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Bioproduction of styrene from renewable feedstocks using ionic liquids as solvents for *in situ* product removal

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Abstract

Styrene is a large volume commodity chemical which is used as a precursor for a diverse range of useful polymers and co-polymers. The conventional styrene synthesis is performed through dehydrogenation of petroleum-derived ethylbenzene, an energy intensive process. Its natural production has been identified in a few organisms, but extraction efficiency is very low. Microbial fermentation for the synthesis of styrene from renewable resources has been previously developed, but styrene inhibits bacterial growth at very low concentration, therefore styrene must be removed *in situ*, whilst the fermentation is being carried out, to an extractant organic phase. The conventional solvents generally used to generate a biphasic system exhibit volatile and flammable characteristics, making them hazardous when used in industrial large-scale processes and are usually toxic towards the biocatalysts. Therefore, it is of great interest to find non-toxic alternatives with high extraction capacity. Room temperature ionic liquids have arisen as potential alternatives due to their exceptionally manipulability, making possible to design almost infinite possible useful combinations of cation and anion. The possibility to tune their physical and chemical properties by altering functional groups or through selection of the cation and anion, allow ionic liquids to match the specific requirements of a biological system whilst also providing low toxicities.

In this thesis, the potential use of ionic liquids as alternatives to traditional organic solvents for the extraction of styrene from the culture media during its biocatalytic production was investigated. A wide range of ionic liquids were tested for their toxicity towards *E. coli* MG1655 using a high throughput screening method. Four biocompatible ionic liquids were then chosen with respect to their chemical structure and their capacity for styrene extraction was evaluated. All the investigated ionic liquids extracted close to 95% of the styrene present in the aqueous phase and achieved high distribution coefficients. Finally, styrene production using a previously metabolically engineered *E. coli* strain co-expressing PAL2 and FDC1 was performed in the

presence of a selected phosphonium-based biocompatible ionic liquid, successfully achieving increased concentrations of styrene in comparison to the production in the absence of ionic liquid as a secondary extractant phase

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This thesis is dedicated to my dear uncle Faco

I'm pretty sure you are sitting down on your favourite star

looking and smiling at us with your telescope;

the same one you used to teach me about the infinite blue sky.

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List of abbreviations

μ	Specific growth rate
ABC	ATP binding cassette
ABS	Acrylonitrile-butadiene-styrene
ATP	Adenosine-5'-triphosphate
cAMP	Cyclic adenosine 3'-5'-monophosphate
CE	Crude extract
CER	CO_2 evolution rate
CM-PDT	Chorismate mutase/prephenate dehydratase
D	Distribution coefficient
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
DCM	Dichloromethane
DCW	Dry cell weight
dH_2O	Deionised water
DMAc	N,N-dimethylacetamide
dNTPs	Deoxyribonucleotides
DO	Dissolved oxygen
E4P	Erythrose 4-phosphate
E. coli	*Escherichia coli*
EE	Extraction efficiency
FDC1	Ferulic acid decarboxylase
GC-FID	Gas chromatography – Flame ionisation detector
GC-MS	Gas chromatography – Mass spectrometry
GHGs	Greenhouse gases
Gram-	Gram-negative

Gram+	Gram-positive
HAPs	Hazardous air pollutants
H^{CC}	Henry's law constant
HSPs	Heat-shock proteins
IC_{50}	Inhibitory concentration
ILs	Ionic liquids
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	Internal standard
ISPR	*In situ* product recovery
Kbp	Kilo-base pair
KOAc	Potassium acetate
K_P	Partition coefficient
LB	Luria Bertani
log*P*	Logarithm of the partition coefficient
$\log P_{crit}$	*Critical* log*P* of the biocatalyst
Lrp	Leucine-responsive regulatory protein
MATE	Multidrug and toxic efflux
MDR	Multidrug resistance
MF	Major facilitator
MFP	Membrane fusion protein
NMR	Nuclear magnetic resonance
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OD	Optical density
OECD	Organisation for economic co-operation and development
OMP	Outer membrane protein
P	Partition coefficient

PADC	Phenylacrylate decarboxylase
PAL	Phenylalanine ammonia lyase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Polydiacetylene
PDC	*p*-hydroxycinnamic acid decarboxylase
PEP	Phosphoenolpyruvate
pHCA	*p*-hydroxycinnamic acid
pHS	*p*-hydroxystyrene
P. Putida	*Pseudomonas putida*
PspA	Phage-shock protein A
PTS	Phosphotransferase transport system
RBS	Ribosome binding site
RND	Resistance nodulation division
rpm	Revolutions per minute
SAN	Styrene-acrylonitrile
$scCO_2$	Supercritical CO_2
S. cerevisiae	*Saccharomyces cerevisiae*
SCFs	Supercritical fluids
SMR	Small multidrug resistance
TAE	Tris-acetate-EDTA
TAL	Tyrosine ammonia-lyase
tCA	*trans*-cinnamic acid
TPPB	Two-phase partitioning bioreactor
Trp	Tryptophan

TSILs	Task-specific ILs
VOCs	Volatile organic compounds
WCW	Wet cell weight

1. Introduction

For over a century, most commodity chemicals and conventional fuels have been produced from crude oil-based sources, which large availability and low prices have allowed the development of many different products derived from petroleum feedstocks. However, over the last decades the interest in the industrial production of commodity chemicals and liquid fuels using bio-renewable resources and sustainable biosynthetic chemistry approaches has gained increased support (Bruschi *et al*. 2011). There are several different motivations for the increased interest in bio-based production of chemicals, such as, the depleted availability of oil supply, in addition to its unstable and increasing price; and political instability within the major crude oil producing countries, making its supply uncertain (Kerr 2007). Additionally, the intensification of energy consumption by the emerging industrializing countries, and environmental concerns to reduce greenhouse gases (GHGs) emissions and diminish the carbon footprint of any process have become key incentives to drive the research of new biological routes to key products (International Energy Agency 2008; Becker and Wittmann 2015).

Biocatalytic processes use natural catalysts, or enzymes, to perform chemical transformations on a wide range of raw starting materials into platform chemicals. Today biocatalysis has become an important tool for the production of fine and commodity chemicals with an increase number of biotechnological processes being developed to an industrial scale, in order to meet the increasing demands for these chemicals whilst reducing the dependency on crude oil resources (Liese, Seelbach, and Wandrey 2006). Additionally, biocatalysis, using either natural or metabolic engineered microorganisms, offers a high chemical selectivity in the production of desired chemicals from renewable feedstocks, increasing the efficiency and improving the environmental profile of the manufacturing processes. Unfortunately, in many microbial fermentations, the desirable product might have inhibitory/toxic effects towards the biocatalysts, and its accumulation might inhibit cell growth

or result in death (Nicolaou, Gaida, and Papoutsakis 2010). Accumulation of such chemicals, whether desirable or not, during the fermentation process affects the microorganism's performance, limiting the product titres, which severely impacts process economics. Therefore, the toxic product needs to be removed from the aqueous phase before causes any harmful effects to the biocatalysts.

In situ product recovery (ISPR) methods haven been shown to have a great potential in enhancing the productivity of many bioprocesses (Dafoe and Daugulis 2014; Daugulis, Tomei, and Guieysse 2011; Lye and Woodley 1999; Malinowski 2001). ISPR involves the selective removal of the toxic end-product from the reaction medium, while the fermentation is still in progress, by using an appropriate and biocompatible separation mechanism; hence avoiding its accumulation to inhibitory/toxic concentrations. Examples of successful ISPR methods of inhibitory biomolecules include, adsorption, absorption, gas stripping, membrane pervaporation, and liquid-liquid extraction (van den Berg 2010). Perhaps the simplest of all the above-mentioned mechanisms to remove a toxic product from a microbial fermentation is by means of liquid-liquid extraction (Van Hecke *et al.* 2014; Malinowski 2001; Gyamerah and Glover 1996; Weilnhammer and Blass 1994; Collins *et al.* 1995; Straathof 2003; Lilly 1982; van Sonsbeek *et al.* 1993; Cull *et al.* 2000), in which the toxic substance is extracted *in situ* into a water immiscible layer. Liquid-liquid extraction presents a high capacity and selectivity for the target product, whilst also facilitates its recovery and purification. However, because of the direct contact between the solvent and the cells, only non-toxic solvents towards the microorganisms must be implemented. Within the area of biotransformation, conventional organic solvents have been commonly used to create biphasic systems due to their high extraction capacities and ability to dissolve a wide range of compounds (Cull *et al*. 2000). However, their volatile and flammable nature makes them hazardous towards the operators and the environment when used in industrial-scale, therefore better alternative solvents as an extraction media are needed.

Styrene is a large volume commodity chemical which is used as a precursor for a diverse range of useful polymers and co-polymers, such as polystyrene, and styrene-acrylonitrile (SAN) and acrylonitrile-butadiene-styrene (ABS) copolymers. The global market is valued at approximately $32.5 billion and is forecast to grow an average of 3.6% per year (U.S. Department of Energy 2012). Commercial styrene is produced by the chemocatalytic dehydrogenation of petroleum-derived ethylbenzene, an energy-intensive process. However, the aforementioned concerns regarding future oil supply and environmental impact associated with fossil fuel-based processes, has incentivised the development of a more sustainable and greener production of styrene based on renewable resources. An engineered microorganism capable of styrene synthesis from renewable resources has been reported (McKenna and Nielsen 2011), yet styrene has been shown to be highly toxic towards the biocatalyst, inhibiting growth at very low concentration (~3 mM), so bioproduction is not economically viable or sustainable at the moment. Therefore, the aim of this project is to develop an integrated sustainable biosynthetic process using the previously reported metabolic engineered strain for the production of styrene from renewable resources and using ionic liquids (ILs) as alternative solvents for the *in situ* extraction of styrene, hence overcoming the end-product toxicity.

2. Literature Review

2.1 Introduction: Biocatalytic production of chemicals – A general overview

The Sun provides continuously The Earth with a tremendous amount of energy of about $1.2x10^5$ TJ per second (Crabtree and Lewis 2007), which drives the ocean currents and powers the complete water cycle that provide us with the fresh inland water so essential for life on Earth, but also triggers natural phenomena such as tornadoes, typhoons and hurricanes that so easily destroy the landscape. This great magnitude of available solar energy largely exceeds the world's rate of total energy needs – total production and consumption per year –, currently of about 13 TW (British Petrolium 2017); and moreover, with The Sun capable of delivering this incredible amount of energy to The Earth in approximately an hour. Its abundance and inexhaustible energy offers a clean, climate-friendly and independent resource to humanity, which could enhance a sustainable development to solve some of the most urgent problems we face as a modern society such as climate change, energy security and universal access to energy for everyone.

The abundance of solar energy is accompanied by its many different possible ways of conversion into useful forms of energy to power the wide range of human activities, such as electricity which can be produced by capturing photons and exciting electrons in a solar cell; chemical fuel, with the sunlight converted into chemical energy, in the form of biomass, by green plants *via* photosynthesis; and solar heat produced by sunlight concentrating technologies used directly for heating or for further conversion to electricity.

Despite its abundance, for several reasons such as its availability is highly dependent on the region's geographic position, weather conditions and land availability (United Nations Development Programme 2000), in addition to the high production cost and conversion capacity limitations, the actual exploitation of the solar energy is much lower than its theoretical potential.

Nevertheless, biomass produced by natural photosynthesis has become the biggest global source of renewable energy on the planet, providing more than 10% of the total energy consumed in the planet (International Energy Agency 2016), being by far the largest use of solar energy as a renewable source, which can be used in its original form as fuel, or can be further processed into solid, gaseous or liquids biofuels and other useful chemicals.

