styrene toxicity was investigated in the same strains; *E. coli* MG1655 (DE3)-pETDuet_MCS1_SrpABC was more tolerant to exogenously added styrene than the control strain. The strain harbouring the SrpABC could tolerate 2.5 mM styrene and recover the growth 2 hours after exposure to 4 mM styrene.

Regarding the tolerance towards other chemicals, the strain harbouring the SrpABC pump could tolerate 10 mM of cyclohexane, cyclohexene and 1- 3, cyclohexadiene and conferred tolerance to up to 100 mM ethylcyclohexane, and was more tolerant than the control strain.

Results

6 AcrABTolC efflux pump

6.1 Overproduction of AcrAB TolC efflux pump

Having shown the production and function of the *Pseudomonas* efflux pump SrpABC, it was of interest to compare it to a different efflux pump from *E. coli*, AcrABTolC. This experiment was done to confirm the protein overproduction of AcrABTolC efflux pump in *E. coli*. For that purpose, the strain *E. coli* BW25113 bearing AcrABTolC (pBAD-AcrABTolC) (Fig 6.1) was obtained (Martin Pos, University of Frankfurt).

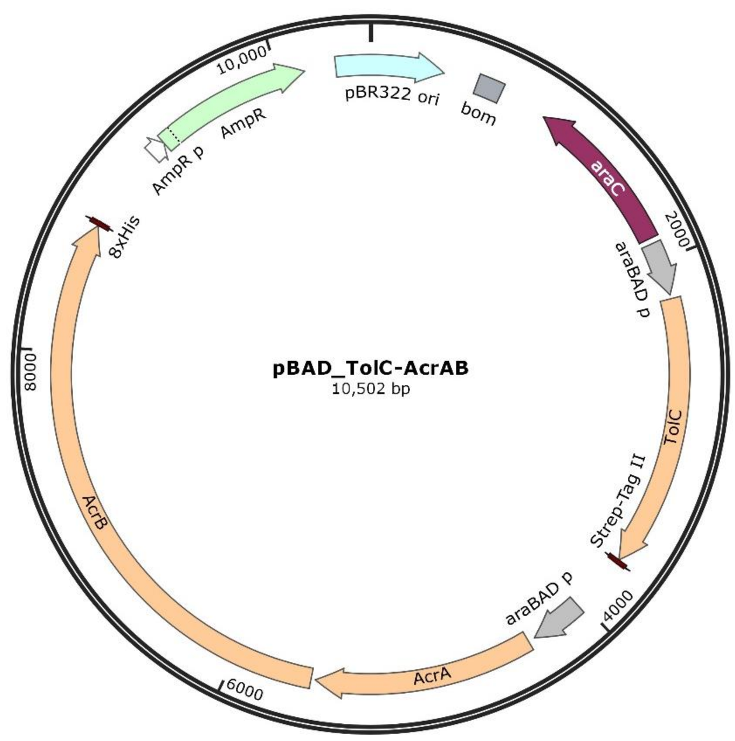


Figure 6.1 pBAD_TolC-AcrAB plasmid map (Snapgene). The plasmid controlled by *ara*BAD promoter, and have pBR322 origin of replication

The *acr*ABtolC genes are under the control of the P_{BAD} promoter which is regulated by the AraC protein. The system is induced by a high concentration of arabinose and repressed by the presence of glucose or by competitive binding of the anti-inducer fucose; these plasmids have very low background levels of expression. In addition, gene expression can be turned on and off rapidly by changing the sugars in the medium (Guzman *et al*., 1995). Thus, this system provides a controllable expression system designed to prevent toxicity resulting from protein overexpression consequences, such as protein misfolding and cytoplasmic aggregates. In order to control the expression, *E. coli* BW25113 pBAD-AcrABTolC cultures were grown in M9 medium (50 mL) using glycerol (2% v/v) as a carbon source with different concentrations of arabinose (0.002 - 0.2% w/v) added at an OD_{600} of 0.5 to determine an arabinose concentration that does not have an impact on growth, but which still shows protein expression. This enabled the selection of an arabinose concentration for the induction (Figure 6.2) in subsequent experiments to examine the effect of AcrABTolC expression on solvent tolerance.

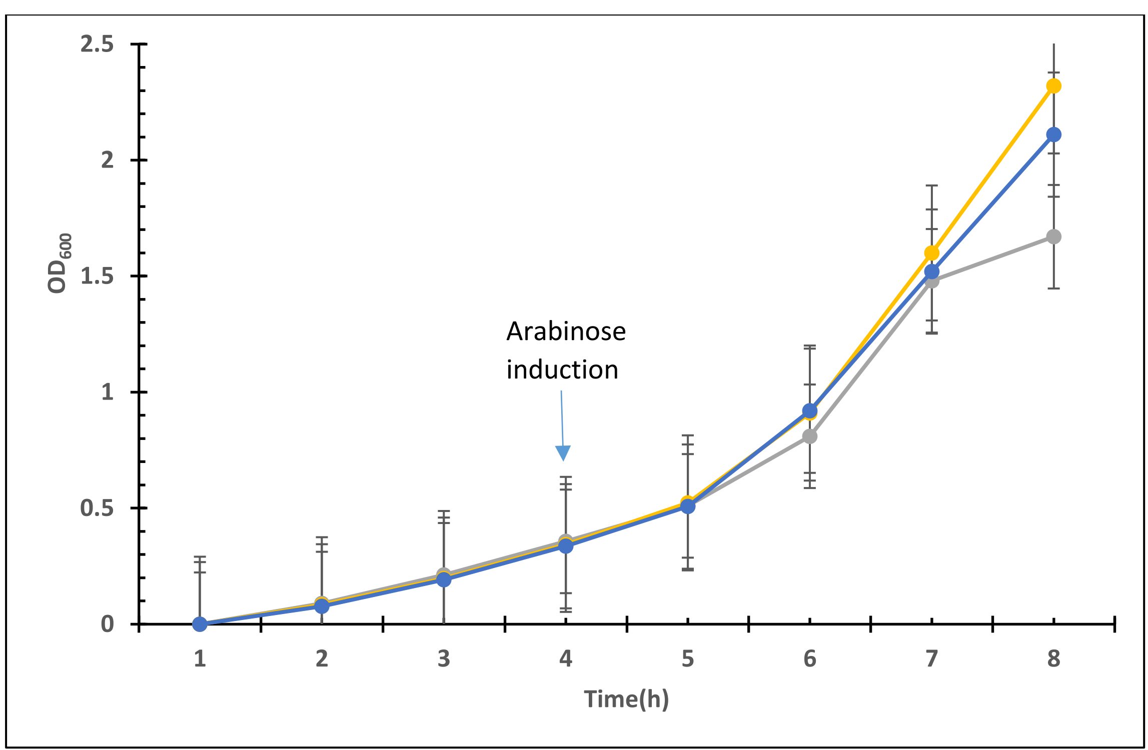
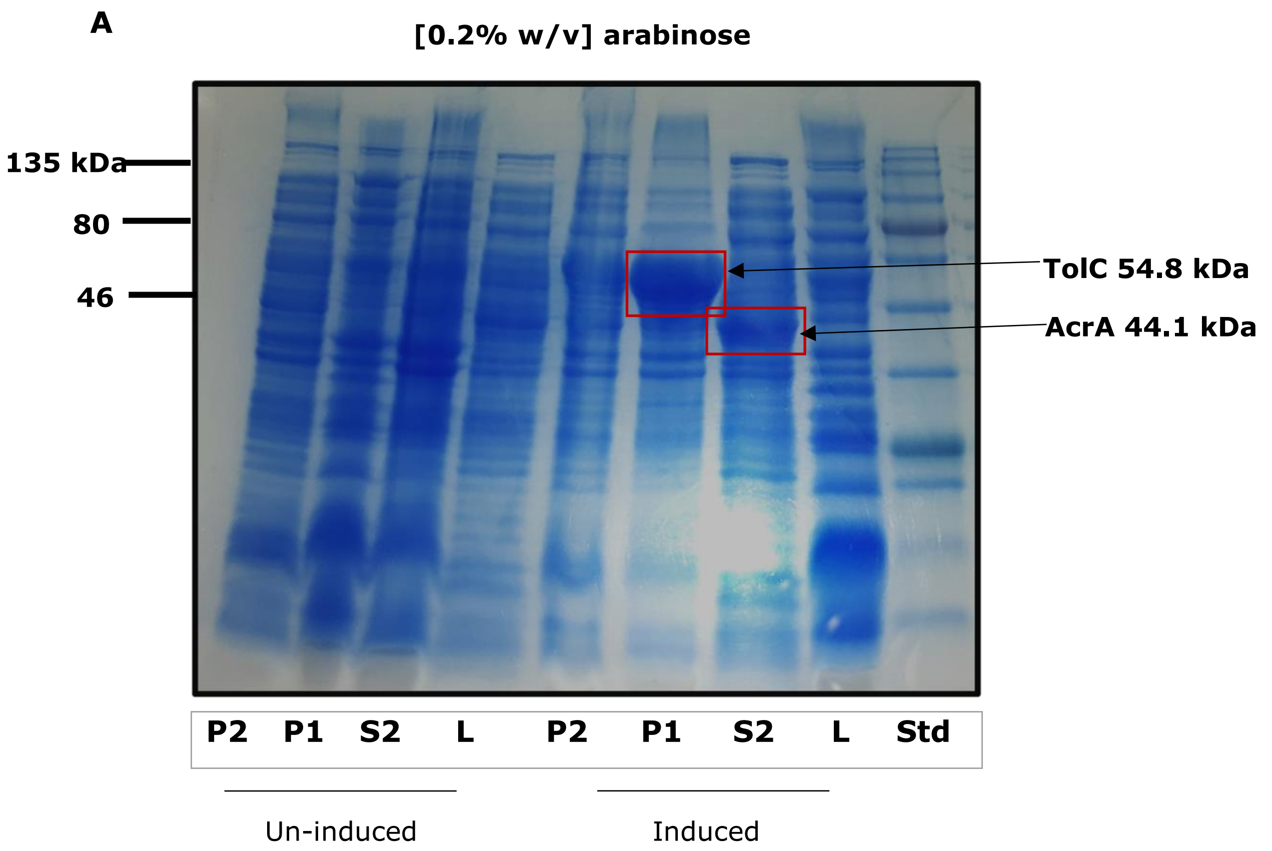


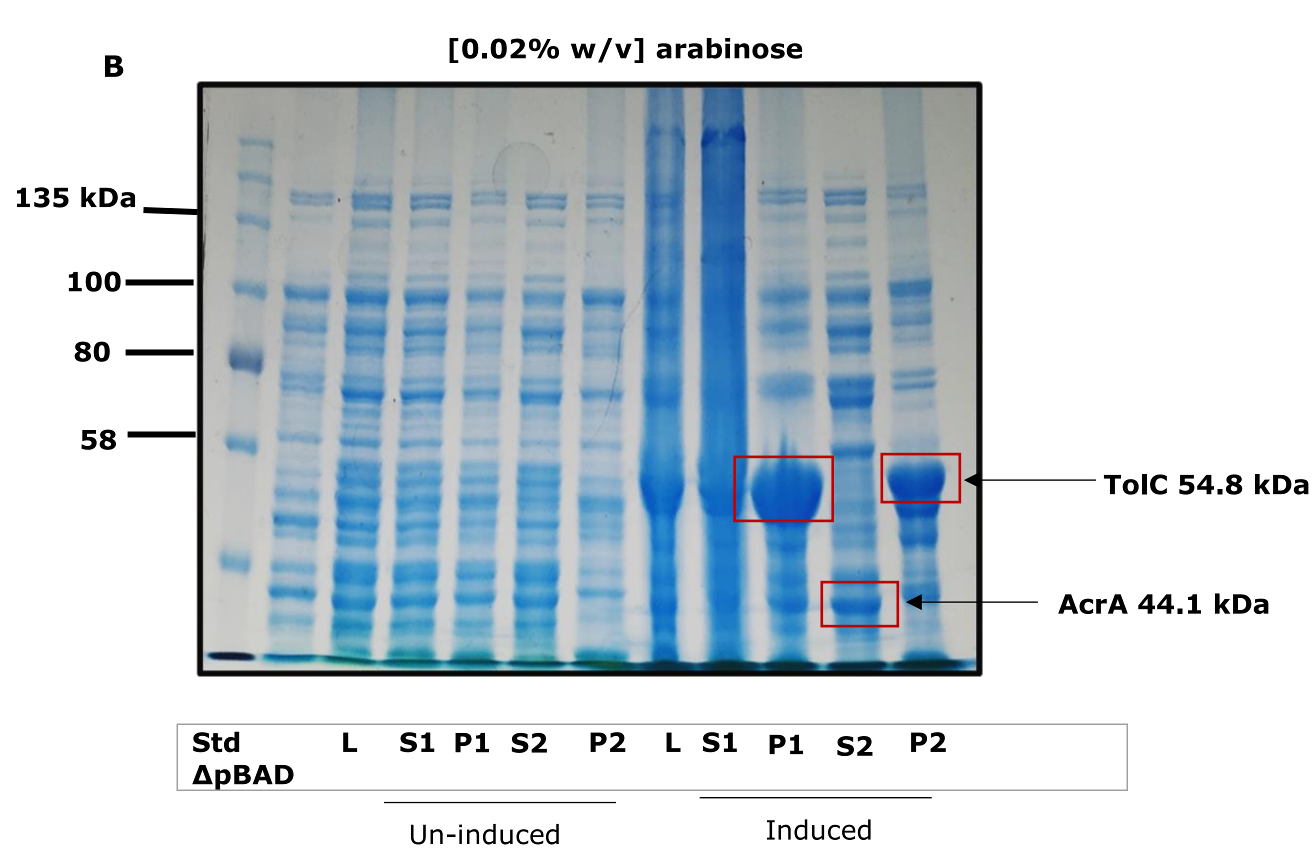
Figure 6.2 Growth curves of *E. coli* BW25113 pBAD_AcrABTolC. Growing cultures of *E. coli* BW25113 were investigated in M9 minimal medium supplemented by glycerol and different concentrations of arabinose (0.002 (blue), 0.02 (Yellow) and 0.2% w/v (Gray)) at 37 °C to choose the suitable arabinose concentration for the induction. Error bars represent one standard deviation from triplicate experiments.

After growing the cultures in M9 minimal medium supplemented with 2% (w/v) glycerol with the induction by different concentrations of arabinose (0.002 - 0.2% w/v) at 37 °C (200 rpm), the OD_{600} readings of the samples were monitored each hour.

The growth curves of the induced cultures by different concentrations of arabinose indicated no difference except that the culture induced with 0.2 % (w/v) arabinose decreased the growth rate 3 hours after induction. This could be explained that the overexpression has limited the culture's ability to divide. Therefore, I have decided to induce the cultures with 0.002% (w/v) arabinose in the chemical-tolerance assay experiments to avoid any overexpression consequences.

Following that, I investigated the protein production of the same cultures by harvesting samples at OD_{600} reaching 1 from both the induced and the un-induced cultures (Fig 6.2). There was an observable expression of TolC and AcrA in the cultures induced with arabinose (0.2% w/v or 0.02% w/v). Thus, the protein expression was increased with the increased arabinose concentration. The expression of TolC (54.8 kDa) was massive in the induced membrane fractions (P1 & P2) compared to expression of the equivalent gene SrpC, which could only be detected by RT-PCR (Figure 5.6). AcrA (44.1 kDa) was detected and located in the soluble fraction in all the SDS gels, consistent with AcrA's localisation to the periplasm. On the other hand, the detection of AcrB (~116 kDa) was weak and not clearly visible by SDS PAGE analysis. The Western blot of the same culture induced with arabinose (0.002% w/v) indicated the expression of the His-tagged AcrB subunit by the appearance of the anti-His antibody in parallel to its molecular weight on the gene ruler in the western blot (Figure 6.3 (B)). In addition, the expression of TolC could be confirmed using Western blot analysis with an anti-Strep tag antibody (Figure 6.3 (C)).


A
[0.2% w/v] arabinose
135 kDa
80
46
TolC 54.8 kDa
AcrA 44.1 kDa
P2 P1 S2 L P2 P1 S2 L Std
Un-induced
Induced


B
[0.02% w/v] arabinose
135 kDa
100
80
58
TolC 54.8 kDa
AcrA 44.1 kDa
Std ΔpBAD L S1 P1 S2 P2 L S1 P1 S2 P2
Un-induced
Induced

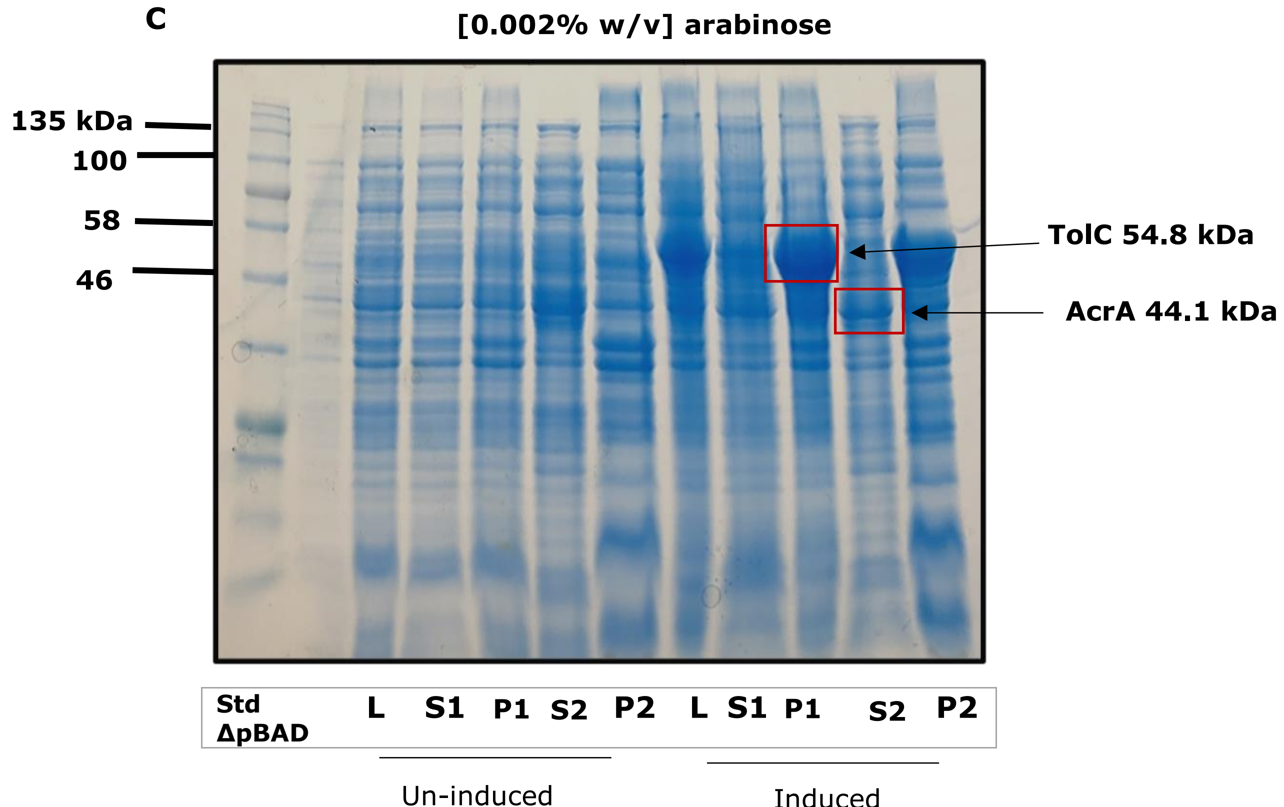


Figure 6.3 Protein production of *E. coli* BW25113-pBAD_AcrAB-TolC. After growing the cultures in M9 minimal broth supplemented with 2% (v/v) glycerol and induced by 0.002 % (w/v) arabinose at 37°C. Cell lysates were centrifuged for 15 min at 4 °C to pellet out any unbroken cells and large debris. The first supernatant (S1) was retained and the first pellet (P1) was re-suspended in the same volume as the original re-suspension. The supernatant was ultra-centrifuged (100,000 g for 1 h at 4 °C). The second supernatant (S2) was retained and the pellet (P2) was re-suspended in a suitable volume of MIB4 and protease inhibitors by shearing initially through a broad needle. A negative control which is the pBAD plasmid carrying no gene (ΔpBAD) was added. After determination the protein concentration of the samples, they were incubated at 90 °C for 10 min with SDS and β-mercaptoethanol to denature the proteins and to break the disulphide bridges respectively. A. with 0.2% arabinose induction, B. 0.02% arabinose, and C. 0.002% arabinose. All SDS PAGE gels were stained with instant blue (Expedeon®).'Std', standard.

Because the detection of AcrB was weak by SDS PAGE analysis, its expression was confirmed by Western Blotting Figure (6.4 A). Besides, the confirmation of TolC protein production was done by the detection of anti-Strep antibody (Fig 6.4 B & C).

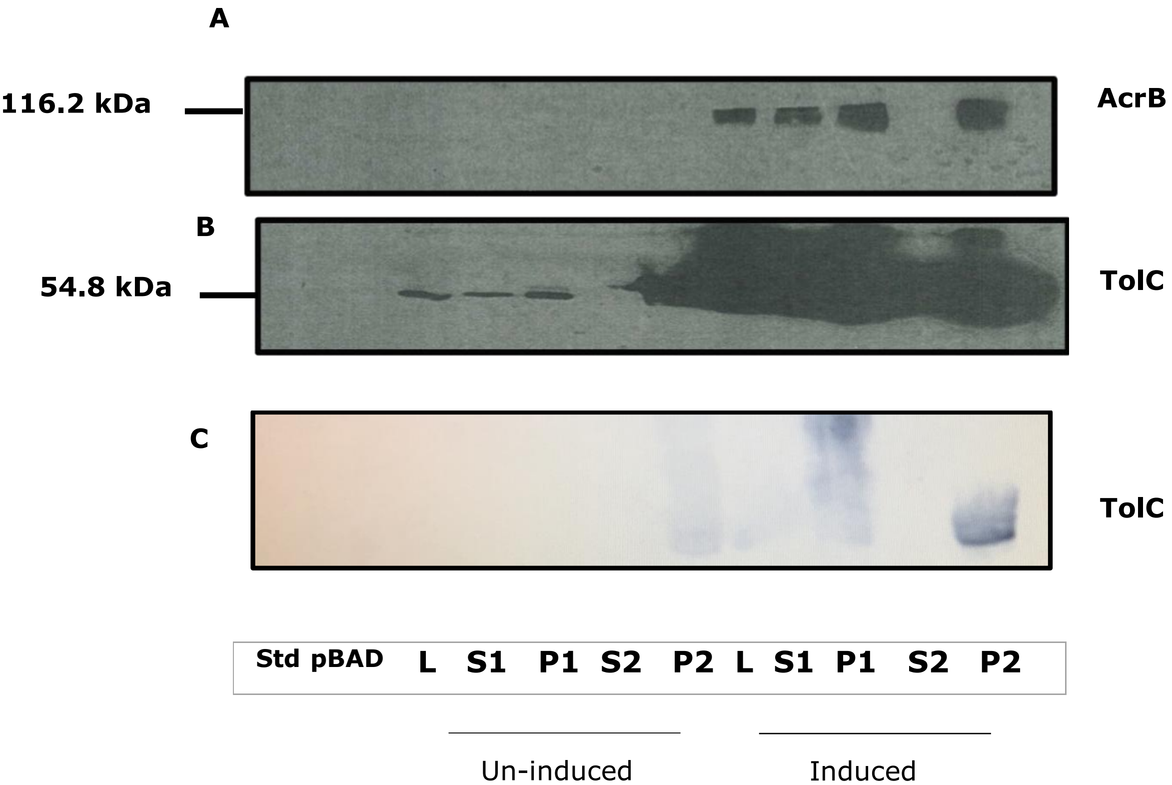


Figure 6.4 Western blot analysis of *E. coli* BW25113-pBAD_AcrAB-TolC. Samples were taken from the growing cultures in M9 minimal broth supplemented with 2% (v/v) glycerol and induced by 0.002 % (w/v) L- arabinose at 37°C. A. Western blot analysis indicating the expression of AcrB subunit (116.2) of the AcrABTolC efflux system in BW25113 using anti-His_6 tag antibody (diluted 1:5000). B. indicating the massive expression of TolC subunit using Anti-Strep antibody (1:2000). C. TolC subunit was detected using (1:20,000) diluted Anti-Strep antibody. 'L' represents lysate, 'P' represents pellet, and 'S' represents supernatant.

6.2 Toxicity tolerance assays in *E. coli* harbouring efflux pump system

Having demonstrated expression conditions for AcrABTolC, toxicity tolerance assay was proceeded to determine the toxicity tolerance of different chemicals in *E. coli* BW25113-pBAD_AcrABTolC and *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC.

For these experiments, the pertinent controls are the strain transformed with an empty pBAD vector and the wild-type strain itself as most previous studies, regarding toxicity tolerance assays used pump-free strains as a control such as *E. coli ΔacrAB* (Dunlop *et al*., 2011); (Mingardon *et al.*, 2015). Initial growth curves were conducted to determine the rate of growth of the three strains in the presence of the inducer arabinose (Fig 6.5).

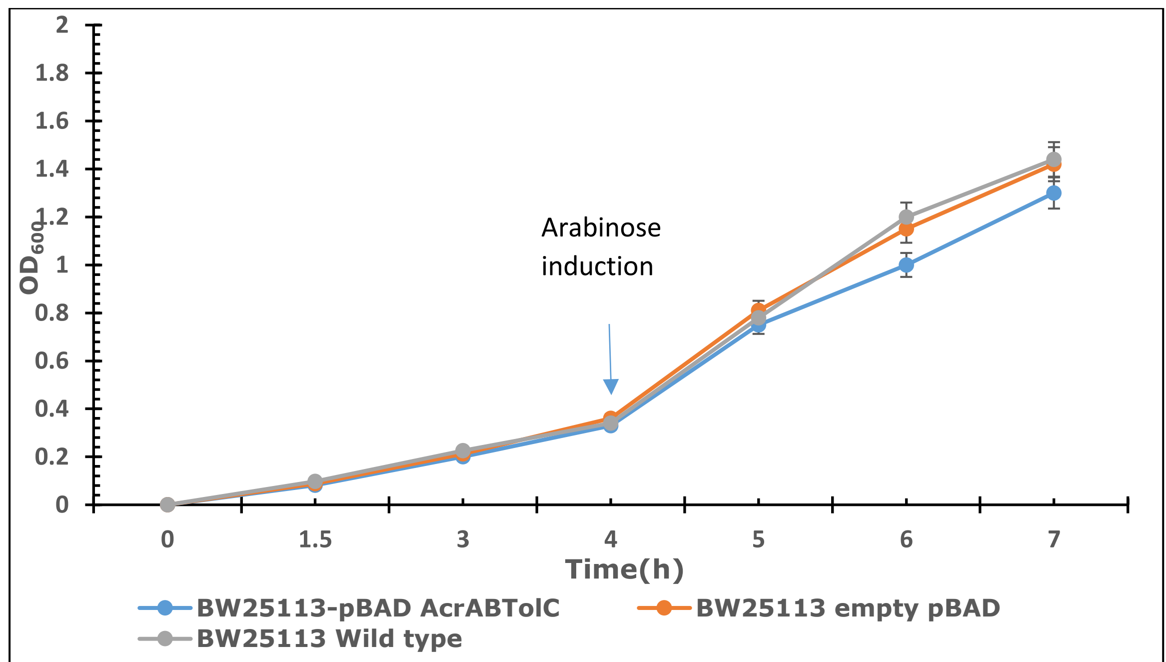


Figure 6.5 growth curves of *E. coli* BW25113-pBAD_AcrABTolC, BW25113-empty pBAD, and the wild-type BW25113 without chemical addition. The growth was in M9 minimal media supplemented with glycerol (2% v/v) and induced by arabinose (0.002% w/v) at 37 °C. Error bars represent one standard deviation from triplicate experiments.

As indicated in Figure (6.5), the growth rate of all the strains was comparable after arabinose induction except that with the efflux pump (*E. coli* BW25113-pBAD_AcrABTolC), which was slightly lower than the control strains.

This is may be explained by that the overexpression of TolC (which is clearly higher than the inner membrane component) could affect the growth rate of the culture and leads to slow the growth.

6.3 Styrene toxicity tolerance in *E. coli* harbouring AcrABTolC system

In styrene toxicity experiments, both empty vector and strain-alone controls were employed to provide some baseline analysis of the effect of styrene on the growth of the strains. As shown in Figure (6.6), the tolerance assays revealed that the strain harbouring the efflux pump, *E. coli* BW25113-pBAD_AcrABTolC, is tolerant to 2.5 mM (260.35 mg/L) styrene and the toxic concentration at 5 mM (520.7 mg/L) which was killing all the growing bacterial cells. The same finding was observed with the control strains. However, the growth rate of the empty-plasmid and wild-type strains (control strains) was higher than in strains expressing AcrABTolC either in conditions without styrene supplementation or in the presence of 2.5 mM styrene (Fig 6.6).

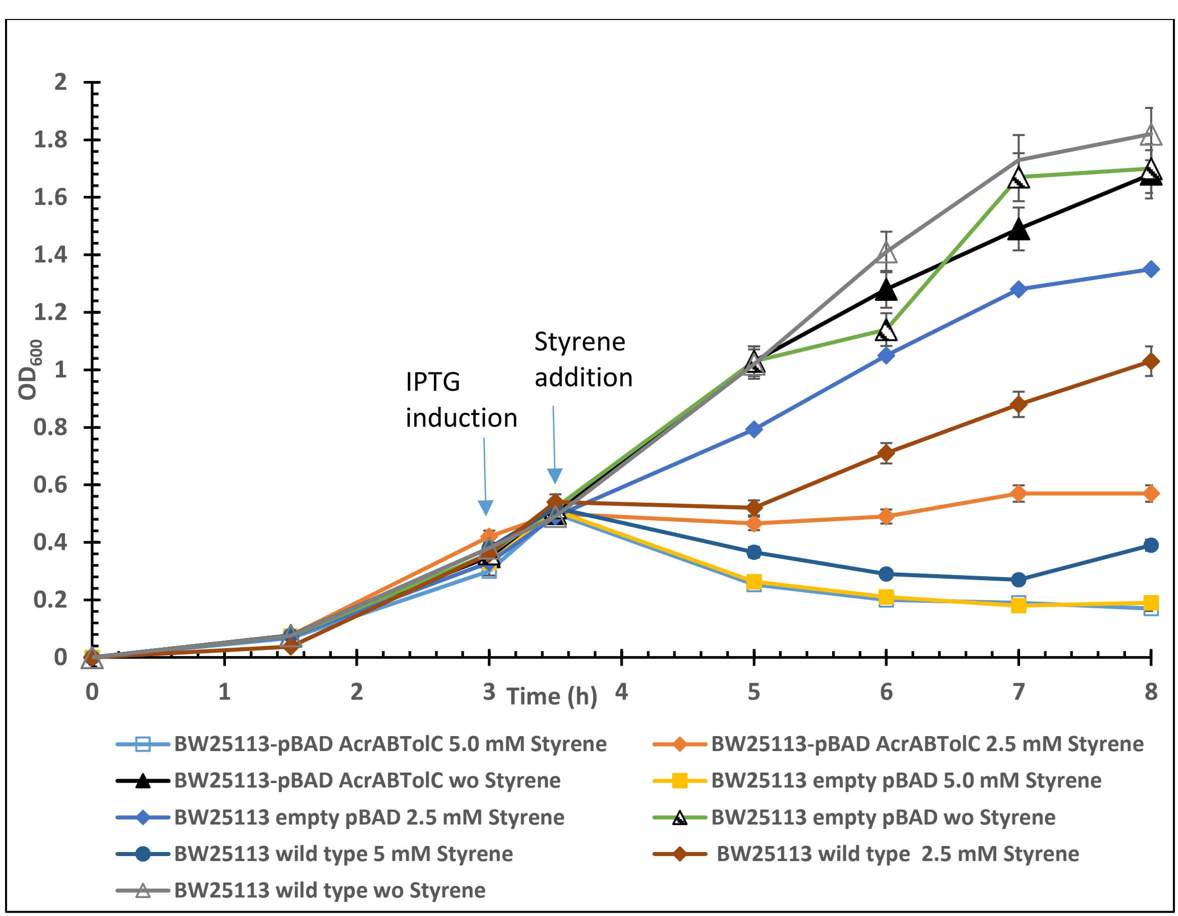


Figure 6.6 Toxicity tolerance of *E. coli* BW25113_pBAD AcrABTolC cells to styrene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, styrene (6.2 mM) was added after 30 min after IPTG induction. Negative control (empty pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored for 4 hours after styrene addition. 'wo' implies without. Error bars represent one standard deviation from triplicate experiments.

As described in the previous section, the reason behind this could be because of the over-expression of TolC subunit in the AcrABTolC system, which would slow the culture growth, perhaps adding to the stress on the cell by the presence of styrene and thus leading to slower growth. Because of that, the overexpression of *acr*AB was acheived by removing the TolC encoding gene from the pBAD_AcrABTolC plasmid and using *E. coli* C43 (DE3) Δ*acr*AB strain for overexpression.

After obtaining the knockout *acr*AB strain, *E. coli* C43 (DE3) Δ*acr*AB (1), the *tol*C gene was deleted and cloned the *acr*AB gene in the pETDuet plasmid. Afterwards,

E. coli C43 (DE3) Δ*acr*AB was transformed with the plasmid and the protein expression was confirmed by SDS PAGE (Section 5.1.2).

6.4 Protein expression of the AcrAB protein.

After the massive overproduction of TolC shown previously and its effect on the decreased overall growth of the strain in the previous tolerance experiments, the *acr*AB genes were overexpressed by cutting TolC-encoding gene from the pBAD_AcrABTolC plasmid and cloning the AcrAB-encoding gene fragment into the pETDuet-1 plasmid. Subsequently, after verification of pETDuet-AcrAB plasmid (Fig 6.7) by DNA sequencing.

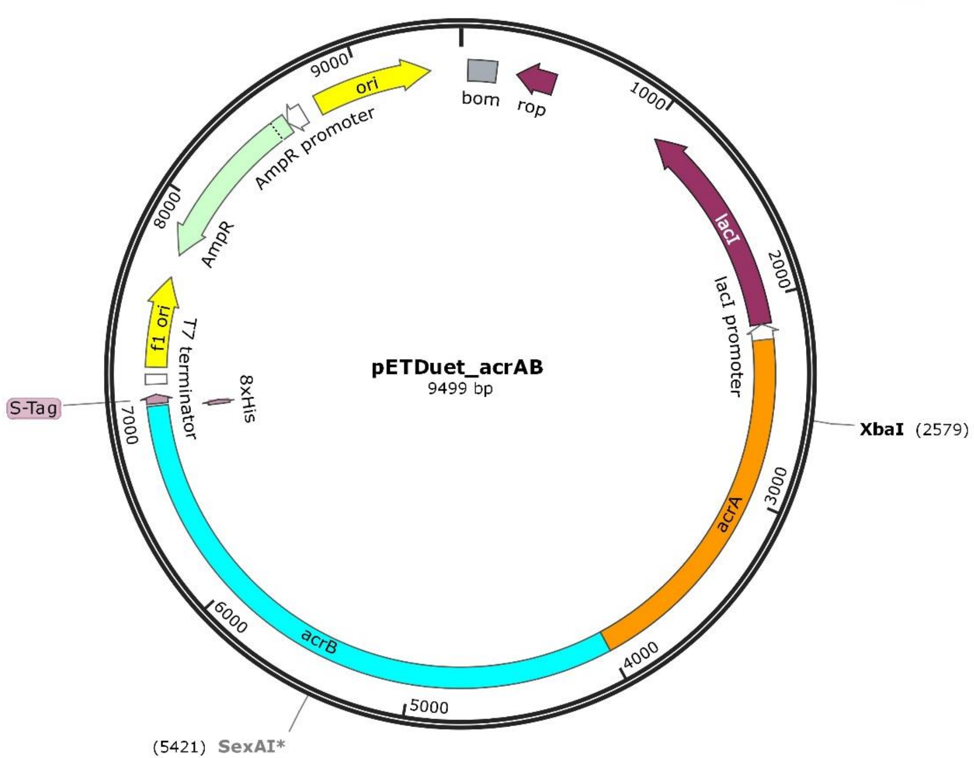


Figure 6.7 pETDuet_AcrAB map. The plasmid is under T7 promoter with Ampicillin antibiotic resistance (I used Carbenicillin instead).

(1) This strain was obtained from Ian Kerr's Laboratory

E. coli C43 Δ*acr*AB was transformed the plasmid and the protein expression was examined by SDS PAGE (Fig 6.8). In detail, after growing the cultures in M9 minimal broth supplemented with carbenicillin (100 mg/L), the culture was diluted (1/100) in M9 minimal broth and incubated at 37°C until OD_{600} reached 0.5. Subsequently, IPTG (0.5 mM) was added to the culture. Samples before and after induction were collected and processed with the previously mentioned membrane preparation protocol (4.11.4.2 section).

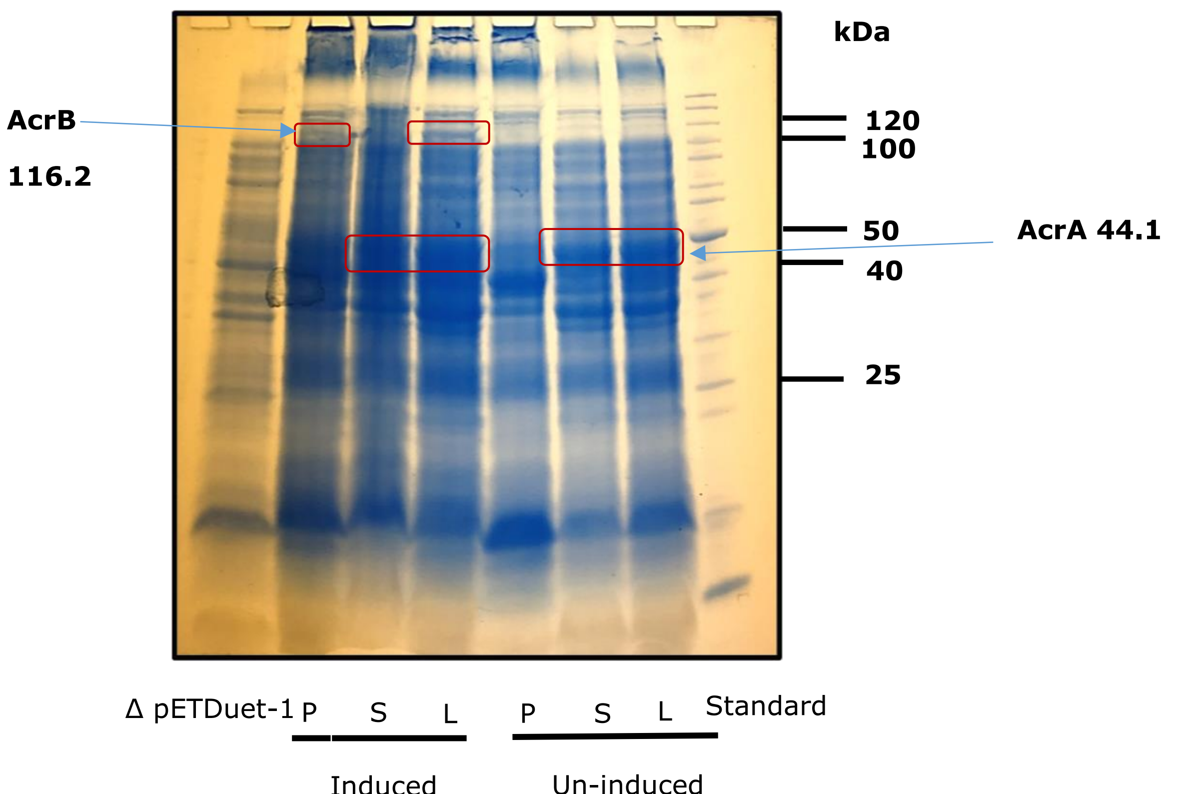


Figure 6.8 Protein expression of AcrAB in C43 (DE3) Δ*acr*AB_pETDuet-AcrAB. The culture of C43 (DE3) Δ*acr*AB_pETDuet-AcrAB was grown in M9 media at 37 °C and induced by 0.5 mM IPTG for 3 hours, the samples were analysed by SDS PAGE. 'L' represents lysate, 'P' represents pellet, and 'S' represents supernatant.

The SDS gel of C43 (DE3) Δ*acr*AB_pETDuet-AcrAB (Fig 6.8) confirmed the protein production of AcrB (~116 kDa) subunit in the IPTG-induced lysate and pellet suspension fractions and this was expected because the AcrB subunit is the inner membrane part of the pump which will be present in the pellets fraction.

AcrA (44.1 kDa) protein was produced in both induced and the non-induced lysate and supernatant fractions. Although the AcrA subunit is found in the periplasmic part of the pump corresponding the soluble protein, but in this expression it is also present in the non-induced fractions because the expression vector (pETDuet-1) is controlled by the T7 promoter which is a leaky promoter (Baneyx, 1999).

The toxicity of styrene of the strain bearing *acr*AB gene was investigated by growing cultures of the cells in M9 minimal media until OD_{600} becomes 0.5, then the culture was induced by 0.4 mM IPTG. Afterwards, styrene was added 30 min after IPTG induction. Styrene at concentration 5 mM was avoided in this experiment due to high toxicity to the cells in the previous experiments. A negative control (empty pETDuet-1 plasmid) was also grown at the same conditions (Fig

6.9).

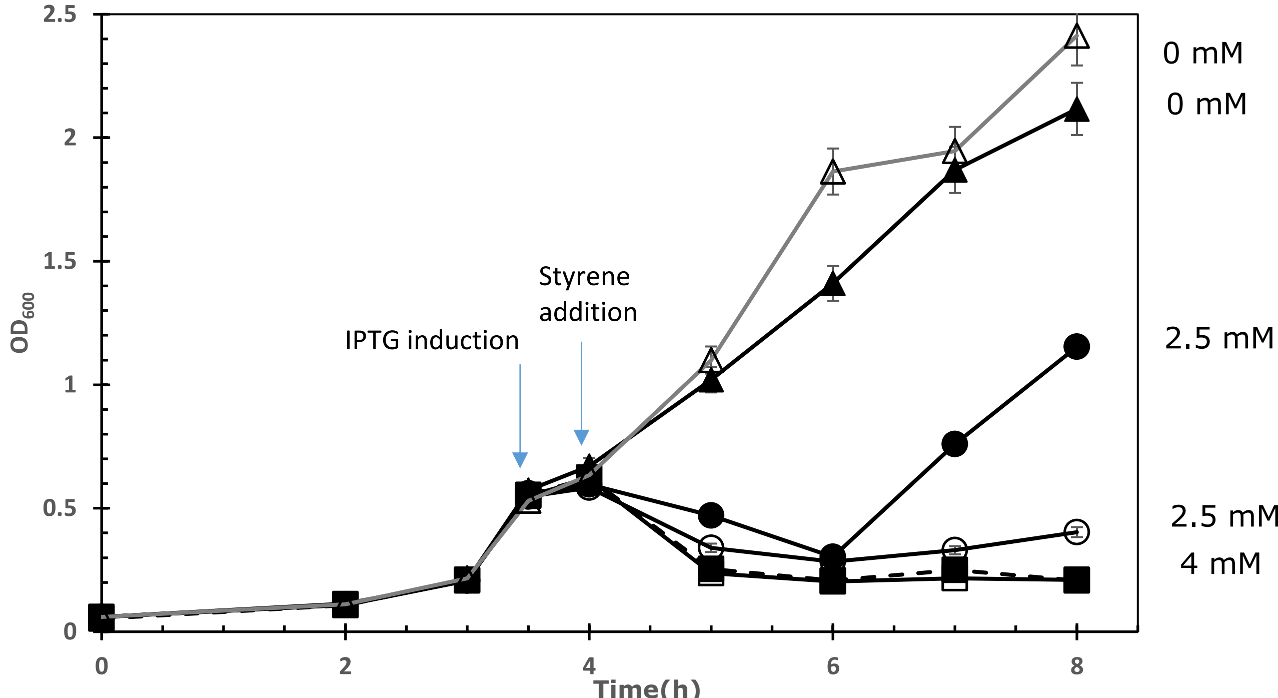


Figure 6.9 Toxicity tolerance of *E. coli* C43 (DE3) Δ*acr*AB_pETDuet_AcrAB cells to styrene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} reached 0.5, then induced by 0.4 mM IPTG. Afterwards, styrene (0 (Triangles), 2.5 (Circles) and 4 mM (Squares)) was added 30 min after IPTG induction. The open shapes represent the negative control (*E. coli* C43 (DE3) *Δacr*AB-ΔpETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after styrene addition. Error bars represent one standard deviation from triplicate experiments.

The strain bearing *acr*AB was more tolerant to styrene at a concentration 2.5 mM than the control strain whereas when using 4 mM styrene, the growth of both strains was inhibited. Therefore, 4 mM styrene is toxic to the cells and the pump was unable to prevent toxicity of styrene above 2.5 mM.

<u>Conclusion</u>

The strain bearing the AcrAB pump was tolerant to 2.5 mM over the control. Styrene at 4 mM concentration is toxic to both the control and strain bearing the AcrAB pump (*E. coli* C43 (DE3) Δ*acr*AB_pETDuet_AcrAB).

6.5 Cyclohexane toxicity tolerance

The same experiment was also conducted in *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB to determine the toxicity tolerance of cyclohexane with the exclusion of 100 mM concentration of cyclohexane due to this showing high toxicity in the previous experiment with the SrpABC pump (Fig 6.10).