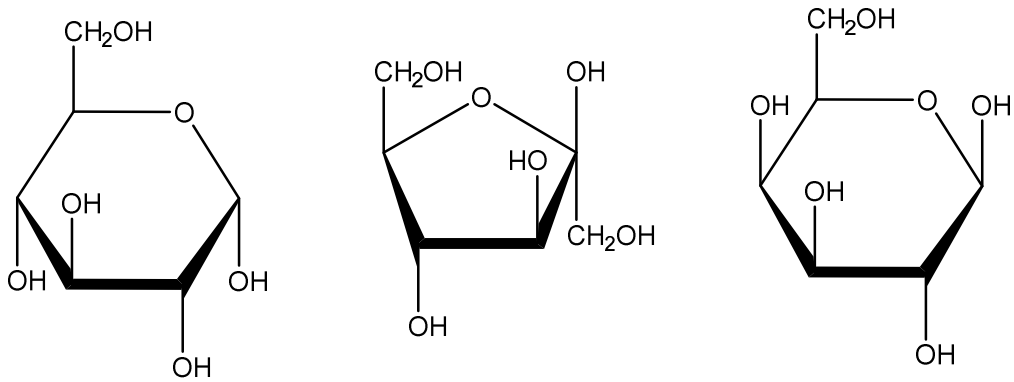
Biomass feedstocks are biologically-derived materials from plants, microorganisms and the bio-degradable wastes resultant from their processing (*e.g.* household and forestry wastes, and agricultural residues). In addition to its renewable features, biomass is usually produced through photosynthesis, by which plants simultaneously collect atmospheric CO_2 and solar energy to produce oxygen and chemical energy stored as biomass (Klass, 1998). Hence, since this CO_2 removal counterbalances the CO_2 emitted when the energy stored within the biomass is released upon combustion, theoretically, no additional atmospheric carbon dioxide is produced.

On the other hand, one of the major concerns regarding the use of biomass as a renewable alternative source of energy and chemicals, especially against the large-scale bio-fuel industry, is the competition for fertile lands for the production of food, the destruction of vast native habitats, increase of food prices and final small reduction in greenhouse gases (GHG) emission (Cherubini 2010; Srirangan *et al*. 2012; Berndes, Hoogwijk, and Broek 2003; Crutzen *et al*. 2008). However, credible studies show that with plausible technology developments, biofuels could supply some 30% of global demand in an environmentally responsible manner without affecting food production (Koonin 2006).

With the majority of chemicals being produced based on fossil-fuel derived feedstocks, concerns such as the depleted availability of oil supply, intensification of energy consumption by the emerging industrializing countries, and environmental concerns to reduce greenhouse gases (GHGs) emissions, has driven the chemical companies to develop more sustainable

processes aiming to diminish the carbon footprint of the complete production cycle (International Energy Agency 2008; Becker and Wittmann 2015). Therefore, over the last decades the interest in the industrial production of fine chemicals and liquid fuels using bio-renewable resources based on biomass and sustainable biosynthetic chemistry approaches has increased throughout the chemical industry.

Biocatalysis refers to the use of a biological agent, such as free enzymes or whole-cells, to perform a chemical transformation. The increasing tendency in the use of biocatalysis to convert a wide range of fermentable starting materials derived from biomass (*i.e.* monomeric sugars. Figure 1) into high value products in the pharmaceutical and chemical industry is due to several advantages that biocatalytic processes offer compared to conventional chemical transformations (Nestl *et al*. 2014; Urata and Takaishi 2001; Held *et al*. 2000). First, biocatalysis usually operates under mild conditions of pH and temperature, reducing the amount of energy consumed, as well as avoiding the formation of unwanted by-products or waste due to the chemo-, regio-, and stereoselectivity of enzymes, allowing the development of environmentally friendly processes. Additionally, the biocatalysts can be completely degraded in the environment avoiding the need of harmful and hazardous procedures. Lastly, biocatalysis allows the production of chemicals using renewable resources making possible to develop improved cost-effective and sustainable processes for a wide range of chemical transformations.



α-D-Glucopyranose β-D-Fructofuranose β-D-Galactopyranose

Figure 1: The chemical structures of α-D-Glucopyranose, β-D-Fructofuranose and β-D-Galactopyranose.

However, there are still some important barriers that need to be overcome in order to utilise widespread biocatalytic process in industry (Becker and Wittmann 2015; Nestl *et al*. 2014; Lye and Woodley 1999), such as high nutritional requirements, easier availability of cheap and stable catalysts, and toxicity of products and metabolic intermediates. Additionally, low productivities of the biocatalytic systems when compared to the chemical equivalent highlight the need for systematic methods for the development of entire process sequences and to enable rational choices to be made between chemical and biocatalytic routes to the same end-product. For large-scale production processes, typical difficulties such as limiting mixing and fluctuations in the process parameters and concentrations could often stress the producing biocatalysts.

Nonetheless, the rapid development of recombinant DNA tools and synthetic biology technologies have helped achieved remarkable success to overcome the inherent limitations associated with the microbial conversion processes, and have aided the development of biocatalysts and their tailor-designed integration into industrial processes, allowing the construction of novel synthetic metabolic pathways in microbial hosts for the production of a wide range of chemicals, with the most successful cases applied to *Corynebacterium glutamicum, Escherichia coli, and Saccharomyces cerevisiae*, established them

as key microorganisms for the industrial production of chemicals (Becker and Wittmann 2015). Therefore, today biocatalysis has become an important tool for the production of fine and commodity chemicals with the highest number of processes developed in the pharmaceutical sector, followed by the food, agro-chemical and cosmetic sectors, respectively (Liese, Seelbach, and Wandrey 2006).

Lastly, while the thermo-chemical counterparts seem a technology more convenient to apply when compared to the biological reactions; nowadays biocatalytic transformations present effective and viable systems for the production of single and well-defined chemical products with minimum by-products or waste. Modern tools of protein discovery and engineering, as well as advances in metabolic engineering, have helped to achieve remarkable success to overcome the inherent limitations associated with the microbial conversion processes, and have aided the development of biocatalysts and their tailor-designed integration into industrial processes. However, further process improvements are still required in order to increase the still sub-optimal product titres and to make the biocatalytic industry more profitable yet.

2.2 Toxic metabolites and solvents towards microorganisms and their tolerance in biocatalytic processes

Chemical stress is commonly found in bioprocesses to produce target chemicals from renewable feedstocks in whole-cell biocatalysis. All biochemical production processes from renewable biomass-derived sources have two common features that may reduce the production of the target product: (i) as a result of biomass pre-treatment the presence of unwanted molecules might inhibit the fermentation process and (ii) the desirable product might have inhibitory/toxic effects towards the biocatalysts, and its accumulation might inhibit cell growth or result in death (Nicolaou, Gaida, and Papoutsakis 2010;

Straathof 2014). Accumulation of such chemicals, whether desirable or not, during the fermentation process affects the microorganism's performance, limiting the product titres, which severely impacts process economics.

The cell membrane consists of a phospholipid bilayer with embedded proteins whose basic functions are to protect the cells from its surroundings, provide structural integrity and facilitate the transport of substances in and out of the cell (Figures 2-3) (Los and Murata 2004). It has been shown that toxic chemicals affect the cells by altering the biophysical properties of the cell membrane, affecting membrane processes such as transport and energy production, and by damaging and denaturing biomolecules, including protein unfolding, lipid damage and nucleic acids degradation (Nicolaou, Gaida, and Papoutsakis 2010; Ramos *et al*. 2002).

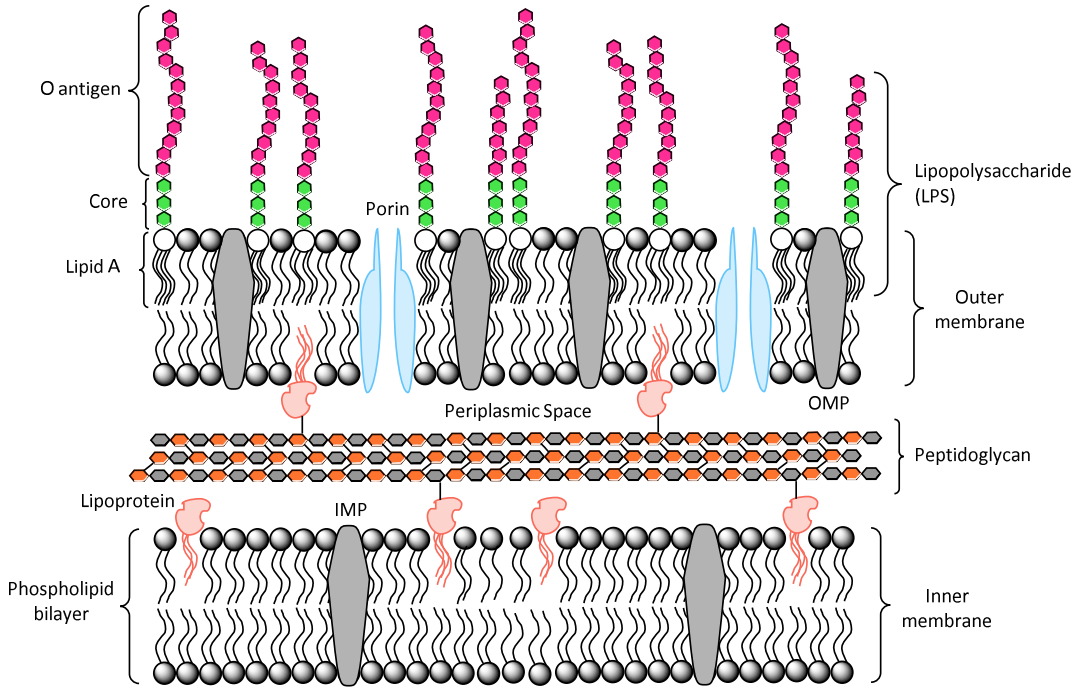


Figure 2: Schematic diagram of a Gram-negative cell wall.

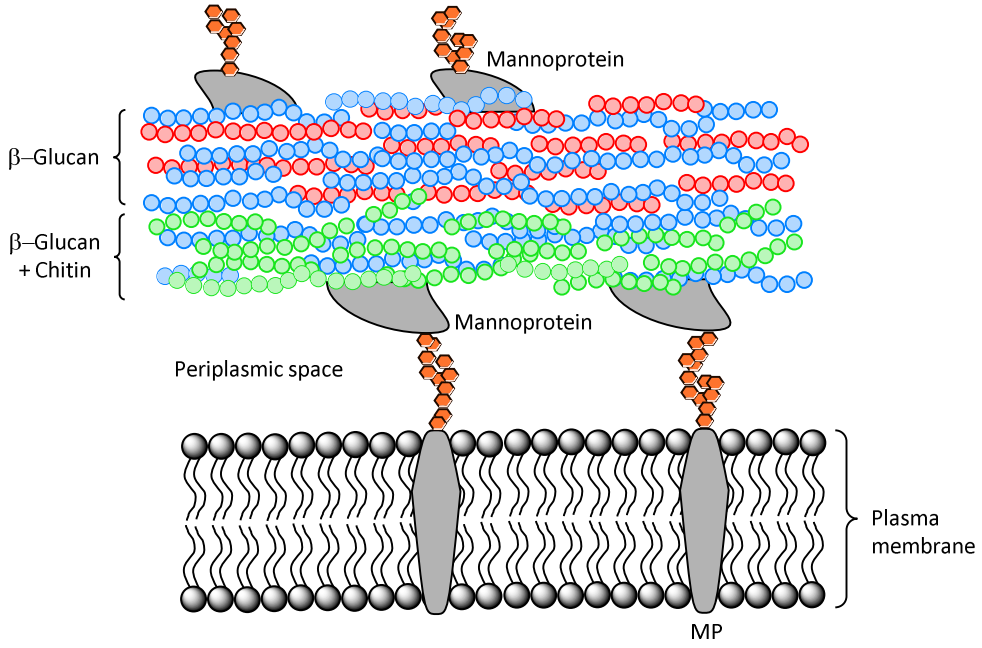


Figure 3: Schematic diagram of a yeast cell wall.