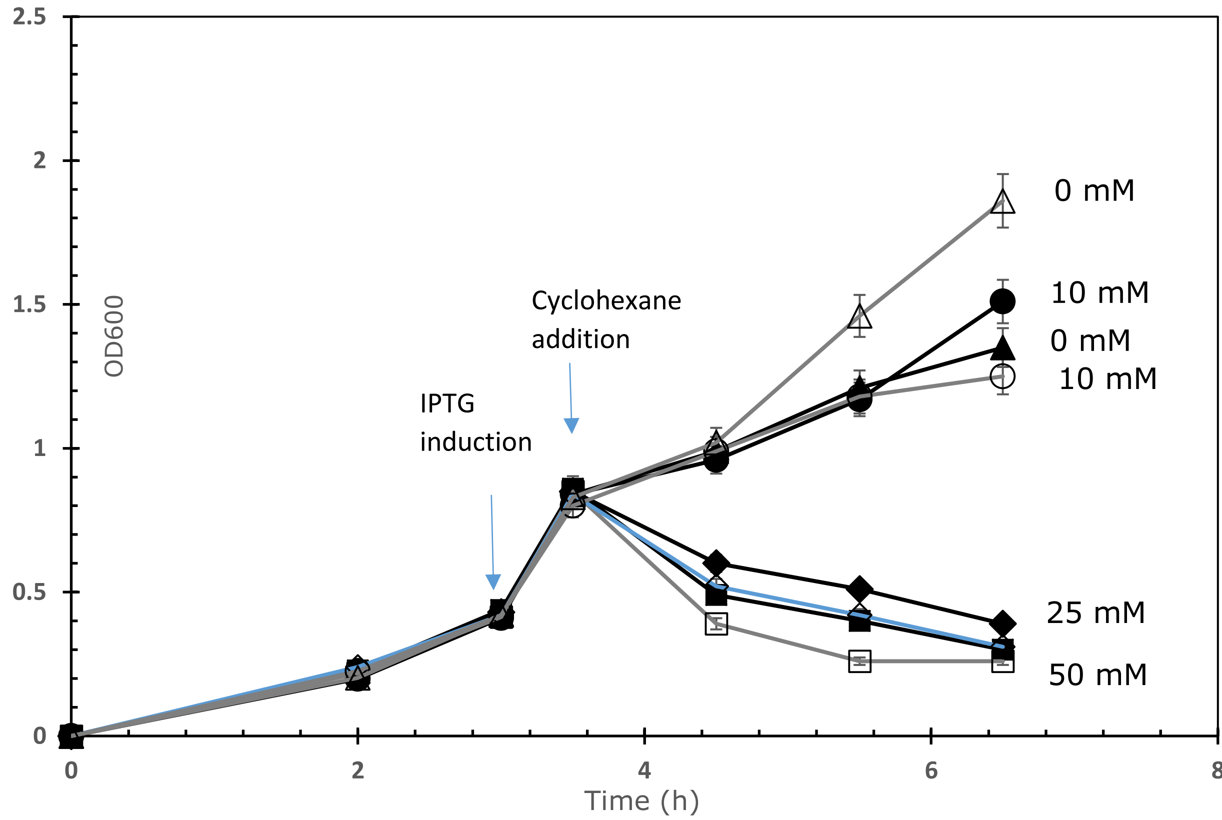


Figure 6.10 The growth curves of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB toward different concentrations of cyclohexane. After growing the cultures in M9 media at 37 °C to OD_{600} 0.5, IPTG (0.4 mM) was added. After half an hour, different concentrations of cyclohexane (0 (Triangles), 10 (Circles), 25 (Diamonds) and 50 mM (Squares)) were added and the ODs were monitored for 4 hours later. Open shapes represent the negative control (Strains with an empty plasmid). 'wo' implies without the chemical. Error bars represent one standard deviation from triplicate experiments.

As indicated in Figure (6.10), all the strains were able to grow up to 10 mM cyclohexane, but higher concentrations of cyclohexane (e.g. 25 mM) resulted in bacterial cytotoxicity.

Conclusion

AcrAB-bearing strain, *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB, could tolerate up to 10 mM cyclohexane over the control strain.

6.6 Cyclohexene toxicity tolerance

Cyclohexene toxicity tolerance was also investigated in *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB. The cultures were grown in identical experiments as previous. Cyclohexene was added after 30 min from IPTG induction. In this experiment, cyclohexene at 100 mM concentration was excluded because it has shown high toxicity to the cells in the previous experiment. Negative control (empty pETDuet-1 plasmid) was also grown in the same condition (Fig 6.11).

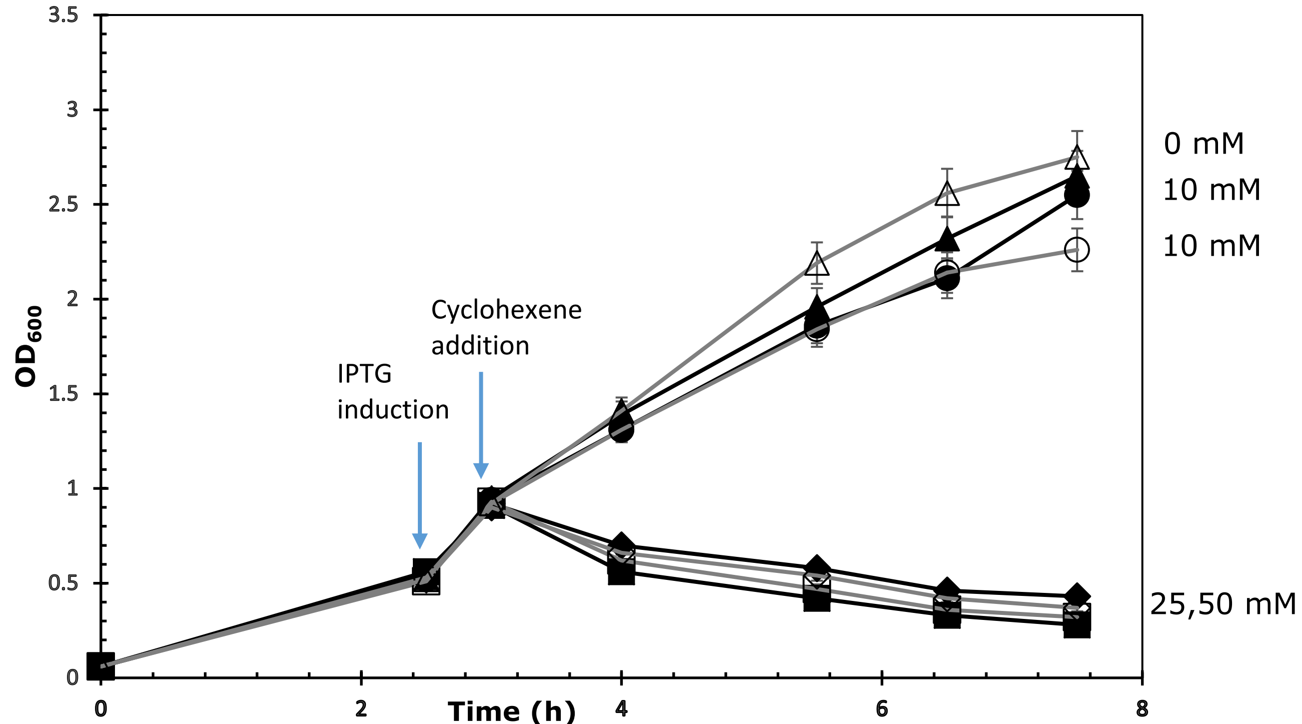


Figure 6.11 Toxicity tolerance of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB cells to cyclohexene. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, cyclohexene (50 (Squares), 25 (Diamonds) and 10 mM (Circles) was added 30 min after IPTG induction. The triangles represent no added cyclohexene. The open shapes represent the negative control (*E. coli* C43 (DE3) *ΔacrAB*-ΔpETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

As shown from the growth graph, the strain harbouring AcrAB pump was more tolerant to cyclohexene at 10 mM than the control strain (with the empty plasmid). A 25, 50 mM cyclohexene the growth of both strains was inhibited, therefore, all these concentrations are no tolerable and killing the bacteria.

<u>Conclusion</u>

AcrAB-bearing strain, *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB, could tolerate up to 10 mM cyclohexene over the control strain.

6.7 Ethylcyclohexane toxicity tolerance

The same experiment was also conducted in *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB to determine the toxicity tolerance of ethylcyclohexane (Fig 6.12). The results have shown that ethylcyclohexane at all concentrations (100, 50 and 25 mM) is toxic to both strains.

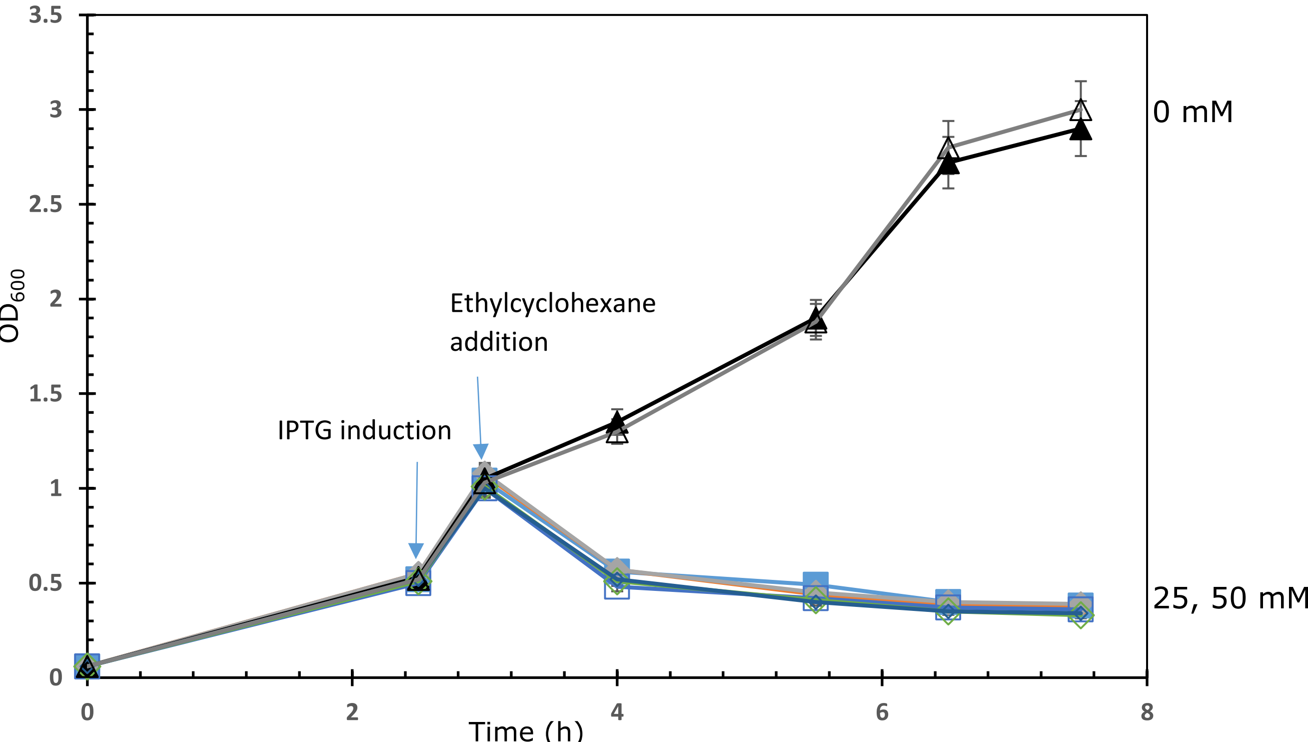


Figure 6.12 Toxicity tolerance of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB cells to ethylcyclohexane. Cultures of the cells were grown in M9 minimal media at 37°C until OD_{600} becomes 0.5, then induced by 0.4 mM IPTG. Afterwards, ethylcyclohexane (100 (Squares), 50 (Circles) and 25 mM (Diamonds)) was added 30 min after IPTG induction. Negative control (Straines with empty pETDuet-1 plasmid) was also grown at the same conditions. ODs were monitored 4 hours after the addition. Error bars represent one standard deviation from triplicate experiments.

6.8 Ciprofloxacin tolerance

AcrABTolC confers resistance to ciprofloxacin in clinical isolates of bacteria (Goswami *et al*., 2016) and thus it was expected that resistance to ciprofloxacin would be observed in *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB.

Antibiotic agar plates were prepared with concentrations 0 to 100 ng /mL of freshly made antibiotic stocks (Table 4.2 (methods section)). M9 minimal broth (5 mL) was inoculated with a colony (C43 (DE3) Δ*acr*AB-pETDuet-AcrAB and C43 (DE3) Δ*acr*AB-empty pETDuet-1) from a freshly inoculated plate (< 1 week) and incubated at 37 °C.

This culture was diluted (1:100) next day in 5 mL M9 minimal media supplemented with the appropriate antibiotics and incubated until OD_{600} reached 0.5 then induced by IPTG (0.5 mM) for 30 min. Culture volume (1 μL) of different diluted bacterial cultures (at OD_{600} equals 1, 0.1 and 0.001) was pipetted onto each antibiotic plate including the antibiotic-free control (Figure 6.13 A and C) and this was incubated at 37°C for 16 hours. The plates were inspected for growth and the MIC was the antibiotic concentration at which there is zero growth on the plate.

The MIC for all strains was 20 ng/mL; there was no difference between the plate count of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB and the control in all CIP concentrations except at 15 ng/mL concentration (Figure 6.13 C and D).

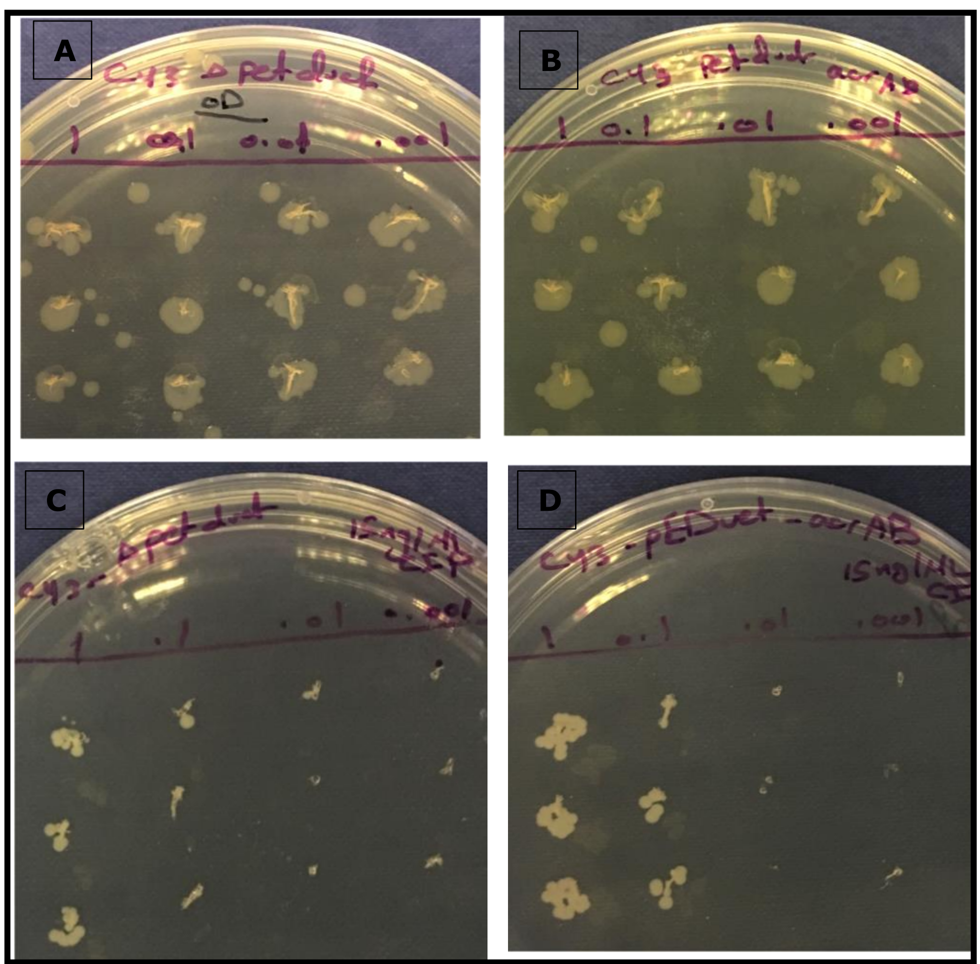


Figure 6.13 Plate colony count of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB and the controls with 15 ng/mL ciprofloxacin. The cultures of *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB and the control (with empty plasmid), in 5 mL M9 minimal media with the addition of IPTG (0.5 mM) at 37 °C and incubated until OD_{600} reached 0.5. Culture volume (1 µL) of different dilutions was pipetted onto each antibiotic plate including the antibiotic-free control and this was incubated at 37°C for 16 hours. The plates were inspected for growth and the MIC was the antibiotic concentration at which there is zero growth on the plate. A, C43 (DE3) Δ*acr*AB-empty pETDuet-1 cells grown on antibiotic-free agar. B, C43 (DE3) Δ*acr*AB-pETDuet_AcrAB cells grown on antibiotic-free agar. C, C43 (DE3) Δ*acr*AB-empty pETDuet-1 cells grown onto 15 ng/mL CIP plate agar. D, C43 (DE3) Δ*acr*AB-pETDuet_AcrAB cells grown onto 15 ng/mL CIP plate agar.

This result could not confirm that the AcrAB efflux pump confers resistance towards CIP in *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB. Thus, the absence of

resistance to this antibiotic indicates that the expression system used was not optimal for examining AcrAB function.

General conclusion

Overproduction of the AcrABTolC was investigated in *E. coli.* AcrABTolC overproduction was confirmed in *E. coli* BW25113 by SDS PAGE and Western blotting of anti-Strep antibody of the Strep-tagged TolC.

During the toxicity tolerance experiments, growth rate of the strain bearing the pump (AcrABTolC) was slower than the control possibly due to the overexpression of TolC. Therefore, the overexpression of only AcrAB was proceeded. Overexpression of the AcrAB pump was confirmed by SDS PAGE. Styrene toxicity was investigated in *E. coli* C43 (DE3) *Δacr*AB-pETDuet_AcrAB, the strain bearing the pump was tolerant to about 2.5 mM of exogenously added styrene, and the tolerance was more the control strain. AcrAB expression seems to provide some resistance to styrene but not to the other organic solvents examined, and the absence of resistance to CIP indicates that the expression system used was not optimal for examining AcrAB function.

Because of the functionality of SrpABC for styrene resistance, styrene production was investigated in SrpABC expressing strains in the following section.

Results

7 Styrene bio-production

As mentioned in the literature section, the main limitation of bio-production of styrene in *E. coli* is its toxicity (McKenna and Nielson 2011). The aim of this chapter was to try to solve the previous problems encountered during styrene bio-production by the co-expression of styrene producing proteins, Pal and Fdc with the efflux pump system (SrpABC). Therefore, this is the first study to use the SrpABC efflux pump in the toxicity tolerance of styrene and its production in *E. coli*.

For styrene bio-production in *E. coli*, McKenna and Nielson (2011) used *E. coli* NST74 with the genotype *aroF aroG tyrR pheA pheAop* (same enzymes as in the shikimate pathway)*,* which can produce L-phenylalanine naturally, the substrate needed for styrene production. The L-phenylalanine over-producing strain, *E. coli* NST74, was co-transformed with pSpal2At and pTfdc1Sc plasmids, expressing PAL and FDC proteins. The plasmids are under *lac* and *trc* promoters, respectively. The recombinant *E. coli* NST74 was able to produce about 260 mg/mL styrene from 1 g/L L-phenylalanine.

In this project, styrene bio-production was conducted using *E. coli* MG1655 (DE3) because it was the strain that confirmed SrpABC pump protein production. In addition, none of the plasmids used previously in styrene-production were compatible to express in *E. coli* MG1655 (DE3). Therefore, L-phenylalanine was added exogenously to the culture medium, because of not using *E. coli* NST74. Moreover, PAL and FDC proteins were expressed in the same plasmid (pETDuet-1 /pACYCDuet1 plasmids), under the control of T7 *lac* promoter.

7.1 Protein expression of Pal and Fdc

As mentioned previously, Pal and Fdc enzymes are required for styrene bio-production.

Scheme 1

L-phenylalanine —PAL→ *t*CA —FDC→ Styrene

McKenna and Nielson (2013) have also cloned PT pal fdc plasmid (Appendix pg 127), which expresses PAL and FDC proteins for the production of (S)-styrene oxide (McKenna *et al.*, 2013), this plasmid is incompatible to express in *E. coli* MG1655 (DE3). Therefore, the plasmid, PT pal fdc was used to clone Pal and Fdc encoding genes into pETDuet-1 and pACYCDuet-1 plasmids by Gibson's assembly protocol (section 4.10.5).

Table 7.1 Gibson's assembly ligation reaction volumes

	Recommended amount of fragments used for assembly	
	2-3 Fragment Assembly	Positive Control
Recommended DNA Ratio	Vector: insert = 1:2	
Total Amount of Fragments	0.03–0.2 pmols X µL	10 µL
Assembly Master Mix (2X)	10 µL	10 µL
Deionized H_2O	10-X µL	0
Total Volume	20 µL	20 µL

The plasmids offer compatibility with our efflux pump systems, and also are compatible to be expressed in *E. coli* MG1655 (DE3) because both plasmids are under the control of T7 *lac* promoter (Fig 7.1).

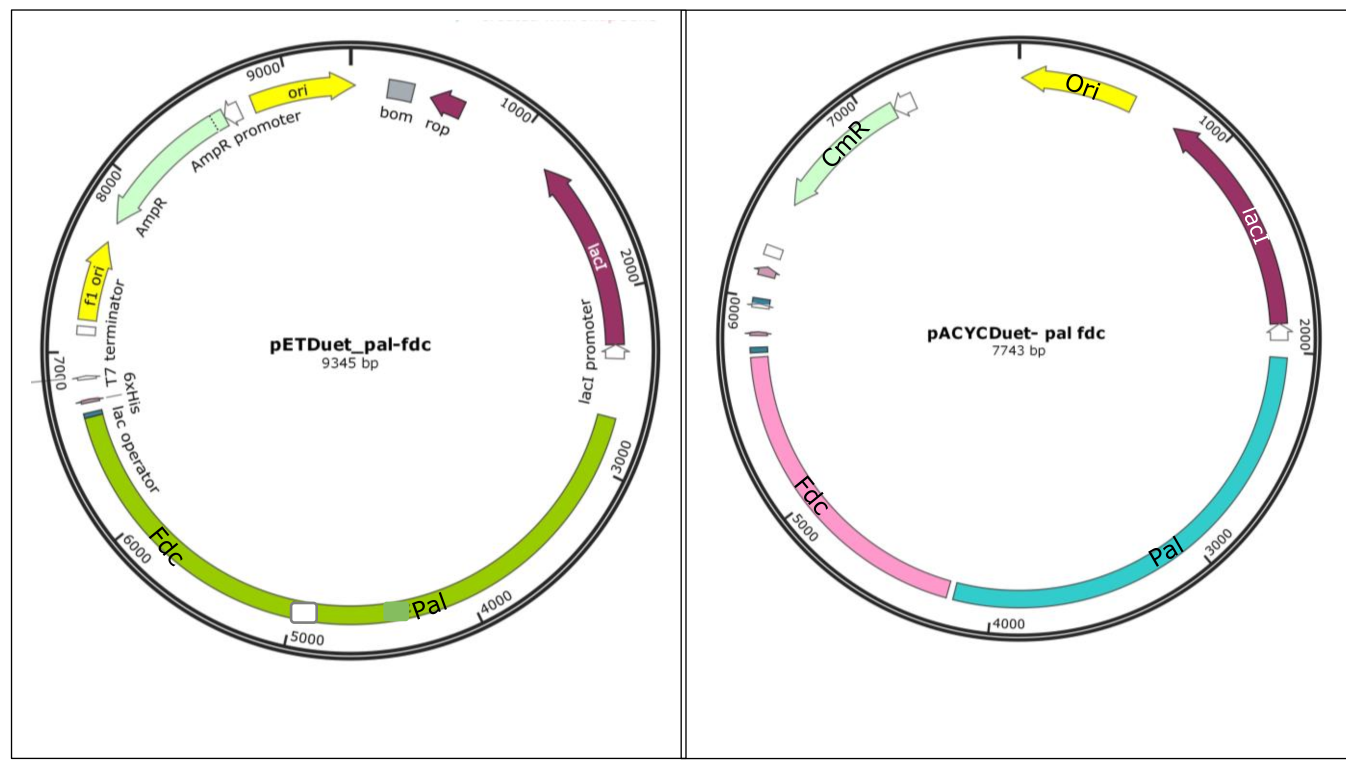


Figure 7.1 Plasmid maps of pETDuet_pal-fdc and pACYCDuet_pal-fdc drawn by Snapgene

Afterwards, the cloning and the verification of the plasmids containing pal and fdc genes (pETDuet_pal-fdc and pACYCDuet_pal-fdc) was confirmed by gene sequencing (Section 4.10.5, pg 42). *E. coli* MG1655 (DE3) transformation and protein production of styrene-producing plasmid, enabling the co-production with the SrpABC efflux pump was confirmed in *E. coli* MG1655 (DE3). *E. coli* MG1655 (DE3) was transformed separately with pETDuet_pal-fdc and pACYCDuet_pal-fdc plasmids. Subsequently, the protein expression was analysed using SDS PAGE (Fig 7.2) and (Fig 7.3), respectively.