Cells respond in many different ways towards solvent effects, and some possible mechanisms involved in solvent tolerance are shown in Figure 4. In fact, several adaptive changes in the structure of the membrane have been observed in reaction to the accumulation of organic solvents in the membrane of microorganism, such as (i) changes in membrane composition and biophysical properties to counteract the fluidizing effects of solvents (*i.e. cis*-to-*trans* isomerization of unsaturated fatty acids, increased ratio of unsaturated to saturated fatty acids, composition changes in the phospholipid head group, and changes in phospholipid chain length); (ii) molecular efflux pumps to drive out the toxic chemicals from the cytoplasm into the extracellular space (*e.g. E. coli* AcrAB-TolC and *P. putida* TtgABC, TtgDEF, TtgGHI); (iii) altered energy metabolism for higher ATP production required for survival and cellular repair processes, and (iv) metabolic detoxification to convert toxic compounds into less harmful chemicals; the latter feature being exploited in soil bioremediation processes (Isken and de Bont 1998; Ramos *et al*. 2002; Sardessai and Bhosle 2002; Sikkema, de Bont, and Poolman 1995). Many of these adaptation mechanisms are elicited by different processes conditions such as temperature or pH, and are independent from one another,

however it is the combination of them that provides the most efficient way to cope with the toxicity of the solvents (Kabelitz, Santos, and Heipieper 2003).

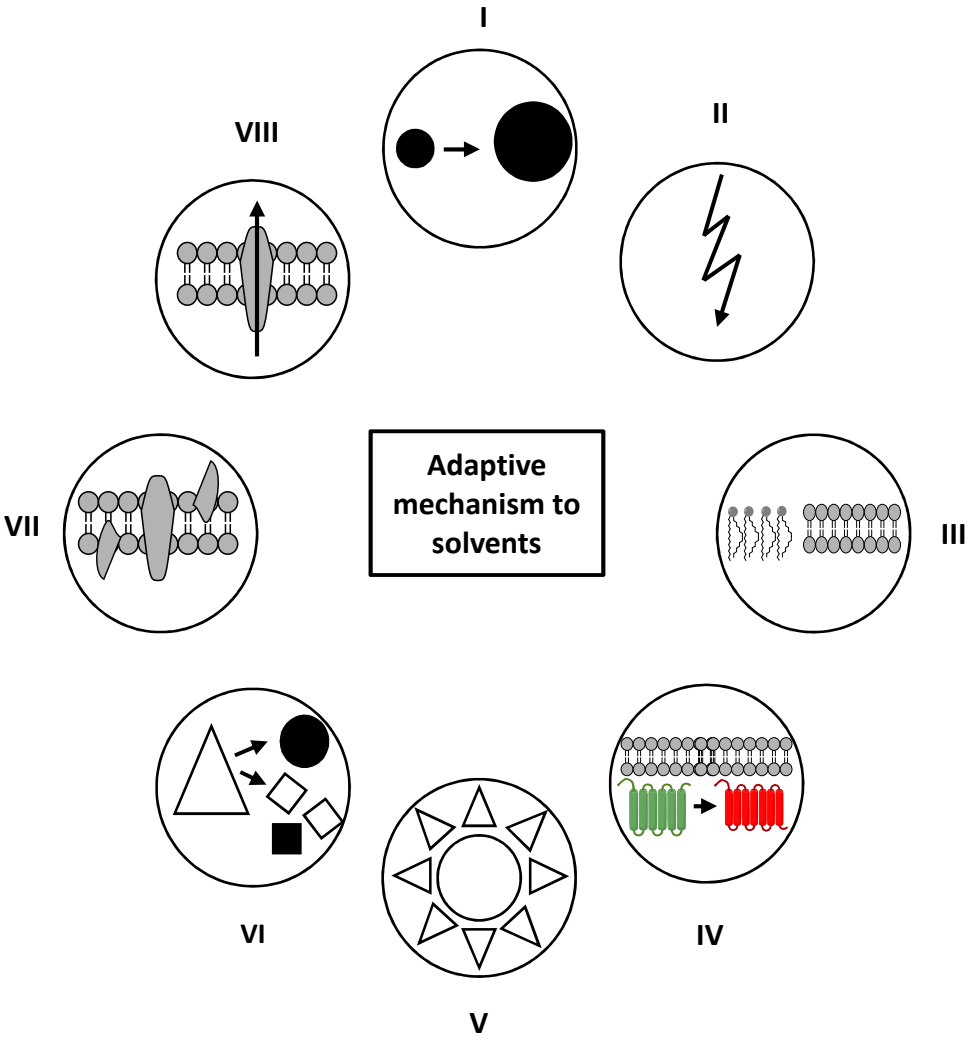


Figure 4: Adaptive mechanisms involved in solvent tolerance.
(i) Morphological adaptation; (ii) changes of the energy status; (iii) modification of the membrane's fluidity; (iv) changes in the cell wall and outer membrane; (v) modifying surface properties (charge and hydrophobicity); (vi) transformation or degradation of the solvent; (vii) modifying membrane proteins; (viii) active export of the solvent. Modified from Isken and de Bont (1998); Sikkema, de Bont, and Poolman (1995); Heipieper *et al*. (2007).

2.2.1 Common metabolites and solvent toxicity

In the context of metabolites produced by microorganisms, either as target molecules or unwanted by-products, common biocatalytic-based substances are organic molecules such as alcohols, aldehydes, carboxylic acids and hydrocarbons. The toxicity of some of these classes of molecules have been extensively studied using model organisms such as *E. coli, S. cerevisiae and P. putida* over the last decades (Bar, 1988; Isken and de Bont, 1998; Ramos *et al*., 2002; Sikkema *et al*., 1995; Weber, Isken, and de Bont, 1994). In particular, organic solvents have been commonly used in biotechnology to enable and facilitate product recovery or substrate delivery in aqueous media. However, many organic solvents have detrimental effects over the cell membrane integrity, and, in some cases, inhibiting completely cell growth.

Different groups have tried to relate the toxicity of different organic solvents to their physico-chemical properties, all related to the polarity of the solvent, such as solubility, dielectric constant, dipole moment and polarizability (León *et al*. 1998). However, the most used and accepted is the correlation between the microbial activity and the logarithm of the partition coefficient (*P*) of the solvent in the octanol-water system (log*P*), demonstrated by Laane *et al*. (1987). They concluded that solvents with log*P* values lower than 2 are relatively polar substances not suitable for biphasic biotransformation systems, and that the cellular activity remains high in apolar solvents with log*P* values above 4. The biological activity varied in solvents with log*P* values between 2 and 4. Additionally, as an extent of the solvent compatibility with the cell, it has been defined the *critical* log*P* of the biocatalyst (logP_{crit}), which indicates the sensitivity of cells to the presence of organic solvents and it must be measured experimentally (Bruce and Daugulis 1991; Yeom and Daugulis 1999). Usually solvents with log*P* values above the logP_{crit} of a cell are biocompatible with the microorganism, therefore the lower the logP_{crit} of a cell, the wider the range of solvents that can be used in the extraction process.

Further work has been developed to investigate the relationship between the toxicity of organic solvents and their log*P* values. Regarding the type of cellular biocatalysts, it has been shown that, in general, the Gram-negative (Gram-) bacteria are more tolerant to solvent toxicity than Gram-positive (Gram+) (Harrop, Hocknull, and Lilly 1989; Inoue and Horikoshi 1991; Vermuë *et al*. 1993). Harrop *et al.* (1989) suggested that the higher tolerance of Gram-negative bacteria is due to the existence of an outer lipid layer, which confers additional protection against hydrophobic compounds. Vermuë *et al.* (1993) found that the transition between toxic and non-toxic solvents towards different Gram-positive and Gram-negative bacteria was in the range of log*P* values from 3 to 5, and in their work with plant cells, Bassetti and Tramper (1994) found that all solvents with log*P* lower than 5 were toxic to the cells. Additionally, Vermuë *et al.* (1993) in their work with homologous series of organic solvents suggested that solvent toxicity is not only determined by their log*P*, but also depends on the molecular structure of the solvent used, as for alkanes, phthalates, and ethers, transition of toxic to non-toxic solvent takes place between log*P* 3 and 4, while for alcohols and alkylbenzenes the transition between toxic and non-toxic solvents takes place at higher log*P* values.

Regarding the organic solvent toxicity mechanisms, even though not fully understood, it is well accepted that the solvents accumulate in the cell membrane causing disruption of membrane functions, breakdown in transport mechanisms, and, at high concentration, lysis of the cell (Vermuë *et al*. 1993; Inoue and Horikoshi 1991; Sikkema, de Bont, and Poolman 1995; Osborne *et al*. 1990; Wilkinson, Woodley, and Ward 1994). Additionally, it has been suggested, that in a biphasic system, toxic effects of the organic solvent towards the microbial cell is due to both phase toxicity and molecular toxicity (Sikkema, de Bont, and Poolman 1995). Phase toxicity is due to the mere presence of a distinct second phase, which may cause the extraction of components from the cell membrane or extract vital nutrients from the aqueous phase, or it may also directly contact the cell in the interface or in an emulsion, limiting the nutrient availability to the biocatalysts. On the other

hand, molecular toxicity is due to the dissolution of solvent molecules in the aqueous phase, and it may cause enzyme inhibition, protein denaturation, and disruption of the lipid membrane.

2.2.2 Microbial tolerance to toxic chemicals in bioprocesses

Toxic solvents induce many different responses by the cell to improve its survival against detrimental chemical compounds. Cellular responses that might impart tolerance to toxic solvents include long term adaptive responses such as change in the cell membrane composition and morphology to counteract the solvent toxicity; induction of detoxification mechanisms to metabolise the toxic chemical, or induction of transporter mechanisms to expel it out *via* molecular pumps; and increase the adenosine triphosphate (ATP) production necessary for repairing processes by induce a higher cellular metabolism (Sardessai and Bhosle 2002; Ramos *et al*. 2002; Isken and de Bont 1998). The following sections briefly described the primary adaptation mechanisms displayed by the cells.

2.2.2.1 *Changes in the cell membrane composition*

Organic solvents accumulate in the cell membrane, disturbing its integrity, displacing proteins and hence inhibiting membrane functions (Sardessai and Bhosle 2002). This accumulation leads to an increase in permeabilisation of the cell membrane, resulting in leaking of macromolecules such as ribonucleic acids (RNA), phospholipids or proteins. As previously described, a clear relationship has been established between values of log*P* and the partitioning of the solvent in membrane-buffer systems (Osborne *et al*. 1990; Sikkema, de Bontt, and Poolman 1994; Heipieper *et al*. 1994), with

solvents having log*P* values between the range 1-5 accumulating in the cell membrane in high concentrations, severely damaging or killing the cell.

Since the solvent's toxic effects target mainly the membrane of the cell, different adaptations at the level of the plasma membrane play a key role in the solvent tolerance of bacteria. The degree of saturation of the fatty acids has been recognised as an important adaptive mechanism against toxic compounds; bacteria respond to effects of solvents with by increasing the saturation degree of the phospholipid membrane by adding saturated long-chain fatty acids (Figure 5-A), neutralising its increased fluidity and maintaining its viscosity and functionality (Ingram 1977). However, since this is an energy-dependent process, only growing cells can perform it. Additionally, upon initial exposure to toxic compounds, cells express genes to increase the ratio of *trans* to *cis* unsaturated fatty acids by isomerizing *cis*-unsaturated fatty acids to the more rigid *trans*-unsaturated fatty acids (Figure 5-C), which reduces the angle formed by their acyl chains at the double bond, hence reducing the steric hindrances between the fatty acid hydrocarbon chains, therefore increasing the membranes' ordering, density and rigidity, and reducing its fluidity. It has been shown that this process does not depend on energy, cell growth or the synthesis of new fatty acids (Heipieper, Diefenbach, and Keweloh 1992). Besides the fatty acid composition, an increase in the amount of membrane proteins and the change of phospholipid headgroups composition have been shown to increase the rigidity and viscosity of the membrane, respectively, counteracting its fluidity (Figure 6). *P. putida* solvent-tolerant strains increase the amount of diphosphatidylglycerol when exposed to toluene (Weber and de Bont, 1996), and *E. coli* mutants with a high resistance to solvents, change the headgroups in a similar fashion (Clark and Beard 1979). Moreover, the dynamic of phospholipid biosynthesis in the membrane also has a key role in tolerance to toxic compounds. It has been shown that the solvent-tolerant bacterium *P. putida* Idaho presented an increase rate of phospholipid synthesis when exposed to xylene (Pinkart and White 1997).

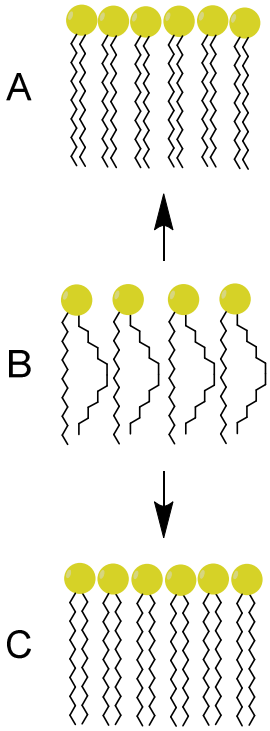


Figure 5: Changes in the conformation of the phospholipids and their effect on the structuring of the lipid bilayer.
(A) Saturated fatty acids; (B) *cis*-unsaturated fatty acids; (C) *trans*-unsaturated fatty acids.

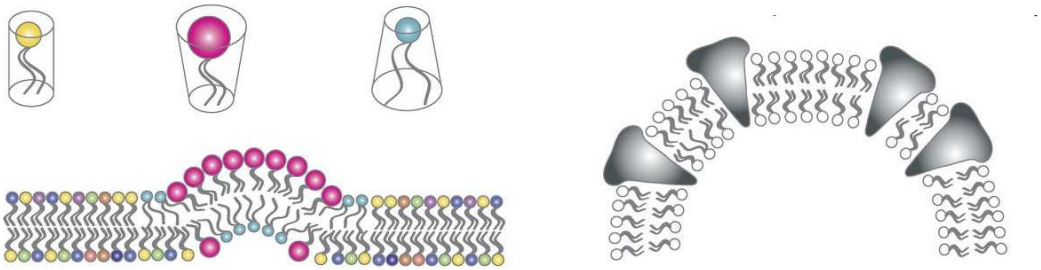


Figure 6: Mechanism of direct membrane protein increase within the lipid bilayer.
(A) Modification of the phospholipids headgroups composition; (B) Insertion of transmembrane proteins into the bilayer in order to increase the amount of membrane proteins. Modified from Jarsch, Daste, and Gallop (2016).

2.2.2.2 *Molecular pumps: active excretion of solvents*

Molecular pumps are unidirectional efflux systems localised in the cytoplasm of the microorganisms and their function is to actively expel toxic chemicals from within the cell to the external medium to protect the cell from their detrimental effects (Webber and Piddock 2003). These pumps comprise the multidrug resistance (MDR) efflux systems, and they may transport many structurally and functionally dissimilar compounds, hence contributing to solvent tolerance. They consist of three components extended across both the inner and outer membrane: a cytoplasmic membrane transporter acting as an energy-dependent extrusion pump, a membrane fusion protein (MFP) situated in the periplasm which generates a channel, and an outer membrane protein (OMP) which facilitates efflux outside the cell (Figure 7) (Ramos *et al*. 2002).

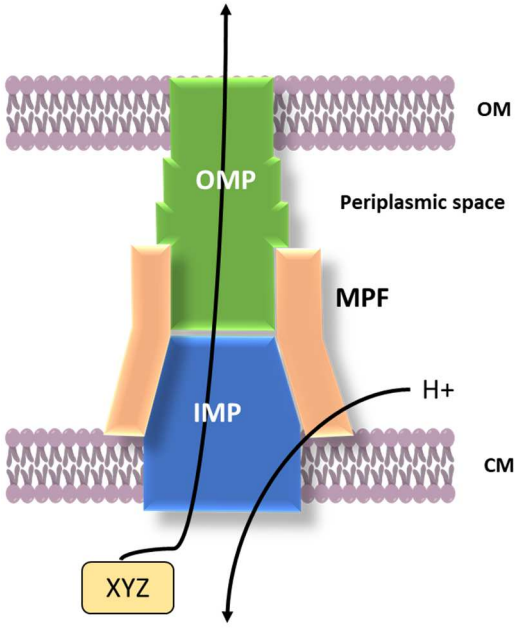


Figure 7: Schematic representation of a tripartite drug efflux complex.
The complex consists of three proteins which span the inner-membrane (CM), the outer membrane (OM), and the periplasmic space. The inner-membrane protein (IMP), *e.g*. AcrB or MexB is responsible for substrate specificity and catalyzes ΔpH dependent drug transport. Examples of the outer membrane protein (OMP) are TolC or OprM. The periplasmic membrane fusion protein (MFP), *e.g*. AcrA or MexA connects the IMP and the OMP. Modified from Nicolaou, Gaida, and Papoutsakis (2010).

Five different families of efflux transporters have been identified across the prokaryotic organisms: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette) (Lomovskaya *et al*. 2001; Webber and Piddock 2003; Higgins 2007). All these efflux systems utilize energy (ATP hydrolysis or proton motive force (H^+/Na^+)) to transport chemicals against an external concentration gradient in antiport mechanisms.

In Gram-negative organisms, the efflux pumps associated with solvent tolerance belong to the RND family, with the AcrAB-TolC system present in *E. coli* (Ramos *et al*. 2002). The AcrAB-TolC efflux pumps consists of AcrB as inner membrane transporter acting as a periplasmic pump, TolC being the outer membrane protein, both linked together by the membrane fusion protein, AcrA. The expression of the different genes that regulate the AcrAB-TolC system have been reported in a number of solvent tolerant *E. coli* strains (Asako *et al*. 1997; Oethinger *et al*. 1998). The transcriptional regulators *marR, marA, soxR and soxS* have been shown to modulate the expression of *acrAB* and other related genes. In this regard, Aono (1998) observed a higher tolerance to cyclohexene when *marA, robA* and *soxS* were overexpressed, resulting in an increased production of AcrA and TolC and, therefore, and increase activity of the efflux pump. Supporting the relationship between the acrAB locus and the resistance to toxic solvents, White *et al*. (1997) observed a lower tolerance to n-hexane and cyclohexene when acrAB was deleted.

Other examples of molecular pumps are the AcrEF-TolC efflux pump of *E. coli*; the Pdr12 transporter in *S. cerevisiae*; the TtgABC and TtgDEF in *P. putida*; and the MexAB-OprM multidrug efflux system of *P. aeruginosa* (Ramos *et al*. 2002; Nicolaou, Gaida, and Papoutsakis 2010). *P. pseudomonas* S12 strain has shown to be resistant to a wide variety of highly toxic and structurally unrelated hydrophobic solvents, including aromatic and aliphatic solvents and alcohols; and, interestingly, it has been shown to be able to be grown in the presence of supersaturating concentrations of styrene and toluene (Weber *et al*. 1993; Kieboom *et al*. 1998). Kieboom and co-workers identified that the SprABC efflux

system confers the solvent-resistant characteristics of *P. putida* S12, which is regulated by the *srpR* and *srpS* genes, by cloning these genes responsible for the solvent efflux pump into *P. putida* strains that have lost their solvent-tolerant phenotype, resulting in the acquisition of solvent resistance. These results suggest the possibility to construct more resistant microorganisms capable of performing biocatalytic production of highly toxic end-products such as styrene.

2.2.2.3 *Heat-shock proteins (HSPs) and other stress proteins*

Heat-shock proteins, also known as molecular chaperones, play an important role in the synthesis, transport, folding and degradation of proteins, and they are known to be expressed under various stress conditions such as heat shock, hyperosmotic shock, or toxic solvents or chemicals (Aono 1998). HSPs interact with other stress proteins in order to confer protection from and tolerance to chemicals and other stresses. Stress conditions, such as heat and harmful substances, denature proteins, causing them to unfold. Once proteins lose their original conformation, they are no longer able to function properly. When the denature proteins are detected by the cell, HSPs are produced and act as molecular chaperones helping proteins to fold back to their original conformation. Thus, HSPs main function is to prevent protein aggregation and assist in refolding of damaged proteins which are crucial for cell metabolism and survival using energy from ATP hydrolysis (Tomas, Welker, and Papoutsakis 2003) (Figure 8). Additionally, since some of these chaperones are inactive at elevated temperatures or oxidative stress due to the inhibition of ATP synthesis, stress proteins whose functions are ATP independent are expressed to minimise the protein aggregation.

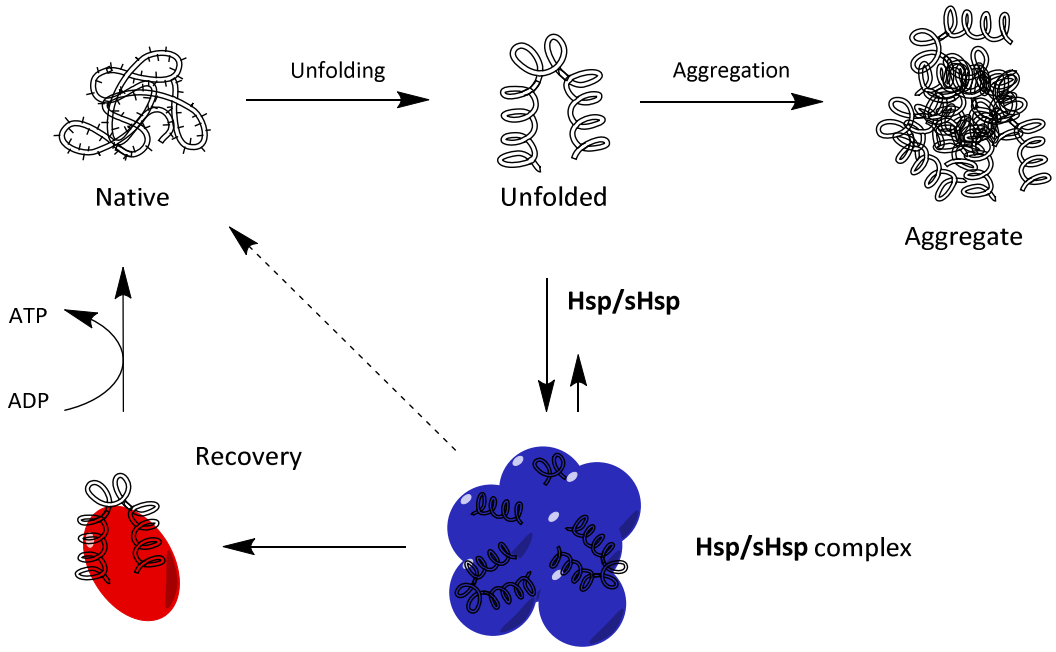


Figure 8: Action mechanism of heat-shock proteins.
HSP, heat-shock protein; sHSP, small heat-shock protein. Modified from Eyles and Gierasch (2010).