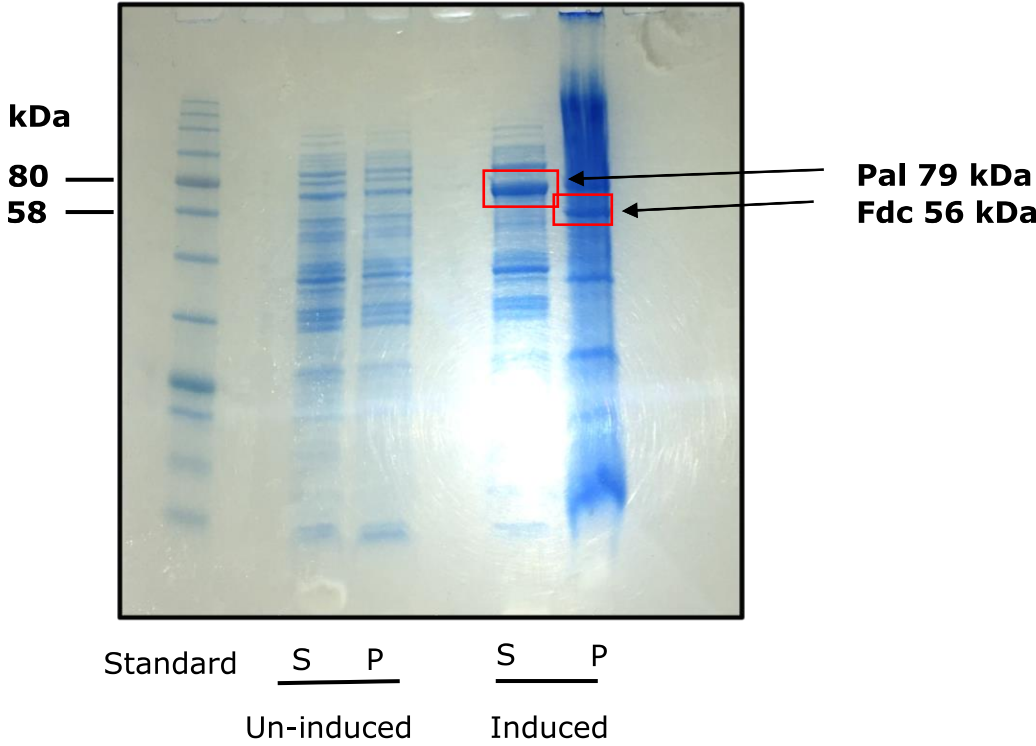


Figure 7.2 Protein production of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc by SDS PAGE. The cultures were grown in M9 minimal media at 37 °C induced by 0.5 mM IPTG for 3 hours, the samples were analysed by SDS PAGE. 'P' represents pellet, and 'S' represents supernatant.

As indicated from the SDS PAGE gel of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc, Pal (79 kDa) and Fdc (56 kDa) proteins were produced in the IPTG-induced cell supernatant and pellet suspensions respectively.

In the case of *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc, both Pal (79 kDa) and Fdc (56 kDa) proteins were produced in the IPTG-induced cell supernatant and pellets suspensions respectively. Pal protein quantity was less than that in *E. coli* MG1655 (DE3)-pETDuet_pal-fdc gel. This may result from using the low copy number pACYCDuet-1 expression vector (Fig 7.3).

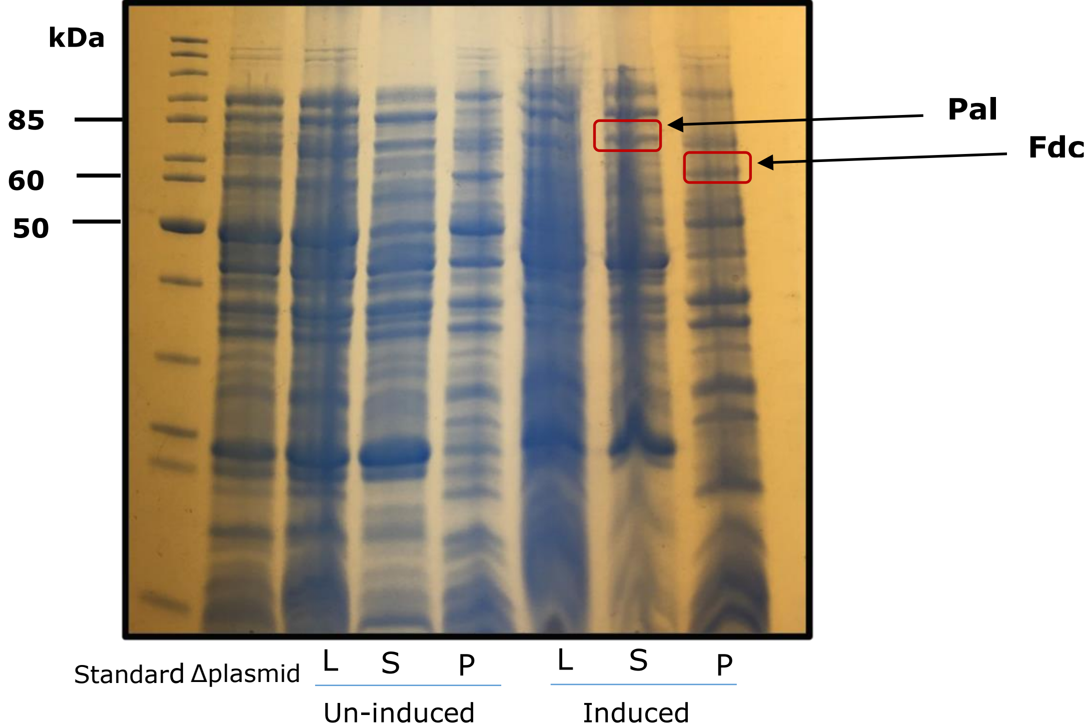


Figure 7.3 Protein production of *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc by SDS PAGE. The cultures were grown in M9 minimal media at 37 °C induced by 0.5 mM IPTG. 'L' represents lysate, 'P' represents pellet, and 'S' represents supernatant.

Protein production of Pal and Fdc proteins was also investigated in *E. coli* C43 (DE3) *ΔacrAB*, to enable the co-production with the AcrAB system, and to understand its effect on styrene production compared to the SrpABC system. Subsequently, *E. coli* C43Δ*acr*AB (DE3) was transformed with pETDuet_pal-fdc plasmid and protein production was examined by SDS PAGE (Fig 7.4).

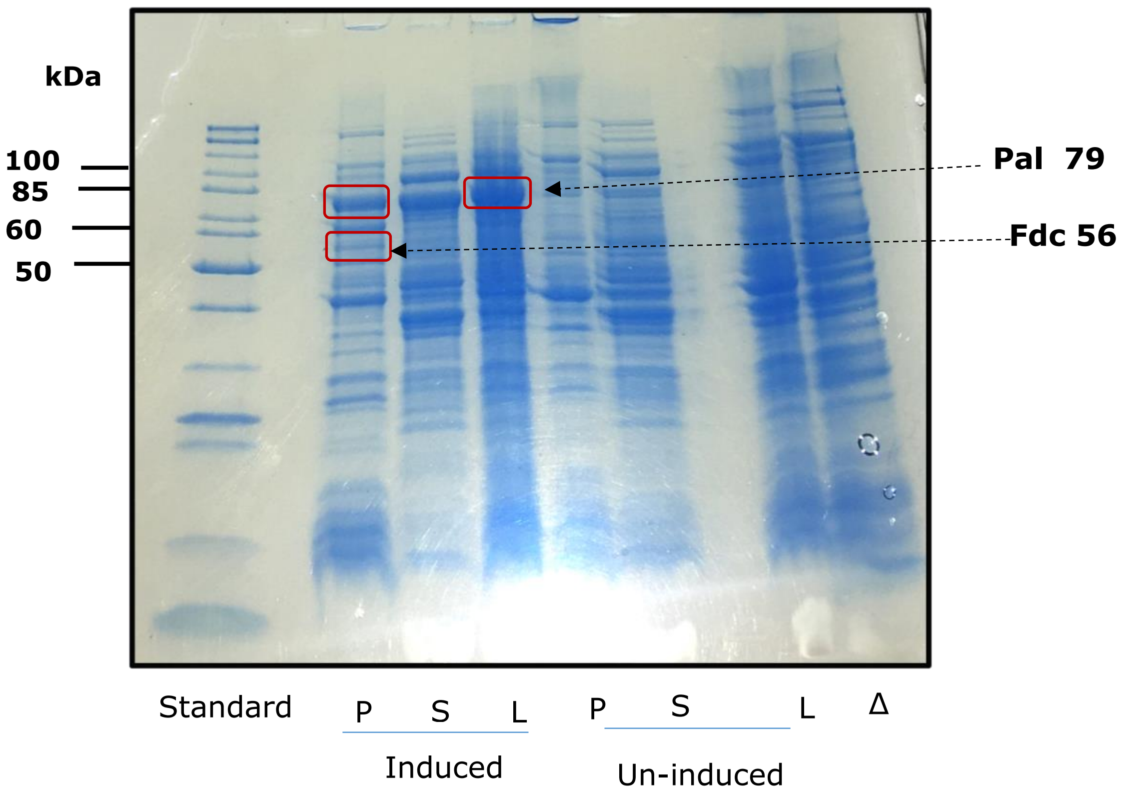


Figure 7.4 Protein production of *E. coli* C43Δ*acr*AB_pETDuet_pal-fdc. The cultures were grown in M9 minimal media induced by 0.4 mM IPTG for 3 hours at 37 °C, samples were analysed by SDS PAGE. 'L' represents cell lysate, 'P' represents pellet, and 'S' represents supernatant.

As revealed from the SDS PAGE gel, Pal protein production was confirmed in all the induced cell fractions, On the other hand, Fdc production was shown in the induced and the non-induced fractions because of the leakiness of the T7 promoter (Mertens *et al*., 1995); (Baneyx, 1999) of the expressing vector (pETDuet-1 plasmid).

Conclusion

The genes encoding styrene-producing proteins, *pal* and *fdc* were cloned into pETDuet-1 and pACYCDuet-1 plasmids to be able to express styrene-producing protein in the same strains overproducing the pumps. The production of styrene-producing proteins, Pal and Fdc, was investigated in *E. coli* MG1655 (DE3) and *E. coli* C43 (DE3) *Δacr*AB. Overexpression of the SrpABC and AcrAB pumps, in *E. coli* MG1655 (DE3)-pETDuet_pal-fdc and *E. coli* C43 (DE3) *Δacr*AB-pETDuet_pal-fdc, respectively was confirmed by SDS PAGE.

7.2 Bio-production of styrene in the presence of expressed efflux systems

As mentioned in the literature section, the main limitation of bio-production of styrene in *E. coli* is its toxicity (McKenna and Nielson 2011). The aim of this experiment is to investigate the bio-production of styrene endogenously in *E. coli* in the presence of overexpressed efflux systems (AcrABTolC and SrpABC).

To improve the rate of the bio-produced styrene, the efflux pump system was co-produced with the styrene-producing system in *E. coli* MG1655 (DE3).

7.2.1 Co-production of SrpABC efflux system with the styrene-producing system

After the evaluation of the bio-produced styrene from *E. coli* MG1655 (DE3)-pETDuet_pal-fdc, the system was co-produced with the SrpABC efflux system (pACYCDuet_MCS2_SrpABC) in *E. coli* MG1655 (DE3).

Protein production of Pal and FDC proteins was confirmed by SDS PAGE (Fig 7.5 (A)), whereas SrpA protein was confirmed by Western blotting of the anti-His antibody of the His-tagged SrpA protein (Fig 7.5 (C)) and SrpB and SrpC proteins were confirmed by RT-PCR (Methods section) (Fig 7.5 (B)).

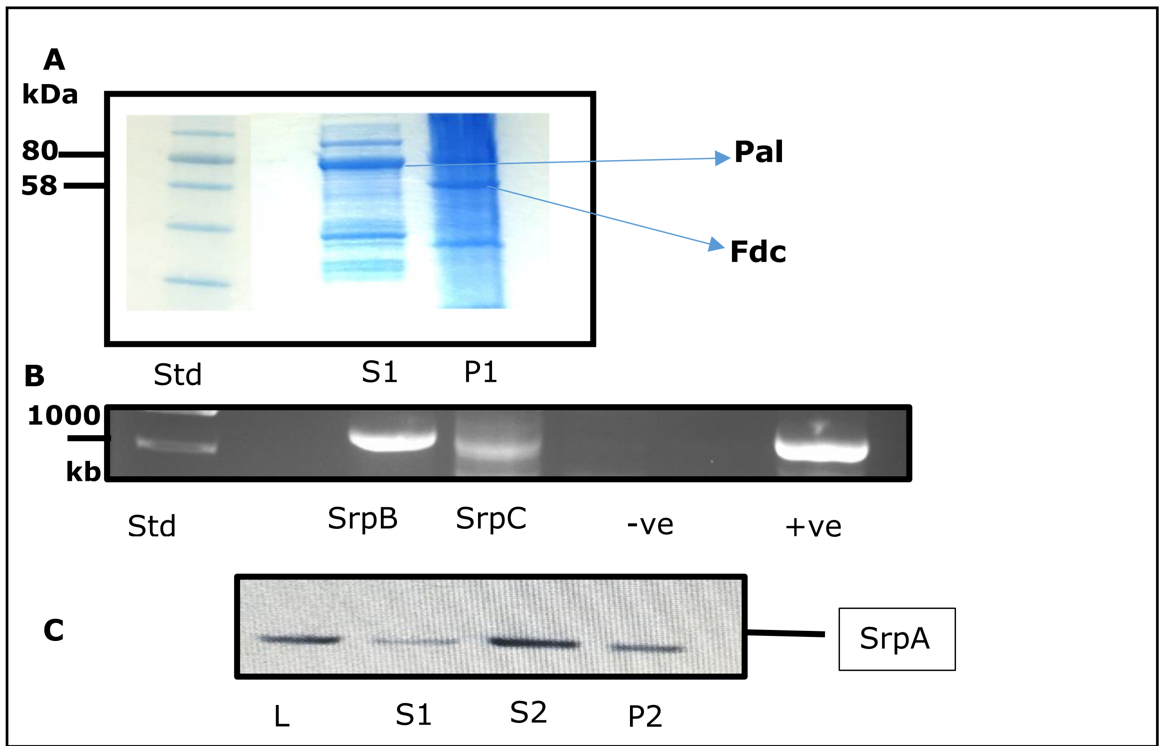


Figure 7.5 Summary of protein production of *E. coli* MG1655 (DE3)-pETDuet_pal fdc_pACYCDuet-SrpABC. Protein expression of Pal and FDC proteins was confirmed by SDS PAGE (Fig 7.4 (A)), whereas SrpA protein confirmed by western blotting using anti-His_6 tag antibody (Fig 7.4 (C)) and SrpB and SrpC proteins were confirmed by RT-PCR (Fig 7.4 (B)). Positive control: psk (+)-SrpABC plasmid. Std: Standard.

Afterwards, the analysis of the bio-produced styrene and its metabolites was proceeded and the results were compared with and without the pump system.

7.3 Bio-production of styrene in shake flasks

Bio-production of styrene in the cells bearing efflux pump systems was performed firstly in shake flasks (250 mL) covered by Suba-seals immediately after IPTG induction (0.5 mM) to prevent the leakage of bio-produced styrene. Samples for the detection of produced styrene and metabolites were collected after IPTG induction and analysed by GCMS and HPLC, respectively.

E. coli MG1655 *(*DE3) was co-transformed with the styrene production plasmids (pETDuet_pal-fdc or pACYCDuet_pal-fdc) and the efflux pump system

(pETDuet_SrpABC or pACYCDuet_SrpABC) producing two combinations, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC. The control strains, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc, were included to compare styrene production with- and without the pump. Cultures of the previous strains with the appropriate antibiotic concentrations were grown as previously (Section 4.8.1, pg 35) in M9 medium with the addition of L-phenylalanine (1 g / L) (because of not using *E. coli* NST74) in 250 mL flasks (Quickfit®) and incubated at 37 °C shaker-incubator until reached OD_{600} 0.5 then induced by IPTG (0.5 mM). The flasks were closed by Suba-Seal® after induction to prevent the volatilisation of bio-produced styrene. Styrene production was analysed by GCMS (Fig 7.6). Other metabolites such as L-phenylalanine and *trans*-cinnamic Acid (*t*CA) were analysed by HPLC.

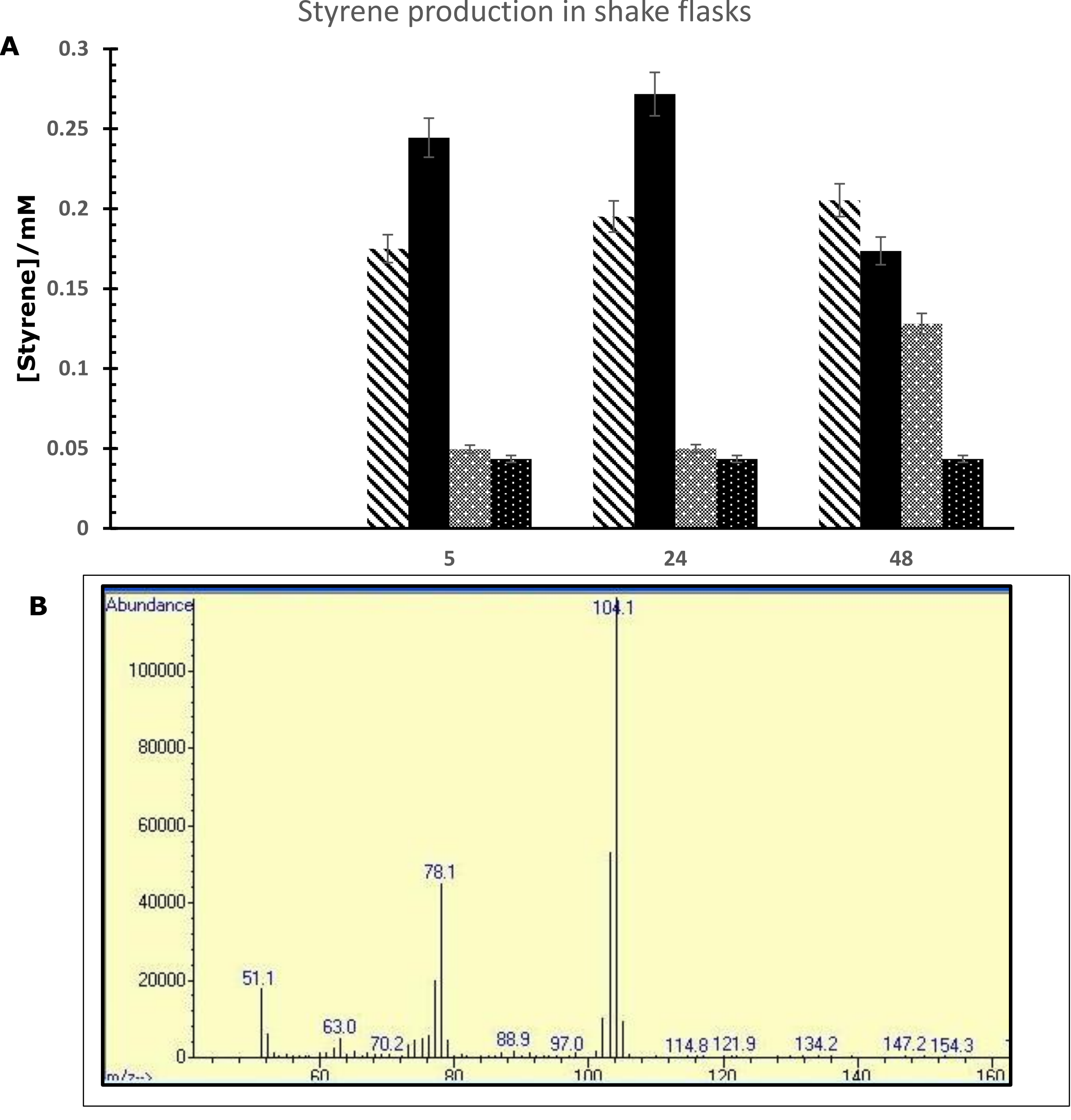


Figure 7.6 Styrene production in shake flasks. A. Overnight cultures of different strains were diluted (1:100) and grown in M9 media with glucose (1.5 % v/v) and L-phenylalanine (1 g/1 L), until OD_{600} reached 0.5 then induced by IPTG (0.5 mM) and closed by Suba seals. Styrene production was analysed by GCMS. Error bars represent one standard deviation from triplicate experiments. ▧ represents *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc, ■ represents *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC, ▒ represents *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc and ▦ represents *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC B. Styrene mass spectrum by GCMS.

Samples for the detection of styrene were taken after induction. As shown by Figure (7.6 A), the highest styrene concentration was produced from *E. coli*

MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC strain, about 0.27 mM styrene 24 h after induction. This concentration was higher than styrene produced from the control strain (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc, without the pump), 0.20 mM. The result could indicate that the SrpABC pump could improve styrene production endogenously in *E. coli*. Styrene production was decreased after 48 h from induction in the strain harbouring the pump, this may be because most styrene production was after 24 h from induction which indicates that most L-phenylalanine was consumed after 48 h. In the other two combinations, *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc (control) and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC, there was no improvement of styrene production in the strain with the pump except that styrene produced from the strain without the pump was higher at 48 h from induction this is likely because low copy plasmid pACYCDuet-1 which was producing styrene production proteins, slowing the production of styrene. In conclusion, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC strain was able to produce more styrene in shake flasks. However, all concentrations of bio-produced styrene were below the tolerable threshold of exogenously added styrene, 2.5 mM which was determined in the toxicity tolerance experiments.

The growth of styrene-producing strains was also monitored and the OD_{600} values were plotted Fig (7.7).