Most microorganisms engage specialized proteins, known as sigma factors, for the regulation of the expression of the HSP genes and other stress proteins that help to diminish cell damage due to solvent stress. The two major regulators of the general stress response in Gram- and Gram+ organisms are sigma S and sigma B, respectively (Nicolaou, Gaida, and Papoutsakis 2010). The sigma factor S, encoded by *rpoS*, affects the transcription of more than 10% of the genes in *E. coli* and it extensively overlaps with other major regulons, such as the cyclic adenosine 3'-5'-monophosphate (cAMP) regulon expressing proteins for intracellular transduction; activates proteins kinases; and also binds to and controls the function of ion channels (Weber *et al*. 2005). Additionally, RpoS interacts with the transcription factor Lrp (Leucine-responsive regulatory protein), thus affecting the cell physiology not only under stress conditions. In *S. cerevisiae*, the stress response is regulated by the *hsf1* heat-shock factor, involving other chaperones in solvent tolerance (Ding, Huang, and Zhang 2009). Additionally, under ethanol stress and other various types of stresses like heat

shock, weak organic acids, cold and oxidative stress, accumulation of Hsp70, Hsp30 and Hsp12 in *S. cerevisiae* have been observed, suggesting that protein repair is a necessary requirement for tolerance, however its mechanism of tolerance is still largely not understood (Ding, Huang, and Zhang 2009; Quan *et al*. 2004; Seymour and Piper 1999; Pacheco *et al*. 2009).

Additionally, *E. coli* has been shown to express the phage-shock protein A (PspA) when exposed to hydrophobic organic solvents (Kobayashi, Yamamoto, and Aono 1998). PspA is a peripheral inner membrane protein known to be induced under ethanol exposure and extreme stress conditions such as heat shock, hyperosmotic shock, protein secretion inhibition, inhibition of fatty acid or ATP synthesis, and prolonged growth in the stationary phase (Aono 1998). It is hypothesised that PspA is involved in solvent tolerance since it plays a major role in maintaining the proton membrane potential under stress conditions, therefore extending the survival of the cells; however the exact mechanism of action is still under study (Darwin 2005).

Finally, it is believed that synthesis and/or uptake of trehalose, proline and glycerol (Figure 9), which are usually accumulated by the cells for protection against osmotic stress, also act as enhancers of chemical tolerance (Kempf and Bremer 1998). There is an increasing number of reports suggesting that the accumulation of these solutes is involved in the protection against solvents. In this respect, it has been established that in *E. coli* trehalose synthesis is also controlled by the sigma factor S, and its accumulation is also a response to heat, salt, starvation and oxidative stress (Strom and Kaasen 1993). In a similar fashion, accumulation of trehalose and L-proline in yeast has been shown to confer protection to cells under ethanol stress (Ding, Huang, and Zhang 2009), and glycerol accumulation has been related to hexane tolerance in *E. coli* (Hayashi *et al*. 2003).

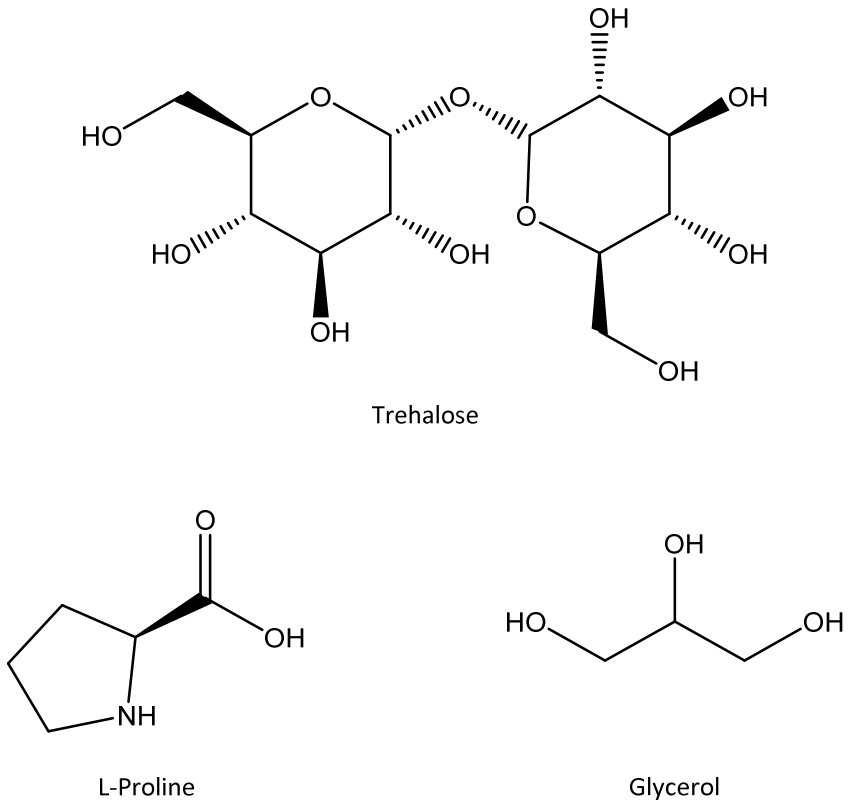


Figure 9: The chemical structure of thehalose, L-proline and glycerol.

2.3 Bio-based production of commodity chemicals: Styrene as a particular case study of a toxic platform chemical in biocatalytic processes

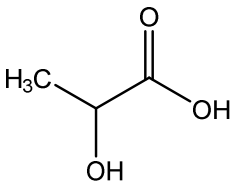
As mentioned earlier in this review, there is an increasing interest to produce commodity chemicals using renewable feedstocks rather than fossil resources across the chemical industry, with this interest mostly driven by the depletion of fossil organic carbon stocks, environmental pollution and climate change through the effects of GHGs. Living systems provide a unique solution for the natural production of a wide range of useful products starting from inexpensive and renewable carbon feedstocks, such as fuels, pharmaceuticals and polymers, however, usually their low production yield makes this process economically unfeasible for industrial production.

Advances in biotechnology and the development of metabolic engineering tools have enable both the efficient design of high yield biocatalytic processes

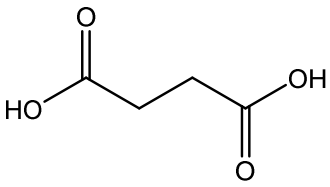
that already exist in nature and the construction of novel pathways using enzymes from different organisms. Hence, through the use of the recombinant DNA technology along with systems and synthetic metabolic engineering tools, scientists have successfully built complete synthetic metabolic pathways in microbial hosts being able to produce important biofuels and fine chemicals.

To the present day, biofuels have been the most prominent application of metabolic engineering, with the main focus on bio-based production of ethanol and butanol (Becker and Wittmann 2015; Jang *et al*. 2012). While the biofuels sector has been attracting the major interest during the last several decades, recent interest among the chemical companies to use metabolic engineering to produce specialty chemicals has developed, with many reported examples of biological production of platform chemicals by metabolically engineered microorganisms, such as organic acids, alcohols, amino acids, and aromatics (Figure 10).

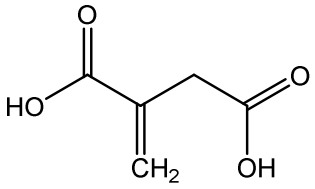
Organic Acids



D,L-Lactic acid

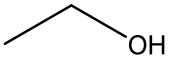


Succinic acid

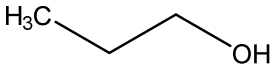


Itaconic acid

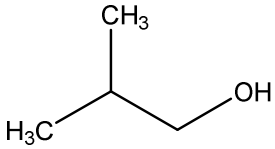
Alcohols



Ethanol

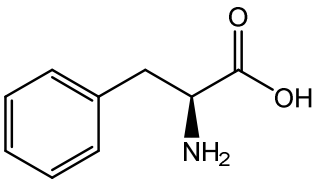


1-Propanol

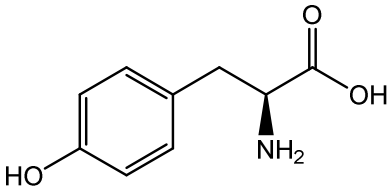


Isobutanol

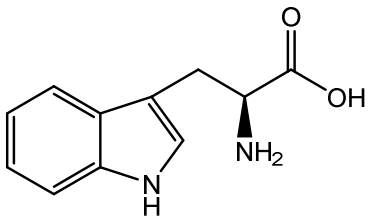
Amino acids



L-Phenylalanine

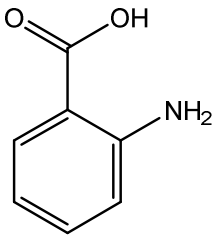


L-Tyrosine

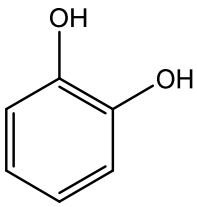


L-Tryptophan

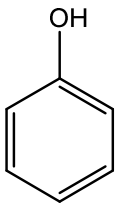
Aromatics



Anthranilic acid



Catechol



Phenol

Figure 10: Selected set of the versatile bio-based product spectrum obtainable using principles of systems metabolic engineering.

In this respect, aromatic compounds represent an important class of platform chemicals with a wide array of applications ranging from use as solvents to monomers for polymer synthesis. To present date, many monoaromatics have been successfully synthesised by microorganisms; for example, anthranilic acid (2-aminobenzoic acid), catechol (1,2-dihydroxybenzene), and phenol (Figure 10) are a group of monoaromatic hydrocarbons derived from petroleum-based feedstocks that have been used worldwide as starting materials for the production of plastics, detergents and pesticides (Jang *et al*. 2012). Engineered *E. coli* strains were used for the production of anthranilic acid (Balderas-Hernández *et al*. 2009), and catechol (Draths and Frost 1995; Li, Xie, and Frost 2005), and the bioconversion of glucose to phenol was achieved using an engineered solvent-tolerant *P. putida* S12 strain (Wierckx *et al*. 2005). However, often the high toxicity of aromatics frequently limits the yields of their microbial synthesis. In this context, this work will focus on the biosynthesis of the monoaromatic styrene, and the efforts to increase the production and to alleviate its toxicity by solvent extraction.

Styrene (Figure 11) is one of the most important monomers produced by the chemical industry today. This cyclic hydrocarbon is widely used for the production of various polymers and co-polymers, most notably polystyrene, styrene-acrylonitrile, acrylonitrile-butadiene-styrene, and styrene butadiene rubber, in addition to produce protective coatings and resins, and as a chemical intermediate (U.S. Department of Energy 2012). In this regard, styrene is an essential precursor to the fine chemicals *(S)*-styrene oxide and *(R)*-1,2-phenylethanediol (Figure 11), which are chemical-building blocks for the production of higher-value chemicals. For example, *(S)*-styrene oxide is used as a precursor of the biocide levamisole and for the production of phenethyl alcohol used in perfumes (Figure 11); as well as a chemical intermediate for cosmetics, surface coatings, and agricultural and biological chemicals (Park *et al*. 2006; Panke *et al*. 2000; U.S. Environmental Protection Agency 2000), and *(R)*-1,2-phenylethanediol is used to synthesise the pharmaceuticals *(R)*-norfluoxetine and *(R)*-fluoxetine (Figure 11), used in psychiatric treatments,

and the β-lactam antibiotics, in addition to a wide range of agrochemicals (Cao *et al*. 2006; Kumar, Upadhyay, and Pandey 2004; Gamenara and Domínguez de María 2009).