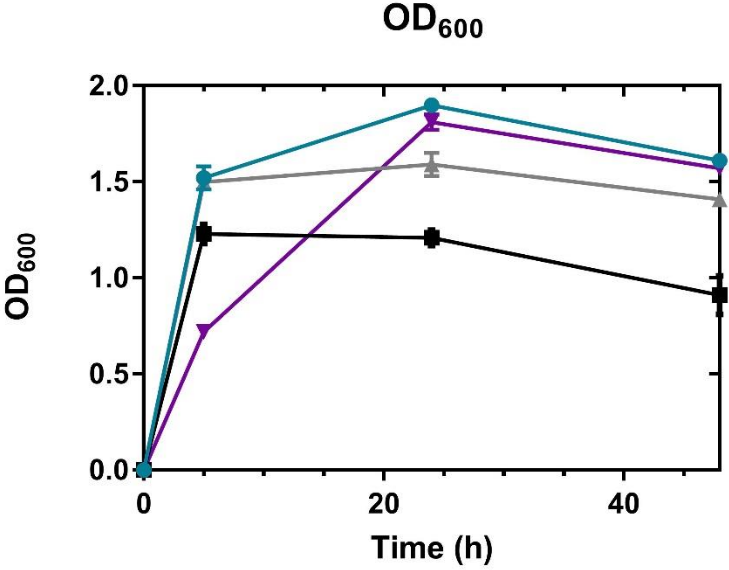


Figure 7.7 Growth of different styrene-producing strains. Cultures of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc (Blue), *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC (Black), *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc (Gray) and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC (Purple) were grown in M9 until OD_{600} reached 0.5 then induced by 0.5 mM IPTG. The $OD_{600(s)}$ were measured after 5, 24 and 48 hours after IPTG induction. Error bars represent one standard deviation from triplicate experiments.

As indicated in Figure (7.7), the growth of the strains harbouring the pump (*E. coli* MG1655 (DE3)_pACYCDuet_pal-fdc-pETDuet-SrpABC and *E. coli* MG1655 (DE3)_pETDuet_pal-fdc_pACYCDuet-SrpABC) was lower than the corresponding strains without the pump; *E. coli* MG1655 (DE3)_pACYCDuet-pal-fdc and (*E. coli* MG1655 (DE3)_pETDuet-pal fdc, respectively. This behaviour could be explained by the growth of the strains harbouring two plasmids generally would be slower than the same strain harbouring only one plasmid. In addition, the growth of most strains decreased 24 h after induction, the explanation behind that the bacterial cells reached the stationary phase at that time.

As previously mentioned, the pathway converts phenylalanine to styrene *via trans*-cinnamic acid intermediate, using PAL2 and FDC1. L-phenylalanine and *t*CA

metabolites of the previous experiment were also measured and analysed using HPLC (Fig 7.8).

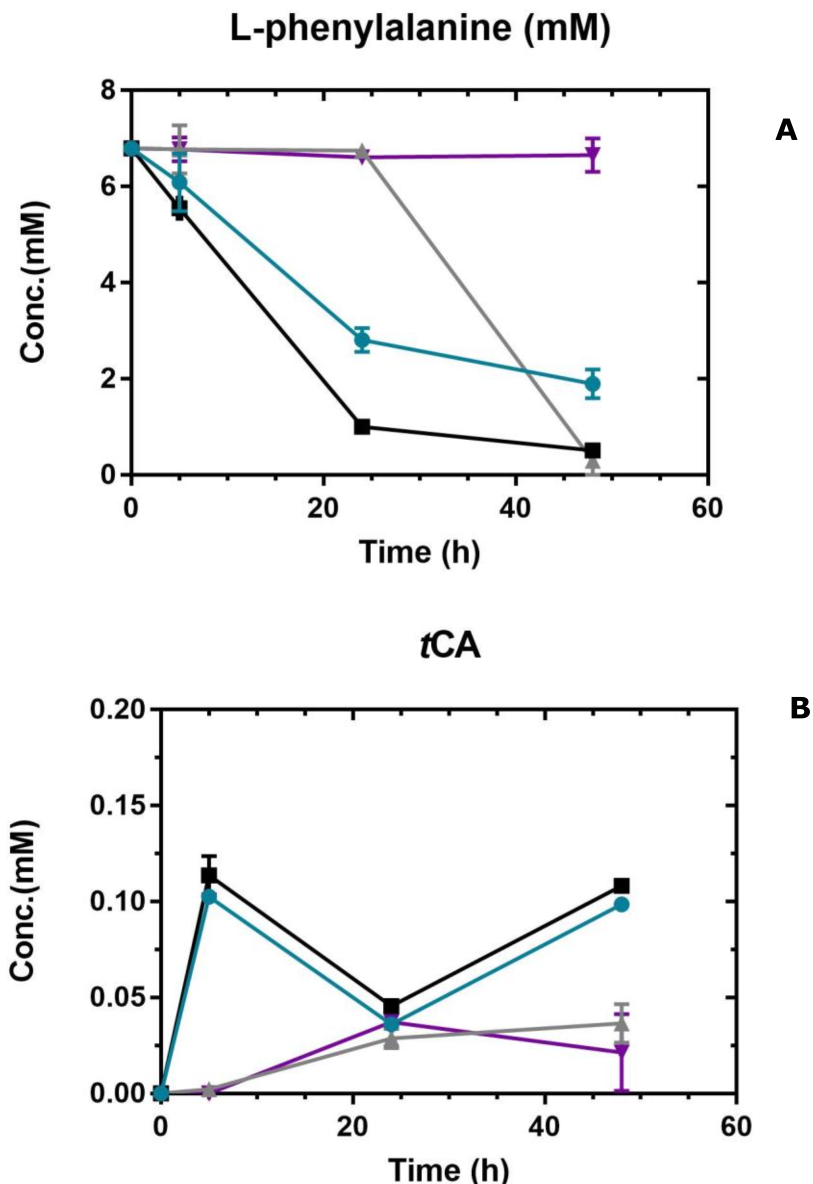


Figure 7.8 L-phenylalanine and *t*CA from different styrene producing strains. L-phenylalanine and *t*CA of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc (Blue), *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC (Black), *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc (Gray) and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC (Purple). Error bars represent one standard deviation from triplicate experiments.

L-Phenylalanine was consumed after IPTG induction (Fig 7.8) to produce *t*CA and styrene and it was inversely proportional to the produced *t*CA and styrene which is logical regarding their reaction equation (Fig 2.7, pg.11). L-Phenylalanine was consumed by *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC approximately at the same rate by the control (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc). Whereas L-phenylalanine was least consumed in the case of *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC, this was because the low copy number, pACYCDuet-1 plasmid, expressing styrene-producing proteins. *t*CA was more produced in *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC *E. coli* MG1655 (DE3)-pETDuet_pal-fdc than other strains, and its value jumped 2h after IPTG induction. *t*CA was optimal 5 hrs after induction and started to decrease after indicating the production of styrene. This explains why styrene production was increasing after induction and reached its maximum value in the same strain 24 h after induction.

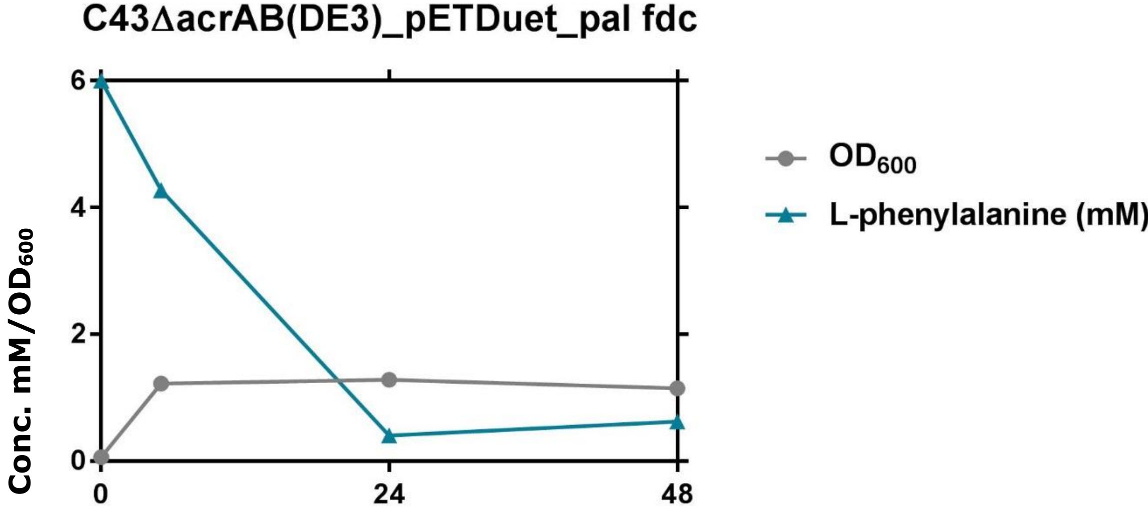
7.3.2 Styrene production in *E. coli* C43 (DE3) Δ*acr*AB

Similar experiments for styrene production were conducted in shake flasks. Styrene production was evaluated from *E. coli* C43 (DE3) Δ*acr*AB_pETDuet_pal-fdc, although the produced styrene was more from this strain ~0.33 mM (Fig 7.9) in comparison to styrene produced from *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC (0.27 mM) and other strains (Table 7.2).

Table 7.2 Maximum produced styrene by different recombinant *E. coli* strains

Recombinant Strain	Maximum produced Styrene (mM)
E. coli C43 (DE3) Δ*acr*AB_pETDuet_pal-fdc	0.33
E. coli MG1655 (DE3)-pETDuet_pal-fdc	0.2
E.coli MG1655(DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC	0.27
E.coli MG1655(DE3)-pACYCDuet_pal-fdc	0.12
E.coli MG1655(DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC	0.04

OD samples were measured, L-phenylalanine and *t*CA results were analysed by HPLC (Fig 7.9)



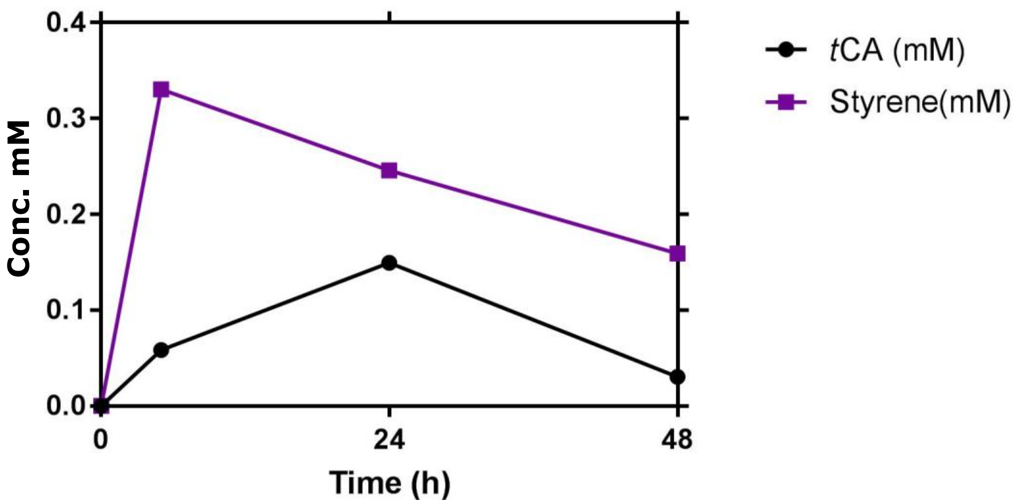


Figure 7.9 Styrene production in *E. coli* C43 (DE3) Δ*acr*AB_pETDuet_pal-fdc and other metabolites.

L-Phenylalanine was consumed after IPTG induction (Fig 7.9) to produce *t*CA and styrene and it was inversely proportional to the produced *t*CA and styrene.

Unfortunately, the effect of the AcrAB pump on styrene production in this strain was not further studied due to a problem encountered several times when co-expression of both the pump and styrene-producing proteins (Pal and Fdc). This was because *E. coli* C43 (DE3) Δ*acr*AB was unable to be transformed with both plasmids at the same time although they are compatible. This could be explained that the strain could lose the plasmids in one or more steps in the double transformation procedure because it is stressful for its growth.

Conclusion

The co-expression of styrene-producing proteins, Pal and Fdc, and the efflux pumps, SrpABC and AcrAB, were investigated in *E. coli* MG1655 (DE3) and *E. coli* C43 (DE3) *Δacr*AB, respectively. Only *E. coli* MG1655 (DE3) was able to co-express both systems (Pal Fdc with the SrpABC).

Subsequently, styrene bio-production in *E. coli* MG1655 (DE3) was investigated in shake flasks. Four different recombinant *E. coli* MG1655 (DE3) strains were used, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC, *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc and *E. coli* MG1655 (DE3)-pACYCDuet_pal-fdc-pETDuet_SrpABC, all the strains were able to produce styrene. The strains holding the pump were able to produce more styrene than strains without the pump. The highest styrene production was from *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC (0.27 mM) from 1 g/ L of L-phenylalanine. However, all concentrations of bio-produced styrene were below the tolerable threshold of styrene, 2.5 mM that determined in the toxicity tolerance experiments.

7.4 Bio-production of styrene in bioreactors

To gain more insights and see the big image of styrene bio-production in our strain, bioreactors were used for the fermentation of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC. Cultures of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc-pACYCDuet_SrpABC were grown overnight at 37 °C shaker incubator. Overnight cultures from the previous strains were diluted to OD_{600} 0.01 (final concentration of the starting batch media in the reactor) with glucose (12 g /L) and L-phenylalanine (2 g / L) in a 3 L-bioreactor (New Brunswick BioFlo®/CelliGen®115). The culture medium was maintained at 37 °C by the chiller and the heater systems in the bioreactor. In addition, the pH was maintained at 7 *via* the automated addition of ammonium hydroxide (30%) and sulphuric acid (2 M) solutions, as appropriate. The oxygen enrichment was also maintained by a continuous regulated inflow of air and agitation. The OD_{600} was monitored until it reached 30 to 40 then induced by adding IPTG (1.5 mM).

Dodecane trap was fixed at the outlet gas flow of the fermenter chamber in order to strip styrene vapour in the solvent and to reduce the toxicity of styrene in the fermenter medium. *n*-dodecane was previously used to extract styrene from the culture medium and was an effective solution for styrene extraction (McKenna *et al.*, 2015). In this study, *n*-dodecane was used as a styrene stripper which was different from previous studies. This method was developed and used by my research group colleague, Patricio Zapata, who was able to strip most the volatile styrene from his bioreactor, therefore he confirmed the effectiveness of this method. The trap was filled with *n*-dodecane (250 mL). Using *n*-dodecane with a different and modified method accompanied by our biological solution (SrpABC efflux pump), to reduce the toxicity and to bio-produce more styrene than previously carried studies. Styrene production inside the fermenter media and in the dodecane trap was analysed by GCMS and the other metabolites such as L-phenylalanine, *t*CA and glucose were analysed by HPLC.

7.4.1 Styrene production without the pump

First, the styrene production was conducted without the pump using *E. coli* MG1655 (DE3)-pETDuet_pal-fdc. Overnight culture of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc was diluted to OD_{600} equals 0.01 (final concentration of the starting batch media in the reactor) with glucose (12 g /L) and L-phenylalanine (2 g / L) in the bioreactor. The OD_{600} of the samples was monitored every two hours (Fig 7.10). The culture medium was maintained at 37 °C and pH equals 7 (Methods 4.12, page 57) with oxygen enrichment until OD_{600} reached 30 to 40 then induced by adding IPTG (1.5 mM). Styrene production was monitored by sampling from the fermenter culture media and from the dodecane trap and then analysed by

GCMS. The dry cell weight (DCW) of the samples were measured and was proportionally related to the OD_{600} values (Fig 7.10).

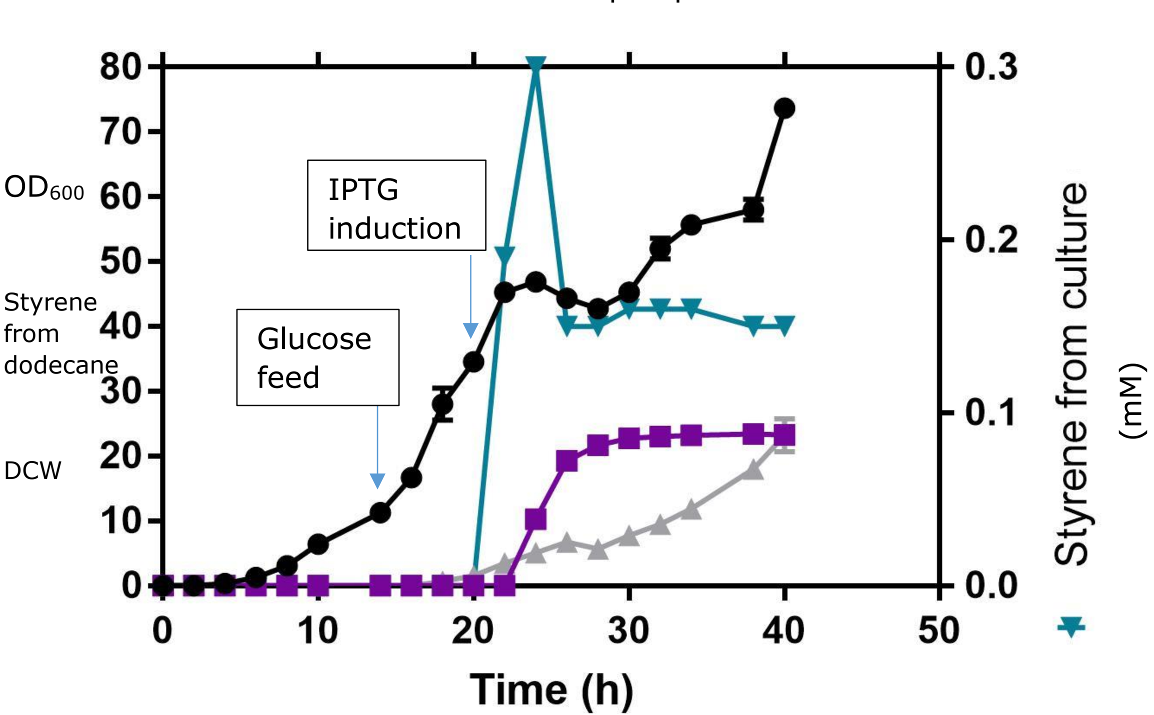


Figure 7.10 Styrene production of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc fedbatch. The overnight inoculum was diluted and added to the bioreactor growth medium, the starting OD_{600} was 0.01. ODs were monitored (Black). When OD reached approximately 30, IPTG (1.5 mM) was added to the culture. Styrene samples were taken from dodecane trap (250 mL) and measured (Purple, mM). Styrene was also detected from the fermenter culture (Blue, mM, Right Y axis). Dry cell weight (DCW) (Gray) was prepared and weighted (g/L). Error bars represent one standard deviation from triplicate experiments.

Samples for styrene detection were also collected from the fermenter medium and analysed by GCMS (Fig 7.10). As indicated from Figure (7.10), the growth of the bacterial culture was increasing proportionally with time until IPTG induction. Subsequently, the growth was decreasing which represents styrene production 4 hrs after IPTG induction, then styrene concentration in the fermenter culture media was increasing again reaching up to 0.31 mM which is tolerable to the bacteria. Whereas most styrene could be stripped into dodecane with up to 5.85

mmoles/ 1L culture (Fig 7.10). The total styrene concentration produced by both dodecane and culture media of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc fermentation was 6.2 mM.

7.4.2 Styrene production in the strains expressing the SrpABC pump

A similar experiment was also conducted with the strain bearing the SrpABC pump. Overnight culture of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC was diluted to OD_{600} equals 0.01 (final concentration of the starting batch media in the reactor) with glucose (12 g /L) and L-phenylalanine (2 g / L) in the bioreactor. The OD_{600} of the samples was monitored every two hours (Figure 7.11).

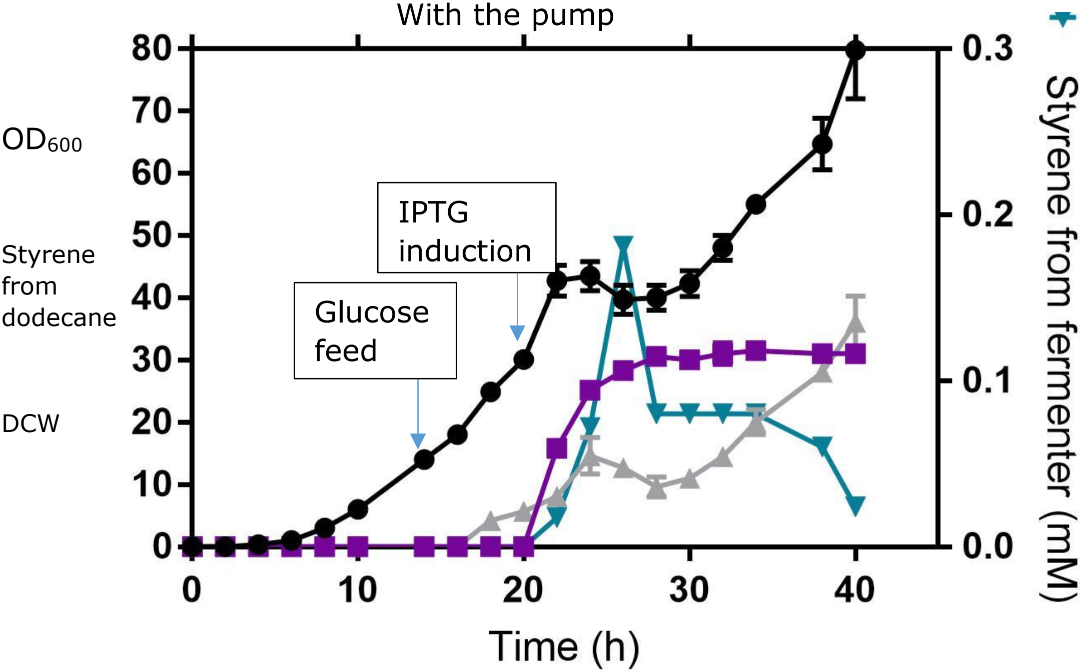


Figure 7.11 Styrene production of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC fed-batch. The overnight inoculum was diluted and added to the bioreactor growth medium the starting OD_{600} was 0.01. ODs were monitored (Black) every two hrs. When OD reached approximately 30, IPTG (1.5 mM) was added to the culture. Styrene samples were taken from dodecane trap (250 mL) and measured (Purple). Styrene was also detected from the fermenter culture (Blue), Right Y axis). DCW (Gray) was prepared and weighted (g/L). Error bars represent one standard deviation from triplicate experiments.

As shown from the curve, the OD_{600} values increased with the time until IPTG-induction then the OD was decreased because of the production of styrene which toxic to the cells. After that, the growth recovered again and increased.

The dry cell weight (DCW) of the samples were measured (Fig 7.11) and was reflected and was directly proportional to the ODs. Styrene production was monitored by sampling from the fermenter culture media and from the dodecane trap and then analysed by GCMS (7.11). Styrene samples was also collected from the fermentor medium and analysed by GCMS (Fig 7.11). As indicated in Figure (7.11), after IPTG induction, styrene concentration in the fermenter culture media was increasing and reached up to 0.18 mM which was lower than produced from the control and tolerable to the bacteria, and after 36 h the styrene concentration started to decrease may be because most of styrene gas was leaving the bioreactor and stripped into the dodecane trap without accumulation in the fermenter medium. Whereas most of the styrene could be stripped into the dodecane trap and reached to 7.87 mM (Fig 7.11).

The reason why the amount of styrene produced from the fermenter of this strain is lower than that produced from the control, may be because most of styrene gas was leaving the bioreactor and stripped into the dodecane trap. In addition, it could indicate that styrene production was high, but the volatile styrene was stripped directly into the dodecane trap as it was produced without accumulation in the fermenter medium.

The total styrene concentration produced from both dodecane and culture media of *E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC fermentation was 8.0 mM. Styrene production from the strain bearing SrpABC pump (*E. coli* MG1655

(DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC) (8.05 mM) was 30 % more than the strain without the pump (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc) (6.2 mM). This could conclude that using combined biological and chemical solution, SrpABC pump and the dodecane trap, respectively could confer the strain the tolerance to produce more styrene and pump it outside the cells.

General conclusion

Styrene bio-production was investigated in bioreactors by the fermentation of the strain bearing the efflux pump, *E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC, and the control strain *E. coli* MG1655 (DE3) - pETDuet_pal-fdc. A dodecane trap was fixed at the outlet gas of the bioreactor to strip the volatile produced styrene. The concentration of styrene stripped from the fermentation of strain bearing the efflux pump, 7.87 mM, was higher than the amount stripped from the fermentation of the control strain, 5.85 mM. However, the styrene concentration monitored in the fermenter culture of strain bearing the efflux pump was lower, 0.18 mM, than the styrene concentration of the fermenter medium of the control strain, 0.31 mM. One possible explanation behind that is may be the strain-bearing the pump was producing more styrene than the control, but most of the styrene gas was leaving the bioreactor and stripped into the dodecane trap, therefore the amount of styrene left in the fermenter medium was lower than the control.

8 Discussion and future work

The aim of this study was to explore the effectiveness of efflux pumps in the improvement of the toxicity tolerance towards styrene and other organic solvents in *E. coli*. Efflux pumps are one of promising biological solutions to the toxicity of bio-produced chemicals in *E. coli* (Dunlop, 2011); (Rojas *et al.*, 2001).

Styrene toxicity tolerance was investigated in *E. coli* using two efflux systems, SrpABC and AcrAB, in two different strains *E. coli* MG1655 (DE3) and *E. coli* C43 (DE3) Δ*acr*AB, respectively. The tolerable styrene threshold concentration was 2.5 mM and the primary difference observed was that at a concentration of 4 mM styrene, there was some growth recovery in cells expressing the SrpABC pump after two hrs from styrene exposure but not with the cells expressing AcrAB. This result reveals that the SrpABC pump is more advantageous than AcrAB pump in terms of conferring styrene resistance. Moreover, this could be explained by the fact that SrpABC pump is originally derived from *P. putida S12* and is specific solely to organic solvents such as styrene (Kieboom *et al.*, 1998). In contrast, AcrAB has a wide range of substrates including antibiotics and solvents. The explanation behind styrene tolerance improvement in the cells overexpressing the SrpABC efflux pump is that styrene tolerance of the host strain (*E. coli* MG1655 (DE3)) may also have been enhanced by the presence of the wild-type AcrABTolC pump.

In previous literature, McKenna and Nielsen (2011) and Mingardon *et al.*, (2015) demonstrated tolerance of styrene at 2.5- 2.9 mM (260 - 300 mg/L) in assays involving 2-8 hrs styrene exposure in *E. coli* NST74. By contrast, our system (SrpABC efflux pump) enabled the strain to recover its growth 2 hours after

styrene addition (4 mM concentration). Consequently, the SrpABC system could improve the tolerance to styrene.

E. coli strains harbouring the same efflux systems, SrpABC or AcrAB also showed similar behaviour regarding cyclohexane toxicity. Both strains were more tolerant to cyclohexane at 10 mM than the control strain. However, at 25 mM cyclohexane, *E. coli* MG1655 (DE3) pETDuet_MCS1_SrpABC was showing more tolerance than *E. coli* C43 (DE3) Δ*acr*AB-pETDuet_AcrAB. Again, this could be explained that the SrpABC pump is more specific to organic solvents than the AcrAB pump. In addition, at 50 mM cyclohexane, the growth of both strains was inhibited, so this concentration is non-tolerable and kills the bacteria. Kieboom *et al.*, (1998) have found that *P. putida S12* having the SrpABC pump can grow with 1% (11.9 mM) cyclohexane concentration.

E. coli K-12 strains are normally tolerant to *n*-hexane and susceptible to cyclohexane (White *et al*., 1997). The deletion of *acr*AB from either wild-type *E. coli* K-12 or a Mar mutant resulted in the loss of tolerance to both *n*-hexane and cyclohexane. Organic solvent tolerance mediated by transcriptional regulators mar, soxS or robA was not restored in strains with *acr*AB deleted (White *et al*., 1997). These results suggest that active efflux specified by the *acr*AB locus is linked to intrinsic organic solvent tolerance and to tolerance mediated by the *mar*A, *sox*S, or *rob*A gene product in *E. coli* (White *et al*., 1997).

Besides, the tolerance towards other chemicals, the strain harbouring the SrpABC pump could tolerate 10 mM of cyclohexene and 1- 3, cyclohexadiene and conferred tolerance to up to 100 mM ethylcyclohexane.

Regarding styrene bio-production, McKenna and Nielsen (2011) have reported that *E. coli* NST74 can produce up to 2.5 mM (260 mg/L) styrene per 1 g/L L-phenylalanine in shake flask cultures. Moreover, they predicted that with the addition of exogenous L-phenylalanine (500 mg/L), the cells could produce up to 500 mg/L styrene but the limiting factor was the toxicity of styrene to the cells [2.88 mM (300 mg/L)]. In addition, they have used an *in situ* product removal (ISPR) by periodic gas stripping into a cold trap, that enabled them to accumulate styrene up to 5.38 mM (561 mg/L) (McKenna *et al*., 2015). In this project, as a first study operating the SrpABC efflux pump for the bio-production of styrene, total styrene production from the strain bearing the SrpABC pump (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc_pACYCDuet_SrpABC) was 8.05 mM using glucose (12 g /L) and L-phenylalanine (2 g / L) which was approximately 30 % higher than the amount of styrene from the strain without the pump (*E. coli* MG1655 (DE3)-pETDuet_pal-fdc) (6.2 mM). In detail, the amount of styrene stripped into dodecane from the fermentation of the strain bearing the pump (SrpABC) was higher (7.87 mM) than the control (5.85 mM), however, the amount of styrene monitored in the fermenter medium was lower (0.18 mM) than the control (0.31 mM). This result had arisen a concern whether the total improvement of styrene production in the strain bearing the pump was because of the pump? Or because of the *in situ* product removal (ISPR) method by the dodecane trap was used? Or due to the combination of the pump with the ISPR. The explanation behind that it may be the strain-bearing the pump was producing more styrene than the control, but most of the styrene gas was leaving the bioreactor and stripped into the dodecane trap, therefore the amount of styrene left in the fermenter medium was lower than the control.

Moreover, styrene produced in the fermenter medium was below the tolerable styrene threshold concentration, 2.5 mM, obtained by toxicity tolerance experiments of exogenously added styrene. These results have spotted the light on how the efflux pumps behave to toxic chemicals produced from inside rather than an exogenously added toxic chemical, or if there are other more important systems on the inner membrane. Indeed, until today, there is a lack of knowledge about how the efflux pumps behave during the production of a toxic product inside the cells and how is it different from their response during the exogenously addition of the same toxic product. Understanding these mechanisms is crucial because, to be effective, the export of toxic chemicals should be able to decrease or maintain the intracellular product concentration at a level where the cell can still carry out processes related to growth.

Although bio-produced styrene titres were below previous studies, this work could generate insights about the role of efflux pumps combined to other ISPR methods to improve styrene bio-production.

Further future work needs to be done to highlight the exact role of SrpABC in *E. coli* MG1655 (DE3) in styrene production. This could be achieved by SrpABC- / AcrABTolC- synergy by the overexpression of genes encoding SrpABC solely in the strain on the production of styrene.

Besides, more work needs to be done to increase styrene production yield by producing SrpABC pump in *E. coli*. The possible routes to increase styrene yield in the same strain are: first by improving the SrpABC effectiveness in styrene tolerance and production. This could be done by directed mutagenesis of different subunits of the pump to determine which part is the most important and reliable in the production process. This could lead us to further investigate SrpABC protein

purification and crystallization, to determine how the three subunits interact with each other, their assembly model and solvent recognition sites. In addition, there is a need to study the detailed bacterial stress responses during the production of styrene is essential to understand the behaviour of the efflux pump during styrene production (Peabody *et al.*, 2014, Kuhn *et al*., 2010). This could be achieved by transcriptomics analysis to determine changes in genes transcription under styrene production and exposure (Lee and Mitchell, Anfelt *et al*., 2013).

Second, to investigate the stability of styrene production by studying plasmid stability. The importance of understanding the plasmid stability in the bioreactor in different growth stages of the bacterial cultures will help to evaluate the optimal styrene production and determine the circumstances that accompanied plasmid instability. Understanding these mechanisms will be very useful to maintain the intracellular product concentration at a level where the cell can still carry out processes related to growth (Dunlop *et al*., 2011).

Third, improving the expression of the styrene-synthetic pathway by changing the pathway by involving the efflux pumps with the addition of styrene synthesis in the same pathway or into the same expression plasmid. Introducing other strategies to improve enzyme expression include tuning the ribosomal binding site (RBS) and translation initiation region, using a stronger promoter (such as *trc*), increasing the copy number of the protein expression system, changing the transcriptional promoter (constitutive versus inducible), or changing the concentration of the inducer (Rosano and Ceccarelli, 2014). In addition, more knowledge about the metabolomics of the two-step styrene-synthetic pathway is

needed to understand the limiting steps for the rate of styrene bio-synthesis (McKenna and Nielsen, 2011).

Besides, solving the bottleneck of the co-expression of the AcrABTolC system with the styrene-producing system will add important information about its effect on the toxicity of bio-produced styrene. This could be done by changing the expressing strain or changing the expression plasmids and promoters. Moreover, styrene bio-synthesis in *E. coli* may be improved by expressing other efflux pumps such as MexAB-OprM and TtgABC of *Pseudomonas aeruginosa* and *Pseudomonas putida* T1E, respectively (Rojas *et al*., 2001; Keith *et al.,* 1996). Therefore, more work needs to be achieved to gain insights about the effect of these efflux pump on styrene production in *E. coli*.

For the production of renewable-styrene derived from microorganisms to become a sustainable alternative and available economically, titres and productivity must be improved. Therefore, if the yields of L-phenylalanine yields were increased could finally outcome into increased styrene production, product toxicity would become the consequent limiting factor for the bioprocess (McKenna and Nielsen, 2011).

If we considered *Pseudomonas putida S12* as an ideal model that withstand styrene at higher concentrations of about 95.1 mM (Weber *et al*., 1993) and comparing it to the lower concentrations of bio-produced styrene by our systems in *E. coli*, this will highlight the importance of the combination of other contributed mechanisms such as *cis-trans* isomerization and vesiculation mechanisms of the outer membrane of *P. putida* S12. This understanding will lead to the conclusion that using efflux pumps solely for the improvement of styrene bio-production maybe not optimal economically, therefore it is worthy to use more than one

biological solution, such as membrane vesicles and Cti protein (Tan *et al*., 2016), with one or more chemical solution to match the increasing global demands of producing styrene.

Another effective approach is investigating other alternative hosts which inherently possesses more tolerance (Fischer *et al*., 2008). The bacterium *P. putida S12* has been engineered as a solvent tolerant host for the bio-production of *p*-hydroxystyrene (Verhoef *et al*., 2009), and this might be employed for the improvement of styrene production.

Previous study has shown that *Pseudomonas sp.* may have improved tolerance to styrene when also grown in the presence of acetate or similar carboxylic acids as the sole carbon source (Weber *et al*. 1993). This developed phenotype may be associated with genetic changes leading to reductions in membrane fluidity, in addition to through the activation of genes believed to be specifically associated with improved tolerance to aromatic compounds.

Finally, using combinatorial procedures such as the global Transcription Machinery Engineering (gTME) (Alper *et al*. 2006); (Nicolaou *et al*., 2010) by reprogramming gene transcription to produce cellular phenotypes important for technological applications. This technology may be employed to improve styrene tolerance in *E. coli* or other alternative hosts. gTME has been successfully applied to enhance glucose/ethanol tolerance in *S. cerevisiae* (Alper *et al*., 2006).

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10 Appendices

10.1 Genes

10.1.1 *srp*ABC gene sequence

*E*coRI and *N*otI gene sequences were added to 5′ and 3′ ends of the *srp*ABC gene respectively.

An extra t was added to keep *E*coRI site in frame with SrpA.

*N*deI (after *E*coRI) and *X*hoI(Before *N*otI) sites were added.

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TGCAACTCCACCACCGGACGTGGGCGTGTACACCGTGCGTGCACAAGCTCTGA
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10.1.2 Fdc1 Gene sequence

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10.1.3 Pal2 (Gene sequence)

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TGCGCCGATTCCTAACCGGATTAAGGAATGTAGGTCGTATCCGTTGTATAGGTTCGTGA
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AGTTTGATAAGGTCTTCACTGCTATGTGTGAAGGTAAACTTATTGATCCGTTGATGGATT
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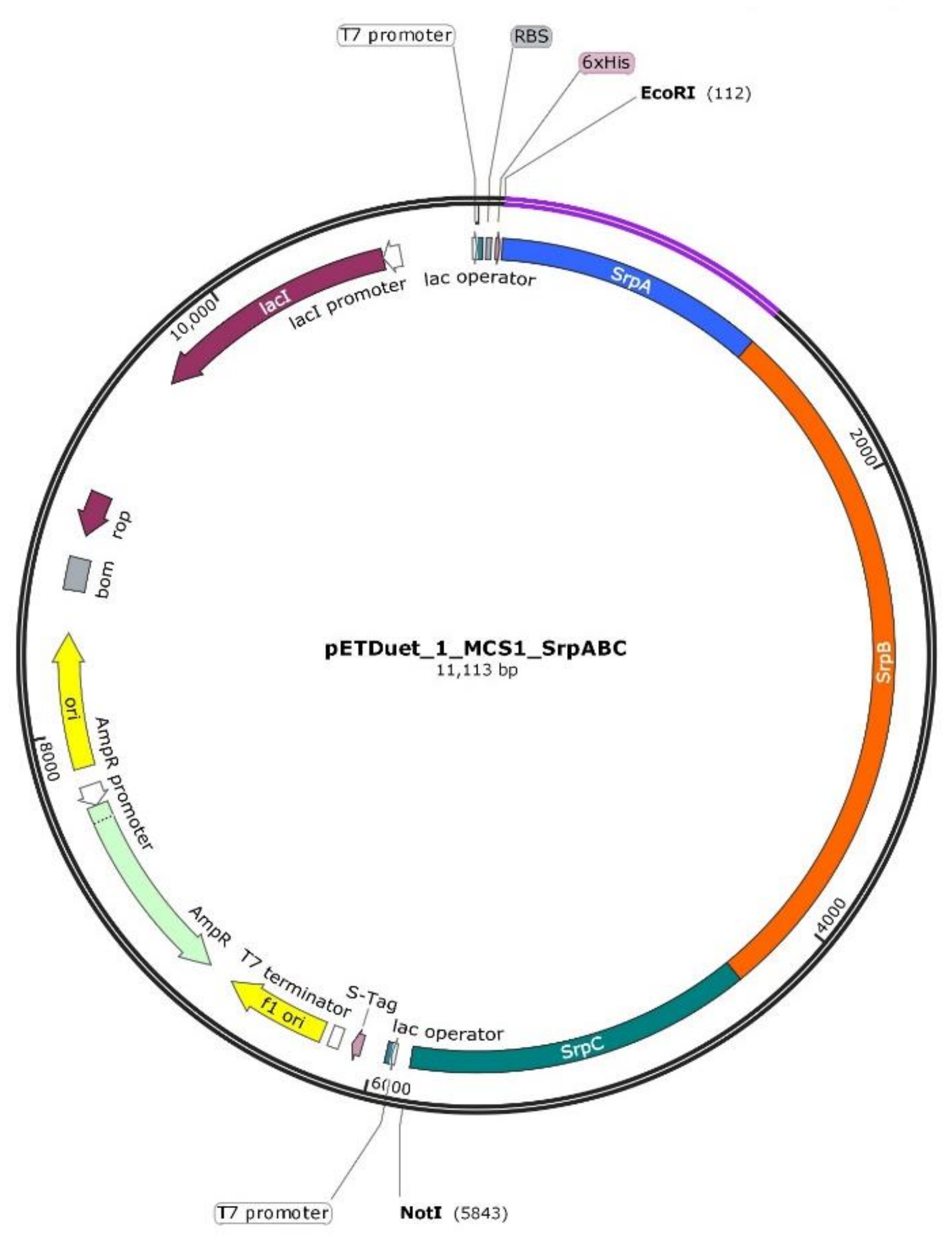
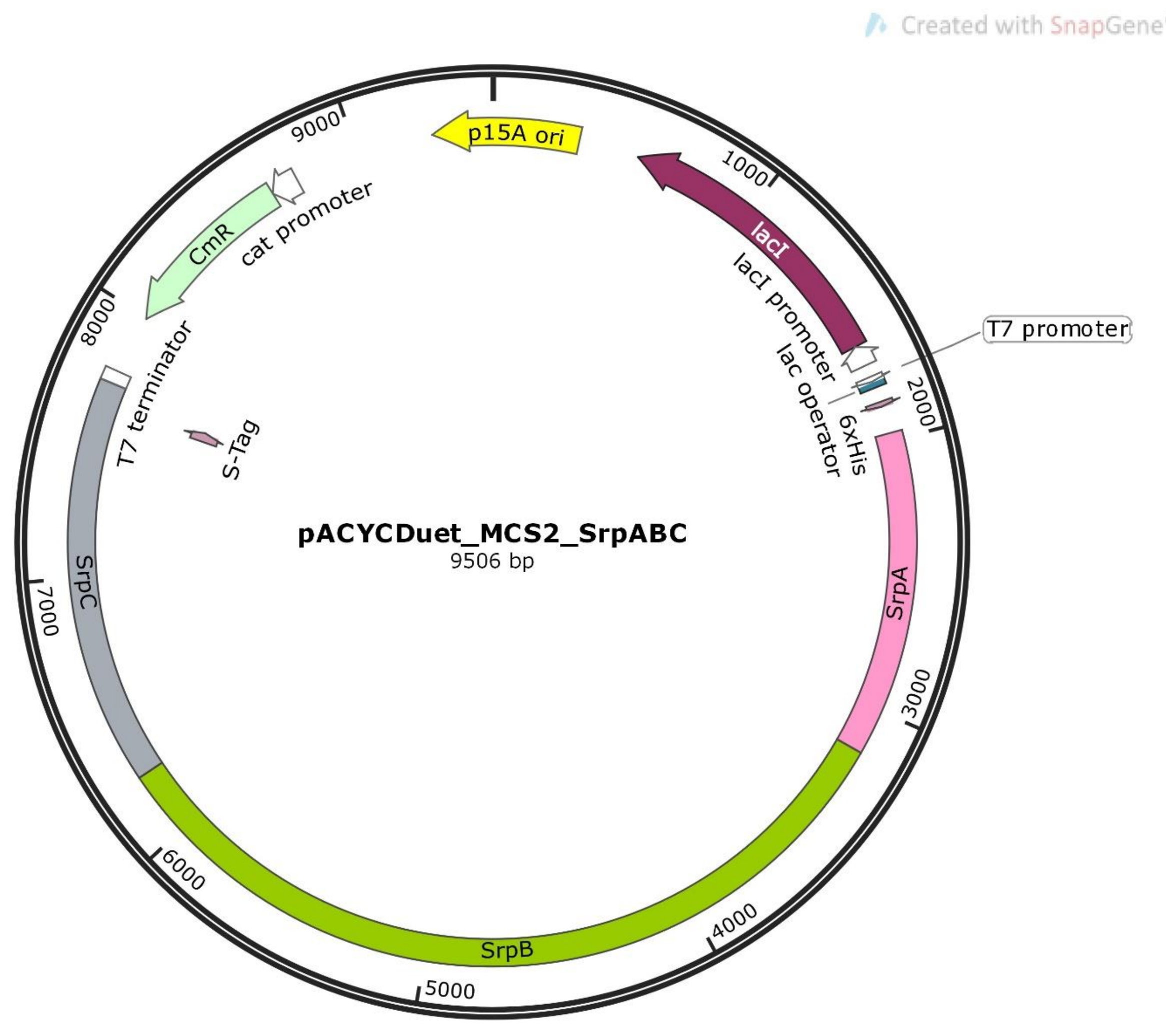
10.2 Plasmid maps