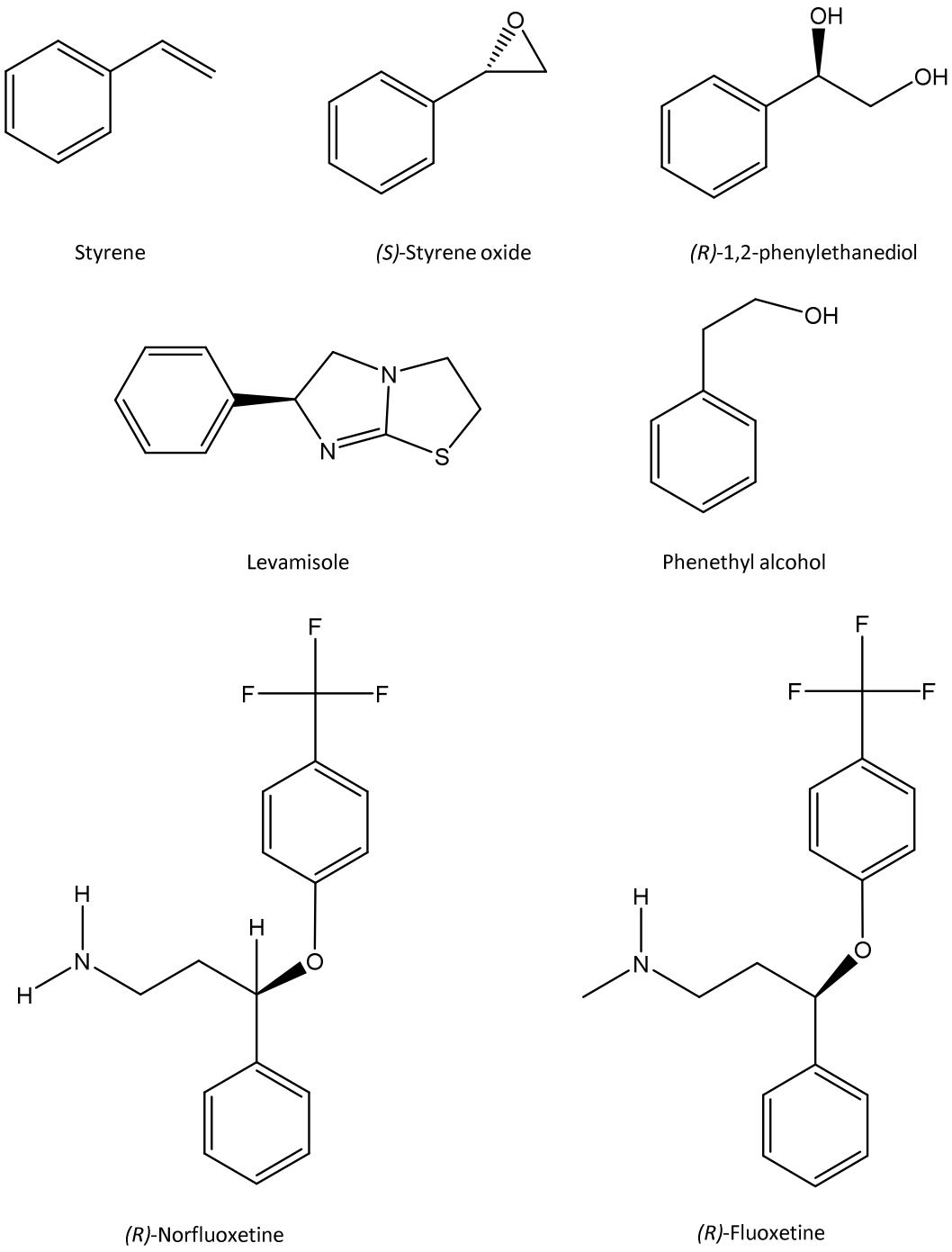


Figure 11: The chemical structure of styrene, *(S)*-styrene oxide, *(R)*-1,2-phenylethanediol, levamisole, phenethyl alcohol, *(R)*-norfluoxetine and *(R)*-fluoxetine.

2.3.1 Current commercial production of styrene

Styrene was discovered by Bonastre in 1827 whilst working with alcohol extracts of Storax balsam, but it was only first isolated in 1831 by distillation of balsam (Tossavainen 1978). Although experimental methods for its synthesis and polymerisation were well-known by the mid-19th century, first commercial successful production of styrene only began in 1925 by I.G. Farbenindustrie in Germany. Ever since, styrene has been recognised as one of the most important monomers for the production of numerous polymers and co-polymers, with an exponential growth of its production over the past century, positioning the industrial process of the catalytic dehydrogenation of ethylbenzene to styrene as one of the 10 most important organic catalytic processes (Ba *et al*. 2015). In 2012 styrene global production exceeded 26.4 million tonnes and analysts forecast the global styrene market to grow at 4.8% over the period 2014-2019 (Merchant Research and Consulting 2013; Research and Markets 2015).

Styrene is mainly produced by the catalytic dehydrogenation of high-purity ethylbenzene in the vapour phase (Figure 12), which accounts for 90% of the commercial styrene production (Ba *et al*. 2015). Due to the endothermic character and equilibrium limitations of this process, high temperatures are needed (*i.e*. 600 - 700 °C), hence the introduction of superheated steam into the feed is indispensable as the thermodynamic driving force of the process and to shift the chemical equilibrium for a higher styrene conversion (Zhao *et al*. 2015). However, the high amount of steam needed for its production (about 3 tonnes of steam per tonne of styrene produced) renders styrene as one the most energy-intensive processes among the commodity chemicals industry, requiring over 6.3x10^9 J/tonne of styrene produced (Mimura *et al*. 1998). Superheated steam is combined with ethylbenzene in the presence of a catalyst to remove hydrogen from the ethylbenzene. Most catalysts are based on iron (III) oxide, promoted by several percent of potassium oxide or potassium carbonate, which enhances the removal of the coke that forms on the iron

oxide catalyst due to the water gas shift reaction. Regarding the role of the steam, besides serving as the heat source for the endothermic reaction, it also dilutes the reagents and products in order to shift the position of the chemical equilibrium towards the products. The output of this process is sent to a distilling tower where additional steam facilitates the separation of styrene from the remaining ethylbenzene

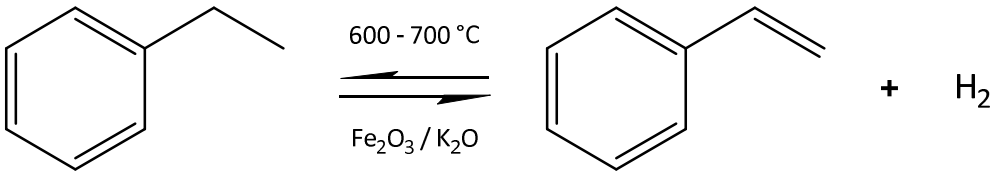


Figure 12: Production of styrene through the chemocatalytic dehydrogenation of ethylbenzene.

The alternative route responsible for a smaller commercial production of styrene also uses ethylbenzene as a starting material, however this process involves a series of multi-step oxidation/reduction reactions (Figure 13). In the first oxidation step (130 °C, 0.2 MPa), ethylbenzene is converted to ethylbenzene hydroperoxide. Then, in the presence of a metal catalyst (110 °C, 4MPa), ethylbenzene hydroperoxide oxidise propylene to propylene oxide and, conversely, ethylbenzene hydroperoxide is reduced to α-methylbenzyl alcohol. Finally, the α-methylbenzyl alcohol is dehydrated to styrene in the presence of a metal oxide catalyst at 250 °C and low pressure (James and Castor 1994).

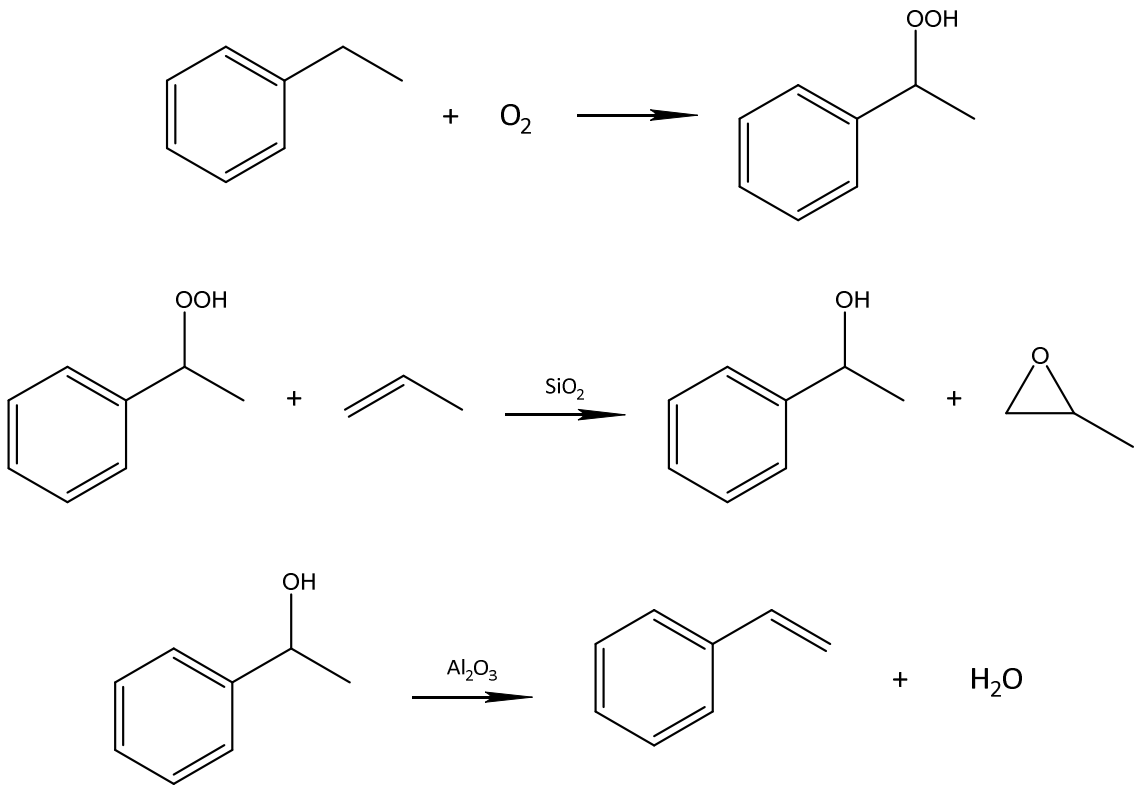


Figure 13: Alternative production of styrene using ethylbenzene as starting material through a series of multi-step oxidation/reduction reactions.

Thus, in the face of the exorbitant energy requirement of these processes and since all commercially-available styrene is based on the world's dwindling petroleum feedstocks, greener and more sustainable production methods based on renewable resources are highly desirable to secure the future needs of consumers.