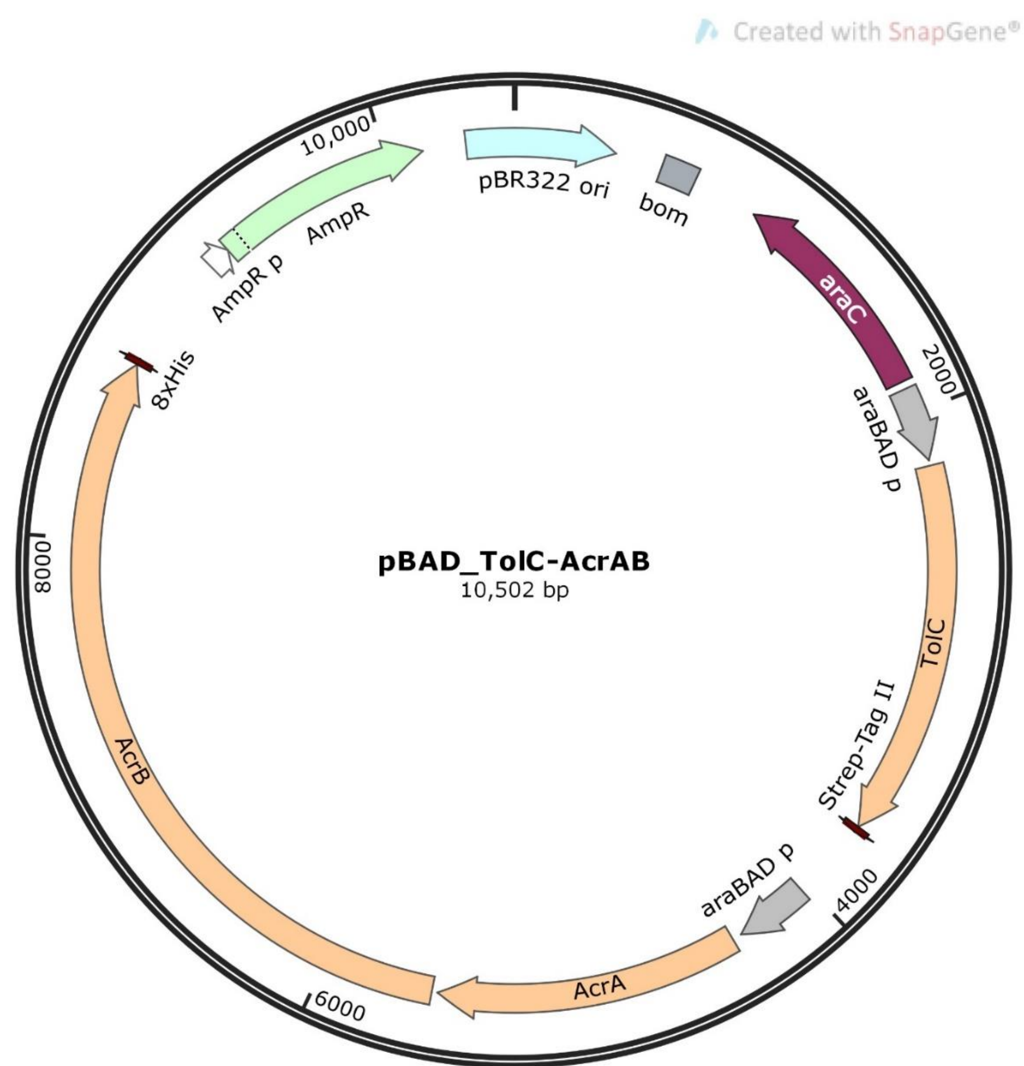
1) **pETDuet_MCS1_SrpABC map**



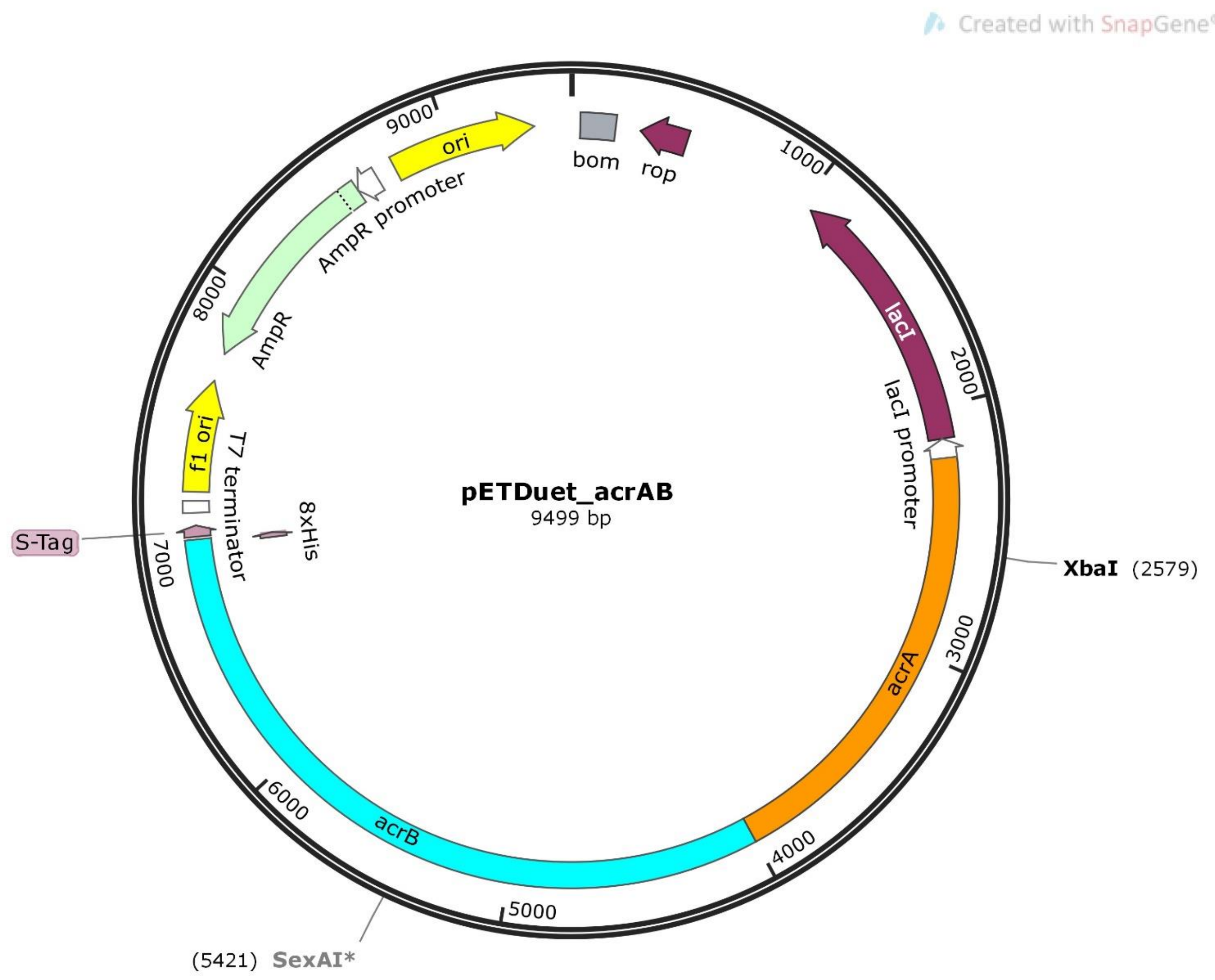
2) pACYCDuet_MCS2_SrpABC map



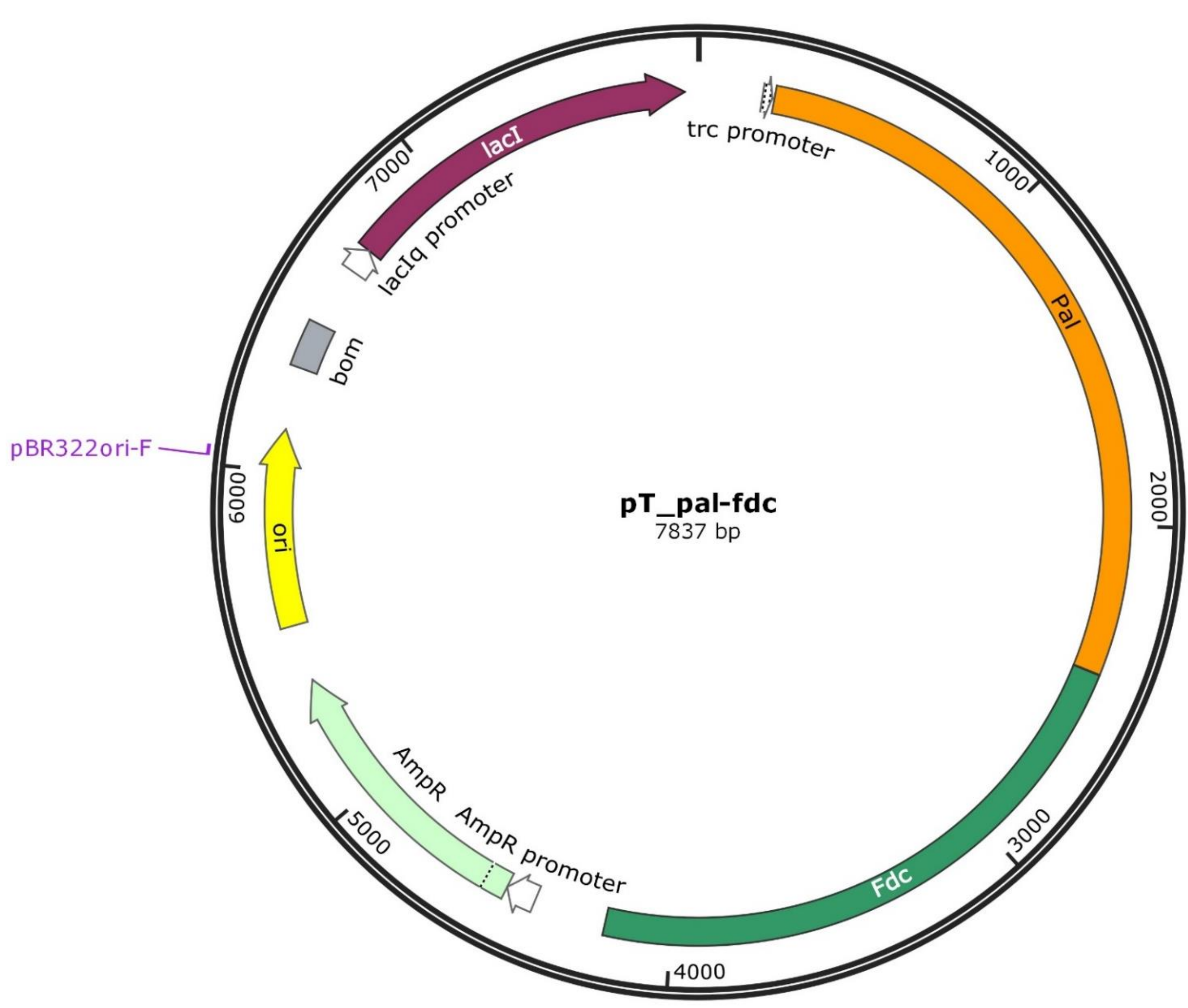
3) pBAD_TolC-AcrAB map



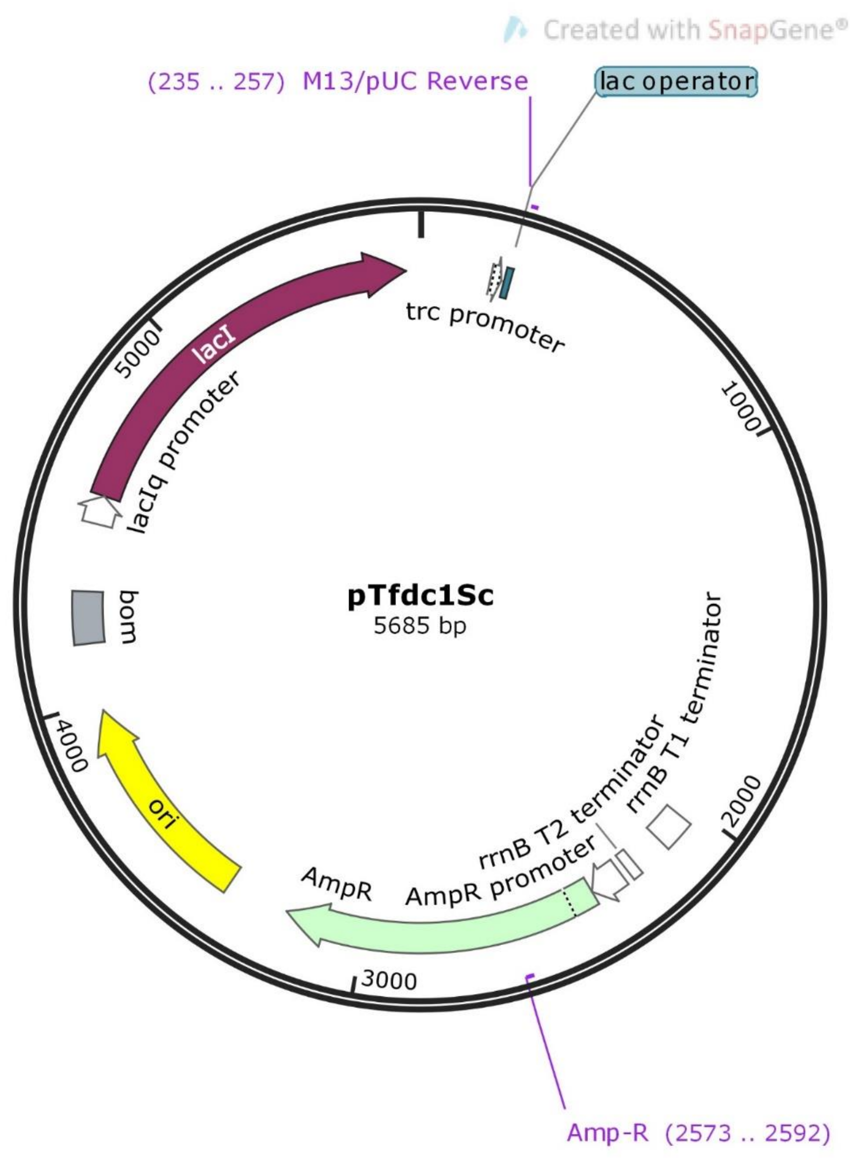
4) *p*ETDuet_acrAB map



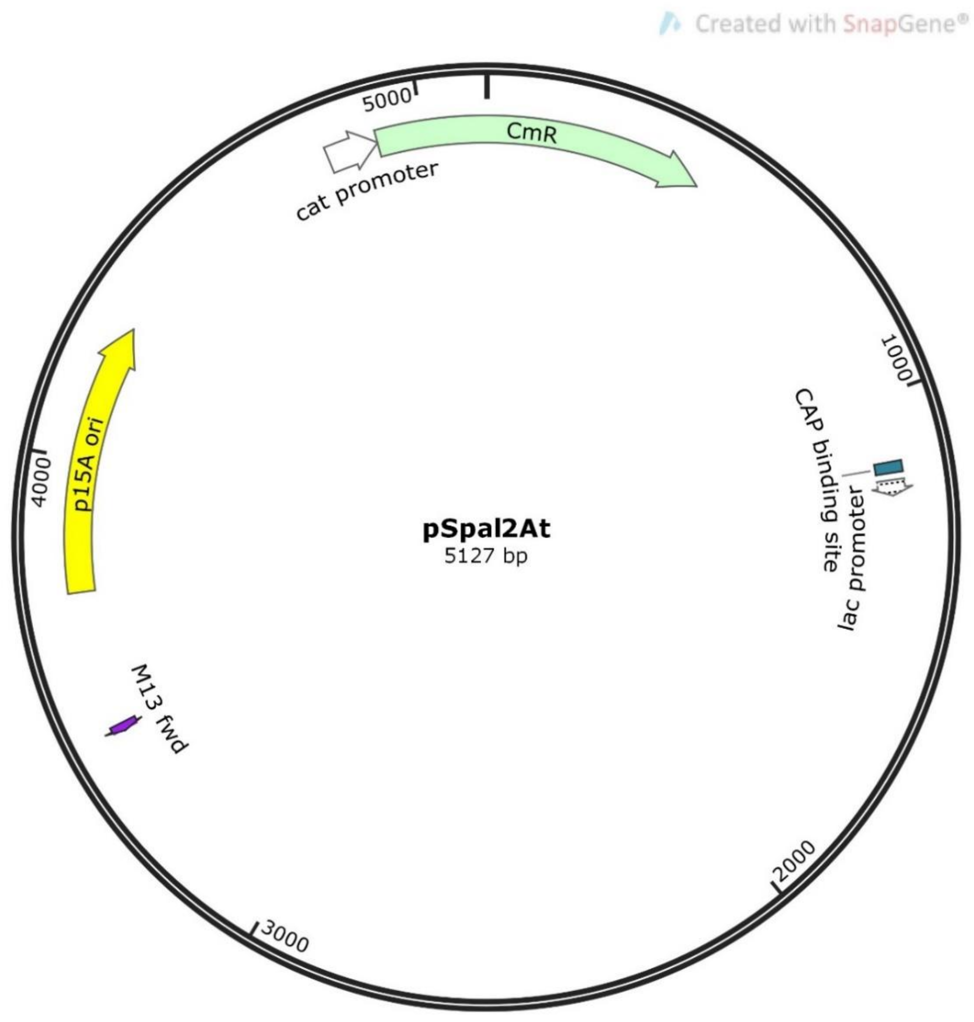
5) PT pal fdc plasmid



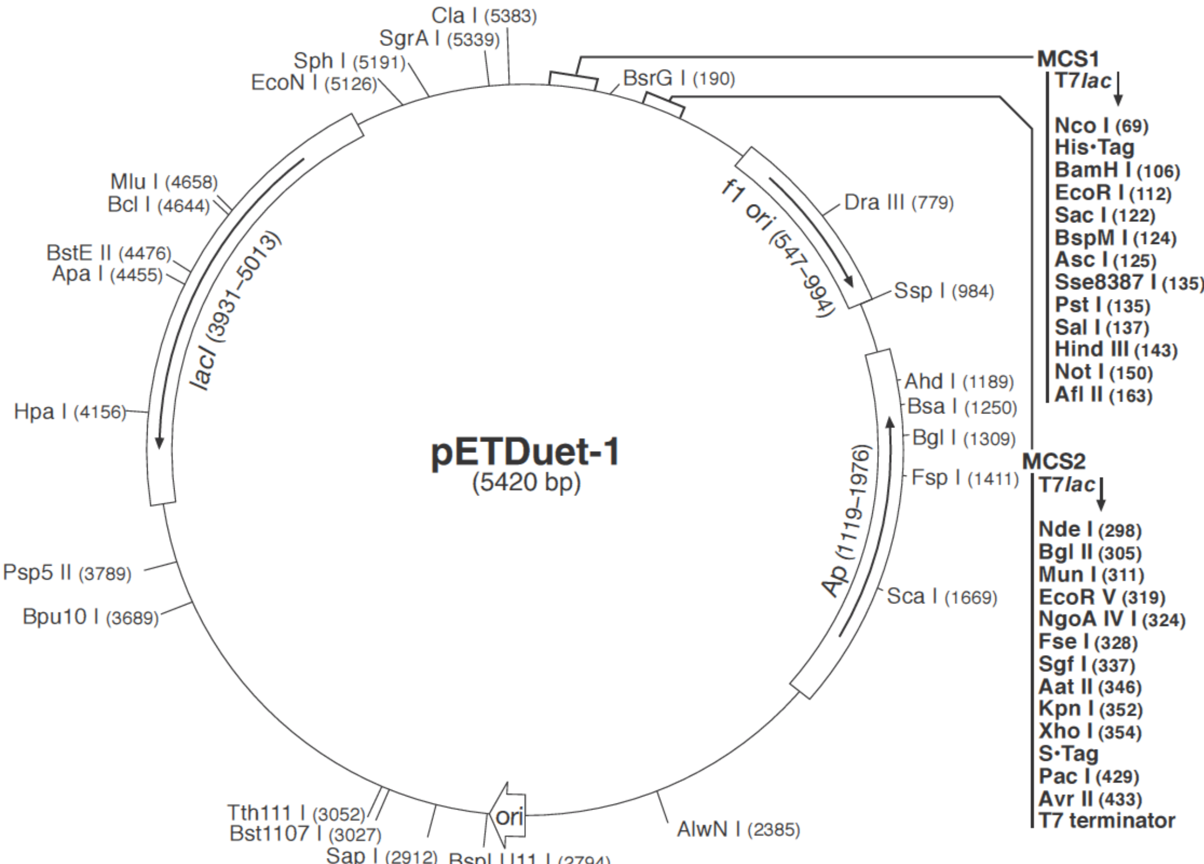
6) pTfdc1 Sc plasmid map



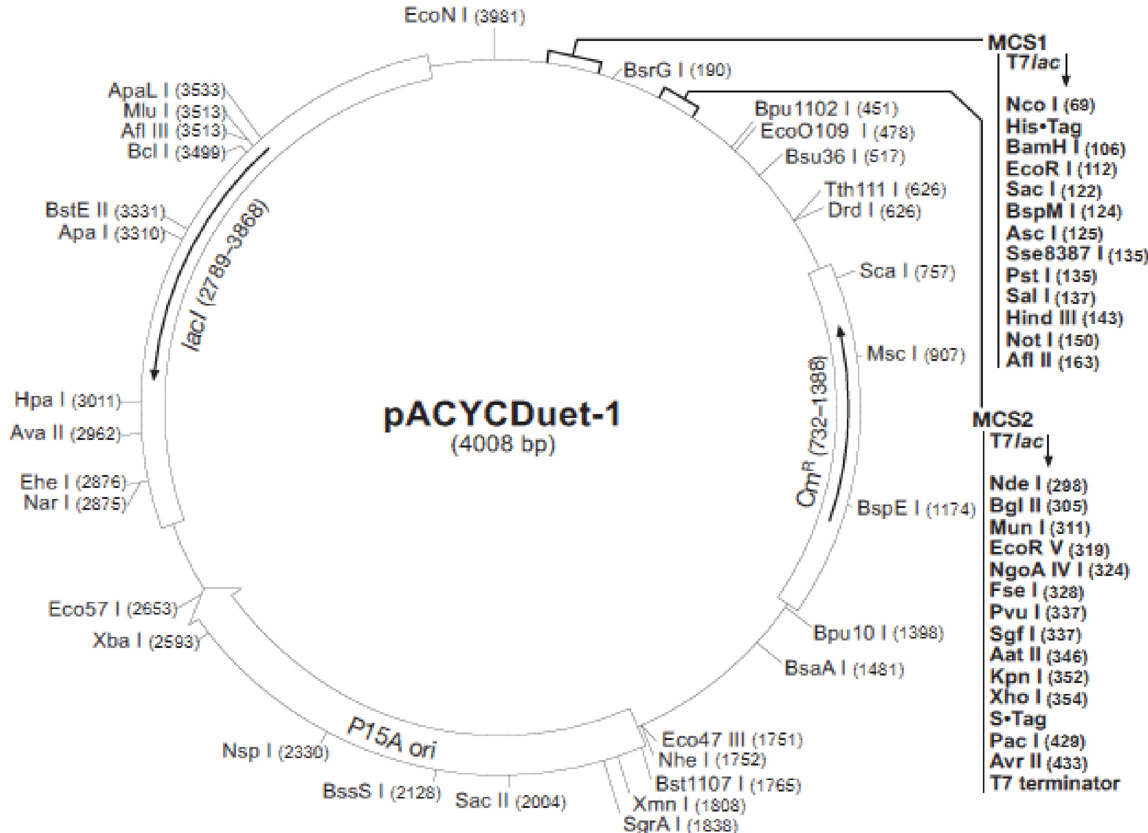
7) pSpal2At plasmid map



8) pETDuet-1 Vector map



9) pACYCDuet-1 vector map:



10.3 Calculations

Styrene formula C_8H_8, MW=104.15g/mol

Density= 0.909g/cm^3

Using these equations:

M= (W/ MW) * (1000/V_{ml})

$$\rho = \frac{m}{V},$$

Where, ρ: density, *m:* mass*, V:* volume

Styrene volumes were calculated using the previous equation (Table 2.1)

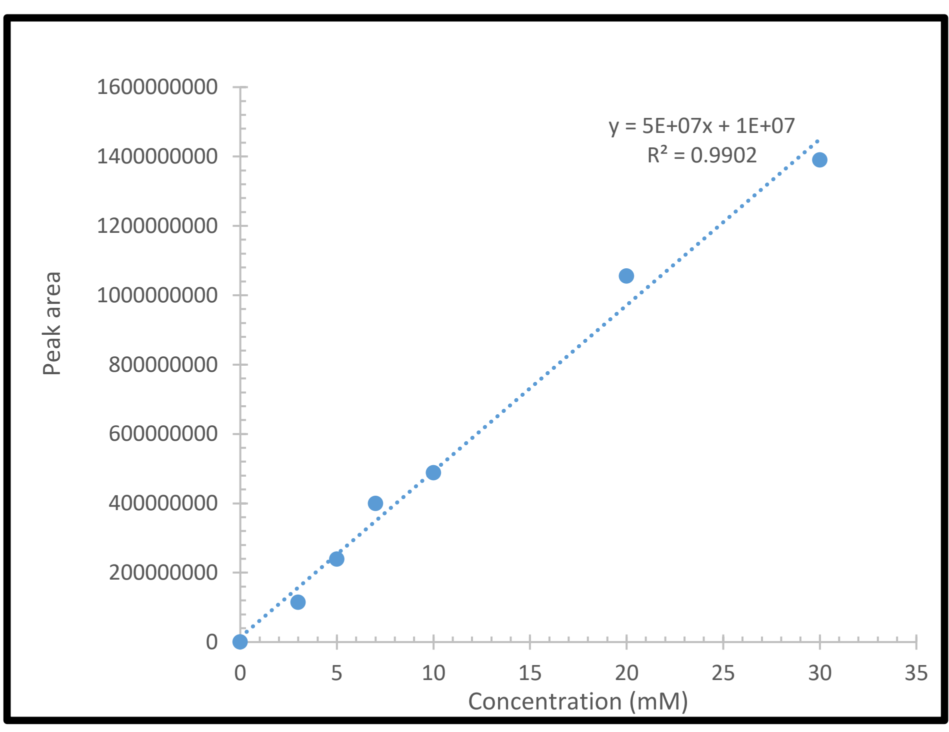
Table 2.1 Styrene Molarity & volume calculations

Styrene Molarity (mM)	Mass(g)	Volume(mL)
2.5	0.013	0.014
5	0.026	0.029

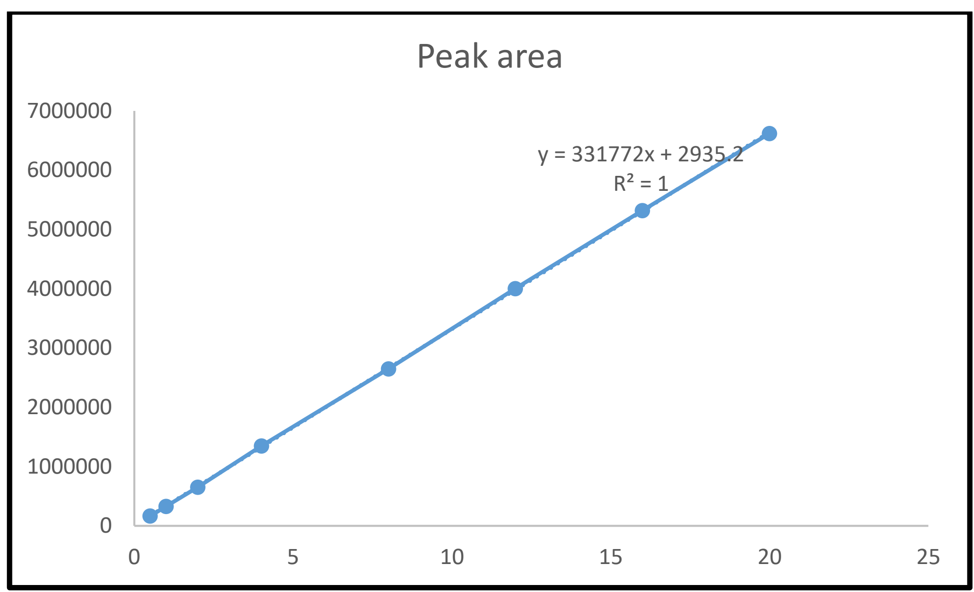
10.4 Calibration curves

Samples for the compounds of interest were always analysed together with test samples every time an assay was performed. A calibration curve was calculated and used for quantification. By doing this, calibration curves were new every time and quantification never relied on dated data. Below are typical calibration curves for all compounds of interest.

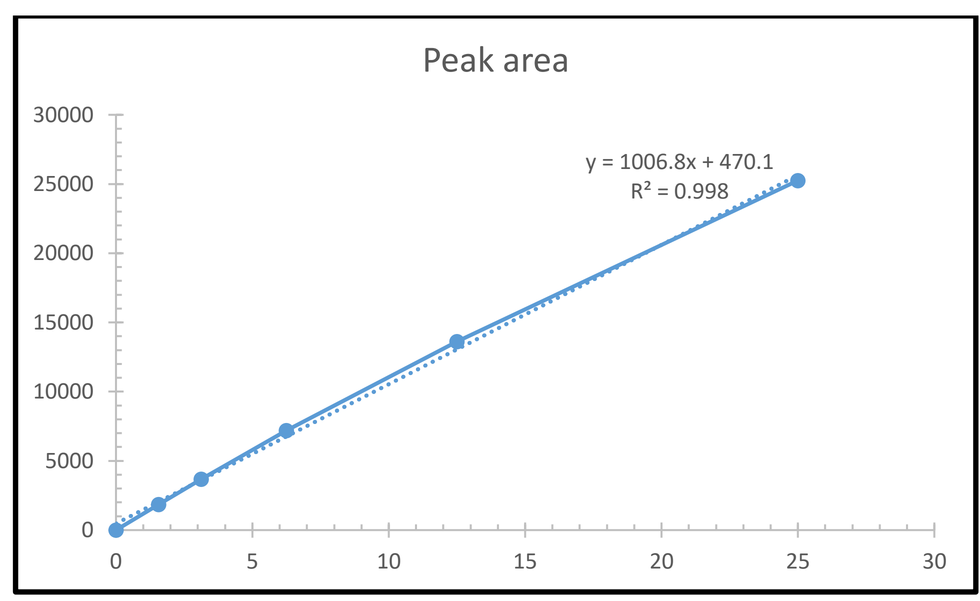
10.4.1 Styrene calibration curve



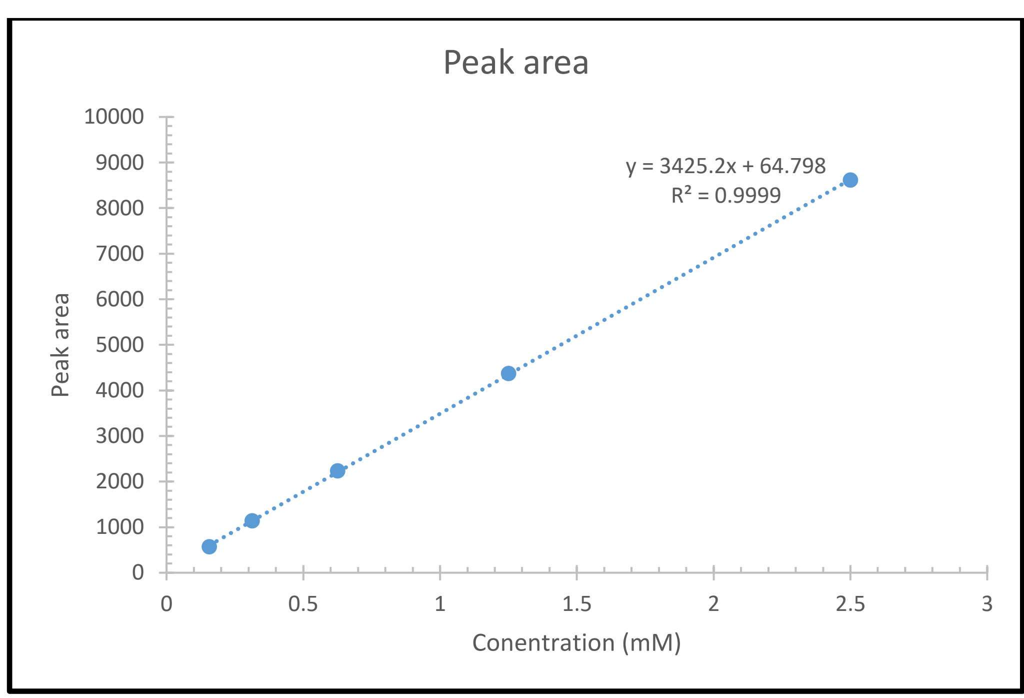
10.4.2 Glucose calibration curve



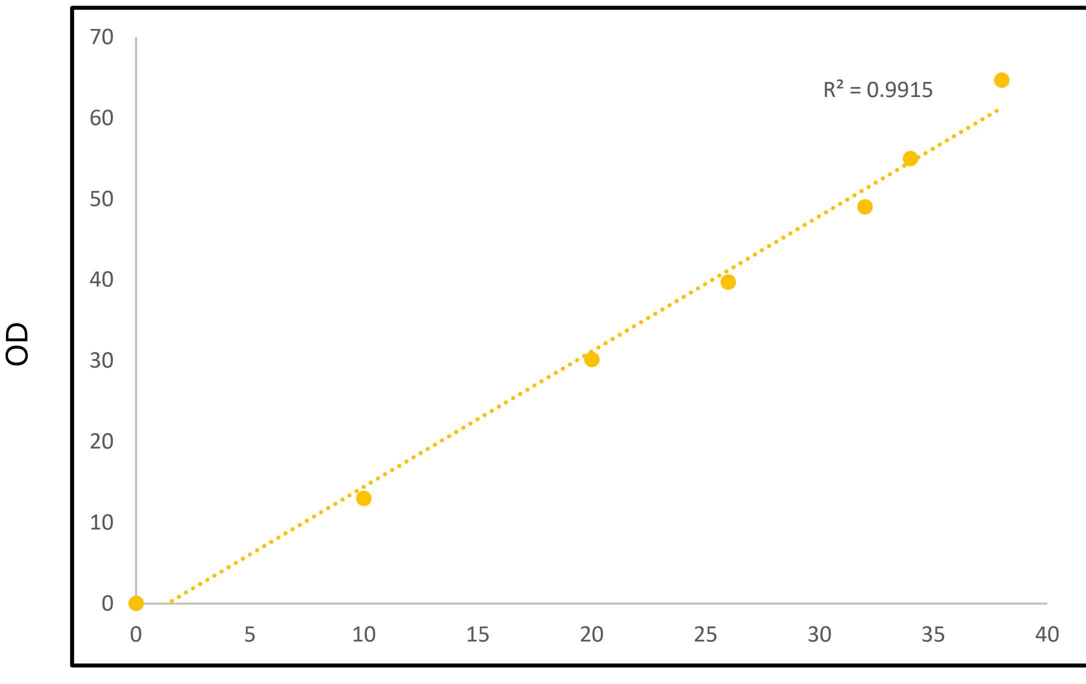
10.4.3 L-phenylalanine calibration curve



10.4.4 *t*CA calibration curve

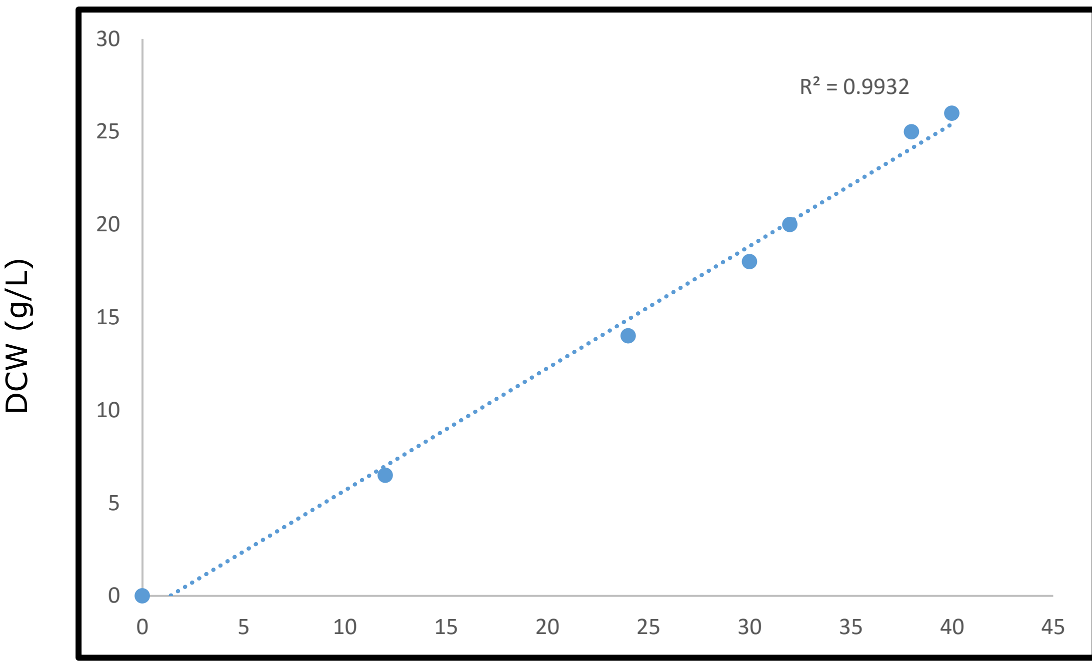


10.4.5 OD calibration curve



Time (hr)

10.4.6 DCW calibration curve



10.5 Other organic solvents

11.5.1 Cyclohexane

Cyclohexane is a cycloalkane with the molecular formula C_6H_{12}. Cyclohexane is mainly used for the industrial production of adipic acid and caprolactam, which are precursors to nylon. Cyclohexane is a colourless, flammable liquid with a distinctive detergent-like odour, reminiscent of cleaning products. It is used as a solvent in some brands of correction fluid (https://libweb.anglia.ac.uk/referencing/harvard.htm).

Cyclohexane is sometimes used as a non-polar organic solvent, although *n*-hexane is more widely used for this purpose. It's frequently used as a recrystallization solvent, as many organic compounds exhibit good solubility in hot cyclohexane and poor solubility at low temperatures. Cyclohexane is also used for calibration of differential scanning calorimetry (DSC) instruments, because of a convenient crystal-crystal transition at -87.1 °C. Cyclohexane vapour is used in vacuum carburizing furnaces, in heat treating equipment manufacture.

10.5.2 Cyclohexene

Cyclohexene is a volatile liquid with a characteristic odour. It is a reactive cycloaliphatic alkene, as a building block used in several different markets, due to the functionality of the double bond which allows a range of chemistries to be applied, and downstream intermediates and products to be derived from it, such as the epoxide, diol and other useful downstream products. Cyclohexene is used in a diverse range of chemical synthesis. It is a building block that is used in pharmaceutical industries e.g. Clidanac, MK 346, Trandolapril and Agchems such as Propargite. It also has industrial and consumer uses in the following: used in

the synthesis of waterproof coatings, LED encapsulation systems, crack resistant films, elastomers, tie binder for coating, adhesives and silanes. It is a precursor to the epoxide, diol and other useful downstream products (https://libweb.anglia.ac.uk/referencing/harvard.htm).

10.5.3 1, 3-Cyclohexadiene

1, 3-cyclohexadiene is an organic compound with the formula C_6H_8. It is a colourless, flammable liquid. Its refractive index is 1.475 (20 °C). A naturally occurring derivative of 1, 3-cyclohexadiene is terpinene, a component of pine oil (https://libweb.anglia.ac.uk/referencing/harvard.htm).

10.5.4 Ethylcyclohexane

Ethyl cyclohexane is an organic compound with the formula $C_6H_{11}C_2H_5$. It has been used as an organic synthetic material, used as a solvent, reference material of chromatography analysis, etc. Also used for metal surface treatment agent (https://libweb.anglia.ac.uk/referencing/harvard.htm).

10.6 Viability test

Table 10.1 No. of colonies after 1 min. styrene addition

Conc. / Dilution	**2.5 mM**	**5 mM**
10^0	TNTC	TNTC
10^{-1}	TNTC	TNTC
10^{-2}	TNTC	>250
10^{-3}	>305	98
10^{-4}	305	29
10^{-5}	39	5
10^{-6}	1	1

-After adding different concentrations of styrene to 50 ml *E. coli* culture, I made serial dilution of the culture by adding 0.5 mL culture to 4.5 mL potassium buffer.

-The number of colonies observed in the plates after 1 min incubation at 37°C was Too Numerous to Count (TNTC) in higher dilutions.

From the data in table (11.1) we were able to calculate the number of viable cells as following:

Table 10.2 No. of viable cells after 1 min. styrene addition

Conc. / Dilution	**2.5 mM**	**5 mM**
10^0	TNTC	TNTC
10^{-1}	TNTC	TNTC
10^{-2}	TNTC	$>2.5*10^5$
10^{-3}	$>3.05*10^6$	$9.8*10^5$
10^{-4}	$3.05*10^7$	$2.9*10^6$
10^{-5}	$3.9*10^7$	$5.0*10^6$
10^{-6}	$1.0*10^7$	$1.0*10^7$

-There was no growth at (25, 50 mM) styrene concentrations after one-minute incubation.

- An inversely proportional relationship between styrene concentration and the viable counts of bacterial cells.

Table 10.3 No. of colonies after 1 min. styrene addition

Conc. / Dilution	0 mM				2.5 mM				5 mM			
	T1*	T2	T3	Avg*	T1	T2	T3	Avg	T1	T2	T3	Avg
10^0	TNTC*	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-1}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^{-3}	>250	>250	>250	>250	>250	>250	>250	>250	181	>250	>250	>250
10^{-4}	$2.28*10^2$	$1.95*10^2$	$2.09*10^2$	$2.10*10^2$	$2.02*10^2$	$1.83*10^2$	$1.72*10^2$	$1.85*10^2$	38	66	47	50.5
SD*	0.15177				0.15177				1.4429			

* Where T: reading number, Avg.: Average, TNTC: Too numerous to count SD: standard deviation

From the data we were able to calculate the number of viable cells

Table 10.4 Mean no. of viable cells after 1 min. styrene addition

Dilution / Conc.	0 mM	2.5 mM	5 mM
10^0	TNTC	TNTC	TNTC
10^{-1}	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	$>2.5*10^6$	$>2.5*10^6$	$>2.5*10^6$
10^{-4}	$2.1*10^7$	$1.85*10^7$	$5.03*10^6$

Table 10.5 No. of colonies after 30 min. styrene addition

Conc. / Dilution	0 mM				2.5 mM					5 mM			
	T1	T2	T3	Avg	T1	T2	T3	Avg	SD	T1	T2	T3	Avg
10^0	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC		0	0	0	0
10^{-1}	TNTC	TNTC	TNTC	TNTC	>250	>250	>250	>250		0	0	0	0
10^{-2}	TNTC	TNTC	TNTC	TNTC	120	109	137	122	0.14	0	0	0	0
10^{-3}	TNTC	TNTC	TNTC	TNTC	48	64	35	49	1.45	0	0	0	0
10^{-4}	>250	>250	51	>250	6	11	7	8	2.64	0	0	0	0

Table 10.6 Mean No. of viable cells after 30 min. styrene addition

Dilution / Conc.	0 mM	2.5mM	5mM
10^0	TNTC	TNTC	0
10^{-1}	TNTC	$>2.5*10^4$	0
10^{-2}	TNTC	$1.22*10^5$	0
10^{-3}	TNTC	$4.9*10^5$	0
10^{-4}	-	$8.0*10^5$	0

10.7 Growth rate calculations

The specific growth rate (μ_t) was calculated for the exponential growth phase as log (OD_t /OD_0) Δt, where OD_0 is the initial OD, OD_t is the final OD, and Δt is the time interval (in hours) between biomass measurements.

1) **Growth rates of *E. coli* BW25113-pBAD_AcrABTolC, *E. coli* BW25113 empty pBAD and *E. coli* BW25113 bacteria without solvent addition**

	E. coli BW25113-pBAD_AcrABTolC	*E. coli* BW25113-empty pBAD	*E. coli* BW25113
μ_5	3.21	3.29	3.25
μ_6	3.50	3.64	3.68

2) **Growth rates of *E. coli* BW25113-pBAD_AcrABTolC *E. coli* BW25113 empty pBAD bacteria towards different concentrations of *n*-Hexane**

	μ_6	
Concentration of *n*-Hexane	*E. coli* BW25113-pBAD_AcrABTolC	*E. coli* BW25113-empty pBAD
10 mM	3.85	4.07
5 mM	3.71	3.75
2.5 mM	3.87	4.08
0 mM	3.77	4.02

3) Growth rates of *E. coli* BW25113-pBAD_AcrABTolC and *E. coli* BW25113-empty pBADbacteria towards different concentrations of Cyclohexane.

Concentration	μ_6	
	E. coli BW25113-pBAD_AcrABTolC	*E. coli* BW25113-empty pBAD
10 mM	3.73	3.92
2.5 mM	3.83	4.08
0 mM	3.80	4.13

4) Growth rates of *E. coli* BW25113 bacteria towards different concentrations of Styrene

Concentration	μ_5		
	E. coli BW25113-pBAD_AcrABTolC	*E. coli* BW25113-empty pBAD	*E. coli* BW25113
5 mM	1.84	1.79	2.19
2.5 mM	2.94	3.75	3.37
0 mM	3.90	4.02	4.04

5) Growth rates of *E. coli* MG1655 (DE3) bacteria towards different concentrations of Styrene

Concentration	μ_5		
	E. coli MG1655 (DE3) pETDuet_MCS1_SrpABC	*E. coli* MG1655 (DE3) empty pETDuet-1	*E. coli* MG1655 (DE3)
5 mM	1.66	2.19	1.94
2.5 mM	2.61	3.27	3.71
0 mM	2.99	3.79	3.87

10.7 Raw Data Bioreactor experiments

Raw Data OD Bioreactor triplicate experiments (with the pump)

Time	OD			Avg	Std
0	0.01	0.01	0.01	0.01	0.00
2	0.04	0.05	0.06	0.05	0.01
4	0.34	0.35	0.4	0.36	0.03
6	1	1.5	1.36	1.29	0.21
8	3	2.8	3.5	3.10	0.29
10	7	6	6.2	6.40	0.43
14	12	11	10.8	11.27	0.52
16	17	17.8	15.4	16.73	1.00
18	25	31.1	27.9	28.00	2.49
20	35.7	34.6	33.4	34.57	0.94
22	44.4	45.4	46.2	45.33	0.74
24	47.3	46.3	47.1	46.90	0.43
26	44.3	45.8	43	44.37	1.14
28	42.8	41.8	43.7	42.77	0.78
30	44	45	47	45.33	1.25
32	50	52	54	52.00	1.63
34	54	56	57	55.67	1.25
38	58	56	60	58.00	1.63
40	74.8	72.9	73.3	73.67	0.82

Raw Data OD Bioreactor triplicate experiments (without the pump)

Time	OD			Avg	Std
0	0.01	0.01	0.01	0.01	0.00
2	0.04	0.05	0.06	0.05	0.01
4	0.34	0.35	0.4	0.36	0.03
6	1	1.5	1.36	1.29	0.21
8	3	2.8	3.5	3.10	0.29
10	7	6	6.2	6.40	0.43
14	14	15	13	14.00	0.82
16	18.1	19	16.9	18.00	1.05
18	25.5	23.4	25.9	24.93	1.34
20	30.1	30.3	30	30.13	0.15
22	43.3	40.1	44.9	42.77	2.44
24	40.9	45.4	44.2	43.50	2.33
26	37.4	39.6	42.1	39.70	2.35
28	38	40	42	40.00	2.00
30	40	43	44	42.33	2.08
32	46	48	50	48.00	2.00
34	54	55	56	55.00	1.00
38	60	66	68	64.67	4.16
40	75.6	74.9	88.8	79.77	7.83

Raw Data DCW (without the pump)

Time (h)	Avg	SD
0	0	0
2	0.01	0
4	0.01	0
6	0.02	0
8	0.02	0
10	0.03	0
14	0.03	0
16	0.05	0
18	0.73	0.78
20	1.57	1.05
22	3.5	0.46
24	5.1	1.45
26	6.77	0.7
28	5.67	0.59
30	7.77	0.31
32	9.47	0.55
34	11.87	0.81
38	18	1
40	23.23	2.55

Raw Data DCW (with the pump)

Time (h)	Avg	SD
0	0	0
2	0.01	0
4	0.015	0
6	0.02	0
8	0.02	0
10	0.03	0
14	0.03	0
16	0.05	0
18	4.2	0.42
20	5.7	0.85
22	8.1	1.27
24	14.6	2.99
26	12.7	0.32
28	9.6	1.65
30	11	1.15
32	14.5	0.32
34	20	2
38	28	1.53
40	36	4.27

Raw Data Styrene from dodecane (mM) after IPTG induction (without the pump)

Time	Avg	SD
0	0	0
2	0.04	0
4	10.28	0.1
6	19.32	0.5
8	21.67	0.6
10	22.75	0.4
14	23	1
18	23.2	0.7
22	23.4	0.5
26	23.3	0.5

Raw Data Styrene from dodecane (mM) after IPTG induction (with the pump)

Time	Avg	SD
0	0	0
2	0	0
4	15.8	0.15
6	25.17	1.05
8	28.32	1.34
10	30.55	0.15
14	30	1
18	31	0.78
22	31.5	0.6
26	31	0.5

Raw Data Styrene from fermenter (mM) after IPTG induction (without the pump)

Time (h)	Avg	SD
0	0	0
2	0	0
4	0.19	0.01
6	0.31	0.02
8	0.15	0.05
10	0.15	0.02
14	0.16	0.03
18	0.16	0.01
22	0.16	0
26	0.15	0

Raw Data Styrene from dodecane (mM) after IPTG induction (with the pump)

Time (h)	Avg	SD
0	0	0
2	0	0
4	0.0175	0.01
6	0.718	0.01
8	0.180	0.02
10	0.08	0.01
14	0.08	0.011
18	0.08	0.015
22	0.06	0.01
26	0.024	0.02