2.3.2 Towards the bioproduction of styrene

2.3.2.1 *Biosynthesis of the aromatic amino acids precursors*

Plants and many bacterial species can naturally produce aromatic compounds through a common pathway for synthesis of aromatic amino acids, known as the shikimate pathway (Gosset 2009) (Figure 14). In *E. coli*, glucose is

firstly converted through a central metabolic pathway into phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), which are the main intermediates and precursors of several aromatic amino acids. These compounds are then condensed into 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) by the DAHP synthase enzymes. This is the first committed step and the most tightly regulated reaction of the common aromatic amino acid pathway, and controls the carbon flux into the shikimate pathway. Then, through seven steps, DAHP is converted into chorismate, which is a branch point for the production of an array of aromatic end-products, including L-phenylalanine (L-Phe), tyrosine (Tyr), and tryptophan (Trp).

Among the constraints limiting the capacity of *E. coli* to produce high levels of aromatic compounds are the highly controlled regulation of genes and enzymes of the shikimate and the different branches pathways (Pittard and Yang 2008). *Escherichia coli* has three 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isozymes, AroF, AroG, and AroH, that catalyse the first reaction of the shikimate pathway, and are sensitive to feedback inhibition by tyrosine, L-phenylalanine and tryptophan, respectively (Smith *et al*. 1962; Zurawski *et al*. 1981). In addition, these DAHP synthases, encoded by each three paralog genes *aroF*, *aroG* and *aroH* are subject to transcriptional regulation (Wallace and Pittard 1969; Davies and Davidson 1982).

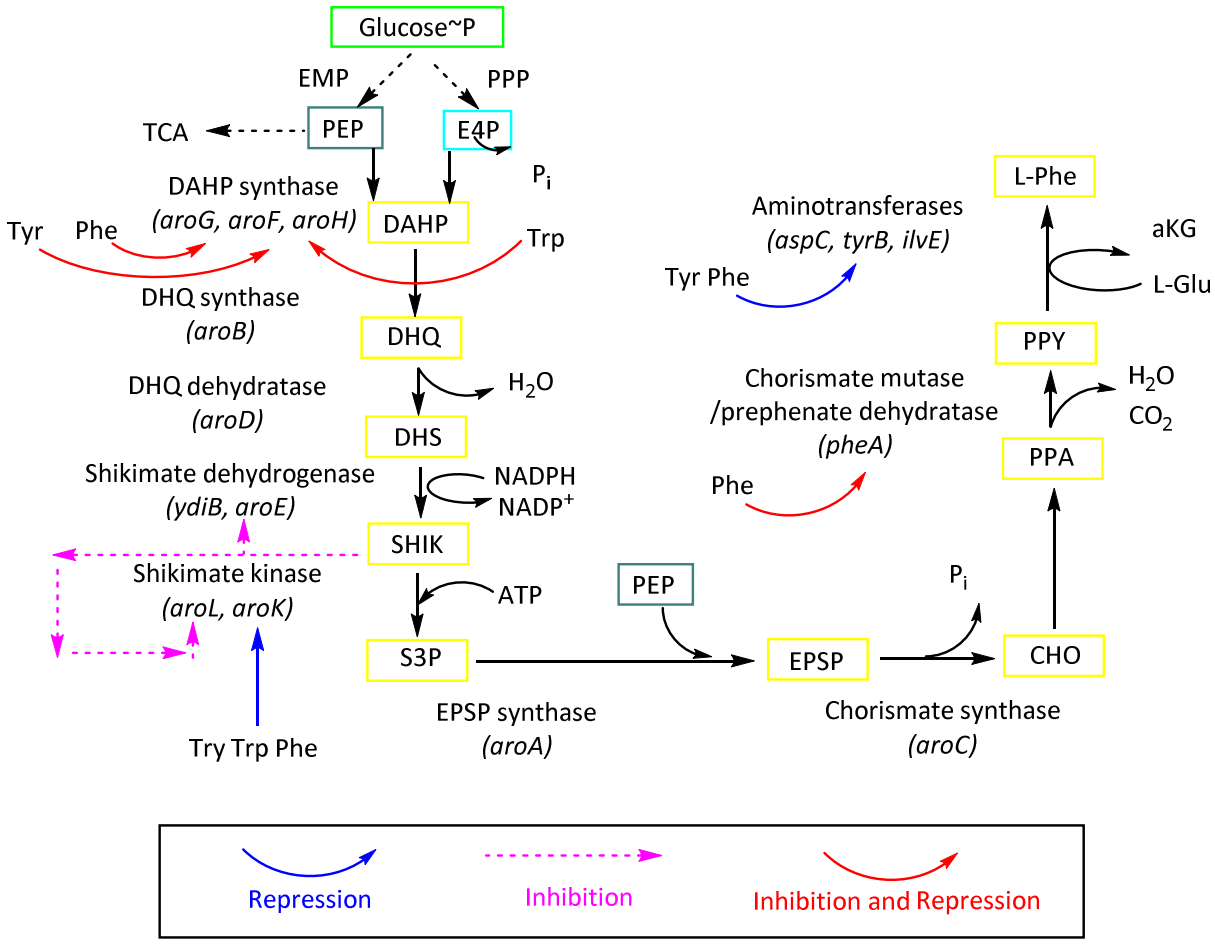


Figure 14: Biosynthetic pathways, regulation, and transport systems of L-phenylalanine biosynthesis in wild type *E. coli.*

Metabolites abbreviations: PEP, phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ, 5-dehydro-quinate; DHS, 5-dehydroshikimate; SHIK, shikimate; S3P, shikimate 5-phosphate; ESPS, 3-enolpyruvylshimate-5-phosphate; CHO, chorismate; PPA, prephenate; PPY, phenylpyruvate; Phe, L-phenylalanine; L-Glu, glutamate; aKG, α-ketoglutarate; Tyr, L-tyrosine; Trp, L-tryptophan.

Firstly, regarding the transcriptional control, both *aroF* and *aroG* are repressed by the polypeptide product of *tyrR*, and *aroH* control is accomplished by the *trpR* protein product, and in each case the respective end-product act as co-repressor forming a complex with their DNA-binding transcriptional regulator, which then binds to the operator sequence of the genes, blocking the RNA polymerase from attaching to the transcriptional promoter region, and thereby inhibiting transcription from occurring. Secondly, as stated earlier, allosteric inhibition of the committed reaction of DAHP synthases occurs as a result of the binding of the end-products with their respective feedback-sensitive DAHP

synthase, which generate a conformational change of the enzyme and alter its ability to bind the substrate.

In addition to regulation at the expression level and allosteric inhibition of the DAHP synthase activity, the flow of substrates along the aromatic pathway is tightly controlled at the level of the metabolite, chorismate, a crucial branch point for the three aromatic amino acids. At this point, the three major enzymes utilizing chorismate, *i.e*. chorismate mutase-prephenate dehydrogenase, chorismate mutase-prephenate dehydratase, and anthranilate synthase, are subject to feedback inhibition by their end-products, namely tyrosine, phenylalanine and tryptophan, respectively (Pittard and Yang 2008). In the case of phenylalanine, of particular interest to this project since it is the necessary endogenous precursor to the production of styrene; the expression of the gene for the enzyme chorismate mutase-prephenate dehydratase (*i.e*. *pheA*) is subject to transcriptional control by a system of attenuation (Gowrishankar and Pittard 1982).

2.3.2.2 *Natural production of styrene and biosynthesis of similar aromatic building blocks*

Besides its petrochemical origins, styrene occurs naturally at low levels in several plant species, and it has been found as a trace metabolite in a variety of foods such as fruits, vegetables, nuts, beverages and meats (Yu 2012). For example, styrene it is known to be present in small amount in benzoin resins, which are naturally synthesised by several species of trees in the genus *Styrax,* belonging to the Styracaceae family (Fernandez *et al*. 2005). Here, even though styrene is the major component of the essential oils, it only accumulates as a minor constituent with less than 0.55% of the total dry weight of the benzoin resins. Additionally, Pagot *et al*. (2007) demonstrated the ability of the *Penicillium camemberti* yeast to catabolise phenylalanine into styrene, although the conversion rate was very low. Furthermore, neither a defined

metabolic route nor the genes associated with said pathway have thus far been identified. Even though styrene can be obtained by distillation of the *Styrax* tree resins, the very low productivity and yields accomplished by this mechanism, and the low inherent value of styrene makes its potential, large-scale biological production by such a mechanism completely unsustainable and economically unfeasible. A more sustainable and inexpensive production method would comprise the development of engineered microorganisms with unique metabolic routes for the synthesis of styrene starting from renewable resources.

To date, the production of a variety of other useful monoaromatic compounds structurally similar to styrene has been achieved through the development of novel biosynthetic pathways which have been engineered in different microorganisms. For example, the conversion of glucose into phenol (Figure 10), one of the most important hydroxylated aromatic compounds, mostly used for the production of bisphenol A and phenolic resins, was achieved by engineering a solvent-tolerant *Pseudonomas putida* S12 (Wierckx *et al*. 2005). In this work, a tyrosine overproducing strain of *P. putida* S12 was modified by introduction of the *tlp* gene from *Pantoea agglomerans*, encoding the enzyme tyrosine phenol lyase, to enable phenol production. However, maximal concentrations were restricted due to end-product inhibition.

Production of *p*-hydroxybenzoic acid (Figure 15), used as a monomer for the production of liquid crystalline polymers and a precursor to form antimicrobial parabens, has been accomplished by the homologous overexpression in *E. coli* of the feedback-insensitive DAHP synthase (encoded by *aroF*), and the *ubiC*-encoded chorismate lyase (Barker and Frost 2001). Nonetheless, modest yields and titres of *p*-hydroxybenzoic acid were synthesised mainly due the toxicity of the acid which negatively impacted *E. coli* metabolism. In a similar fashion, a *Pseudomonas putida* S12 was engineered to produce *p*-hydroxybenzoic acid from renewable carbon sources, either glucose or glycerol, *via* the central metabolite L-tyrosine (Verhoef *et al*. 2007). In this study, a tyrosine overproducing strain of *P. putida* S12 was used as a platform host, and

phenylalanine ammonia lyase (PAL) from the yeast *Rhodosporium toruloides* was introduced for the conversion of L-tyrosine into *p*-coumarate, which is further converted to *p*-hydroxybenzoic acid by endogenous enzymes *p*-coumaroyl-CoA synthase (Fcs), *p*-coumaroyl-CoA hydratase/lyase (Ech) and *p*-hydroxybenzaldehyde dehydrogenase (Vdh). However, when compared to the production system *via* chorismate in *E. coli* the product yield in *P. putida* was slightly lower, which also showed an end-product inhibition.

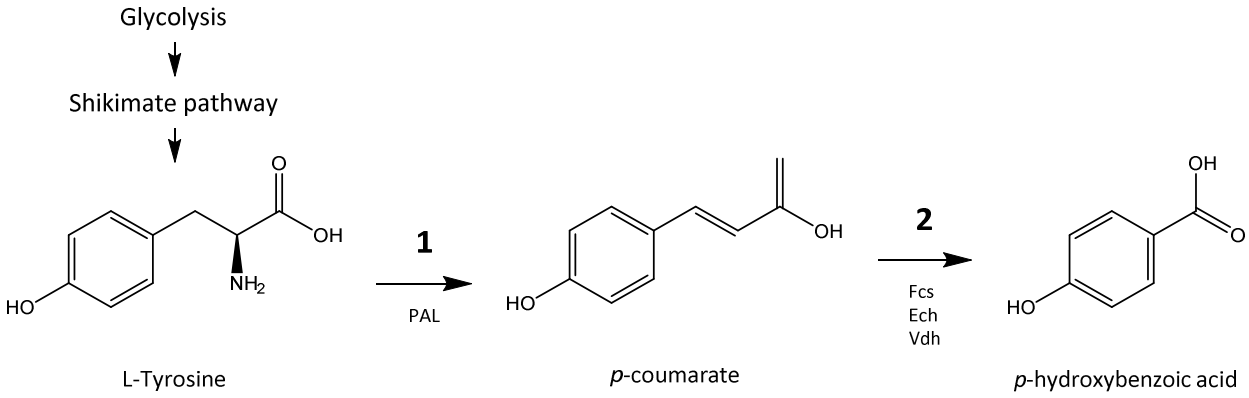
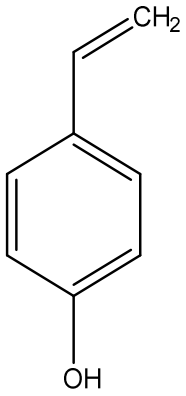


Figure 15: Enzymatic pathway for *p*-hydroxybenzoic acid production from endogenous L-tyrosine in *P. putida*.

Step 1 requires the enzyme phenylalanine ammonia lyase; step 2 requires enzymes *p*-coumaroyl-CoA synthase (Fcs), *p*-coumaroyl-CoA hydratase/lyase (Ech) and *p*-hydroxybenzaldehyde dehydrogenase (Vdh) (Verhoef *et al*. 2007).

The biosynthetic production of *p*-hydroxystyrene (pHS) (Figure 16), a monomer used for producing polymers applied in photoresist materials, resins, inks, elastomers and coatings, has been constructed by simultaneously expressing the bifunctional enzyme phenylalanine/tyrosine ammonia-lyase (PAL/TAL) from *Rhodotorula glutinis* and a *p*-hydroxycinnamic acid decarboxylase (PDC) from *Lactobacillus plantarum* in an aromatic overproducing strain of *E. coli* (Ben-Bassat *et al*. 2003; Qi *et al*. 2007). A similar approach was used to introduce the enzymes PAL/TAL from *Rhodosporidium toruloides* and PDC from *Lactobacillus plantarum* in a solvent-tolerant strain of *Pseudomonas putida* with an optimised flux to the central metabolite L-tyrosine (Verhoef *et al*.

2009). However, in both cases the maximum concentrations of *p*-hydroxystyrene obtained were limited due to the high toxicity of the product to the biocatalytic host.



p-hydroxystyrene

Figure 16: The chemical structure of *p*-hydroxystyrene.

An interesting approach to overcome the *p*-hydroxystyrene toxicity was achieved by decoupling the production of the toxic material from the fermentation process (Sariaslani 2007). In this method, the first step corresponded to the fermentation of glucose to tyrosine by an *E. coli* tyrosine-overproducing strain, followed by the deamination reaction of L-tyrosine to *p*-hydroxycinnamic acid (pHCA) using immobilized *E. coli* cells expressing PAL/TAL enzymes from *Rhodotorula glutinis*. The final step is a high-yielding, base-catalyzed thermal decarboxylation of pHCA into pHS. Potassium acetate was used as the catalyst and N,N-dimethylacetamide as the process solvent (Sariaslani 2007). Although high pHS yields up to 98.6% were obtained using commercially available pHCA in this chemical decarboxylation process, when bioderived pHCA was tested for its applicability to this process, impurities from the bioprocess were found to be detrimental to the decarboxylation performance. Additionally, even though splitting the process helps to overcome the product toxicity constraints, more sequential unit operations are

needed, increasing the cost of process when compared to an integrated biotransformation process.

In recent years, a styrene biosynthetic pathway has been designed by metabolically engineering an *E. coli* strain so that it is able to produce styrene from renewable substrates such as glucose (McKenna and Nielsen 2011). The proposed biosynthesis of styrene utilised endogenously produced L-phenylalanine, which is deaminated to *trans*-cinnamic acid (tCA) by means of expression of a suitable phenylalanine ammonia lyase (PAL). The following step involves the decarboxylation of tCA by a phenylacrylate decarboxylase (PADC) to produce styrene as the final product (Figure 17).

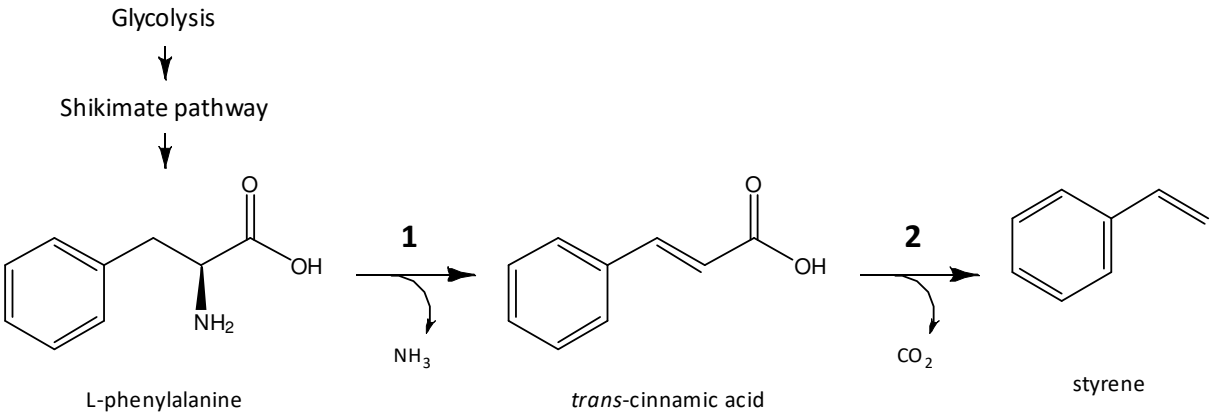


Figure 17: Enzymatic pathway for styrene production from endogenous L-phenylalanine in *E. coli.*

Step 1 requires the enzyme phenylalanine ammonia lyase; step 2 requires a *trans*-cinnamic acid decarboxylase (McKenna and Nielsen 2011).

First of all, as stated earlier, since phenylalanine is the necessary precursor for the production of styrene, high flux and elevated titres of this aromatic amino acid through the shikimic acid pathway are desired, for which it is necessary to alleviate the tight feedback control over the key enzymes in *E. coli*. Therefore, the feedback resistant strain *E. coli* NST74, previously engineered to overproduce phenylalanine (Tribe 1987), was selected as the host platform for

the biosynthetic production of styrene. This strain was obtained by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis with the following isolation of mutants by growth in minimal media containing either the tyrosine anti-metabolite 3-fluorotyrosine or the phenylalanine anti-metabolite β-thienylalanine. In the presence of these anti-metabolites, natural production of the respective aromatic amino acids ceases due to the feedback control imposed on the shikimate pathway enzymes; therefore, in order for the organism to survive in the presences of the anti-metabolites, it must evolve to de-regulate the feedback sensitive enzymes. The evolved strain was developed from the multiple combination of mutations with the following relevant genotype: *aroF394(fbr), aroG103(fbr), tyrR366, pheA101(fbr),* and *pheO352*. These varied mutations removed both inhibition of the enzyme DAHP synthase AroF and AroG by tyrosine and L-phenylalanine, respectively, as well as the inhibition of the bifunctional enzyme chorismate mutase P-prephenate dehydratase by L-phenylalanine. Furthermore, mutations on the DNA-binding transcriptional regulator TyrR and the phenylalanine operator, pheO, resulted in alleviated repression. Later, various candidate isozymes from yeast, plants and bacteria, were screened for the following two enzymatic steps. Finally, PAL2 (phenylalanine ammonia lyase) from *Arabidopsis thaliana* and FDC1 (ferulic acid decarboxylase) from *Saccharomyces cerevisiae* were selected and co-expressed in the L-phenylalanine overproducing strain *E. coli* NST74 to convert endogenously synthesised L-phenylalanine to styrene. However, in a similar way to the previous production of monoaromatic compounds, the styrene concentrations achieved were limited by the styrene toxicity threshold towards *E. coli*.

Product toxicity remains as the major obstacle in order to obtain higher concentrations of styrene in this biosynthetic process. Due to its hydrophobic nature, it is believed styrene accumulates within the cytoplasmic membrane of the cell resulting in swelling of the membrane bilayer and an increase in membrane fluidity, affecting the integrity of the membrane. In this respect, an important tool to cope with toxic products is the development of metabolically

engineered microorganisms highly tolerant to specific chemicals in order to increase the productivity of specialized bioprocessing applications. Current work in our research group involves the development of molecular efflux pumps in *E. coli* strains to increase the tolerance of the microorganism to styrene and, consequently, enhance the overall production of this cyclic hydrocarbon. While finding a more solvent-tolerant host and improving its ability to synthesise styrene remains an important goal, another way to deal with the inhibition of the cells due to toxic products is to design process strategies that may minimise the direct contact of the cells with the toxic compounds, hence protecting the cells from its toxicity. Therefore, an alternative approach to overcome product toxicity by using an *in situ* product removal strategy will be developed in this project.

2.4 Whole cell biocatalysis and *in situ* product extraction

As stated earlier, a common reason for whole cell biotransformation processes to have limited productivity yields is the presence in the reaction media of a desired product that has inhibitory (reversible loss of catalytic activity) or toxic (irreversible loss of catalytic activity) effects toward the microorganisms (Verhoef *et al*. 2009; Wood *et al*. 2011). The toxicity of the target product towards the microorganisms may be an obstacle that needs to be overcome, as it may reduce the productivity and make the process economically unviable. Even though pathway engineered biocatalysts have undoubtedly helped to expand the range of renewable aromatic products, toxicity is still a common challenge for the microbial biosynthesis of most aromatic compounds in order to achieve economically-viable levels of production.

In this regard, *in situ* recovery methods appear to have a great potential in enhancing the productivity of many bioprocesses. *In situ* product removal is a method that involves the selective removal of the product from the reaction

medium by using an appropriate and biocompatible separation mechanism whilst the fermentation is still in progress; hence a low concentration of the toxic product is in contact with the cells in the aqueous phase (Lye and Woodley 1999; Daugulis 2001; Malinowski 2001). This being its main characteristic, ISPR may increase the productivity or yield of the process not only by overcoming the inhibitory or toxic effects of the product, but also by shifting unfavourable reaction equilibria, minimizing product losses due to degradation or uncontrolled release, and, at industrial scale, reducing the total number of down-stream process steps. Examples of successful approaches for the *in situ* recovery of inhibitory biomolecules include, adsorption, absorption, gas stripping, membrane pervaporation, and liquid-liquid extraction (van den Berg *et al.* 2008).

Solid adsorbents (Figure 18), due to their solid nature, are usually mechanically stable and do not dissolve in the fermentation medium, therefore they are generally biocompatible and non-bioavailable. Solid extractants range from natural materials such as zeolites and activated charcoal to synthetic macroporous such as gel-type resins (Zou *et al*. 2013; Hu, Xu, and Nie 2010; Liu *et al*. 2011; Hua *et al*. 2010). Synthetic resins can be designed to have specific chemical properties such as polarity and acid/base characteristics incorporated on the surface of the porous materials in order to enhance the interaction with the target molecule. Additionally, their relatively simple implementation has led to an increasing number of reports of recent extraction experimental researches. However, the extractant selection has usually been made by screening commercially available materials with trial-and-error methods to compare their capacity and selectivity, with no attempt to predict their effectiveness *via* first principle considerations. Furthermore, the performance of these adsorbents is usually decreased due to the presence of other solutes in the media competing for the available sorption sites, and, the formation of biofilm and fouling by cells on the surface area of the macroporous resins (Mirata, Heerd, and Schrader 2009). Moreover, heating or concentrated salt

solutions are needed to regenerate the extractant and recover the adsorbed molecule, adding energy-intensive steps and waste streams to the process.

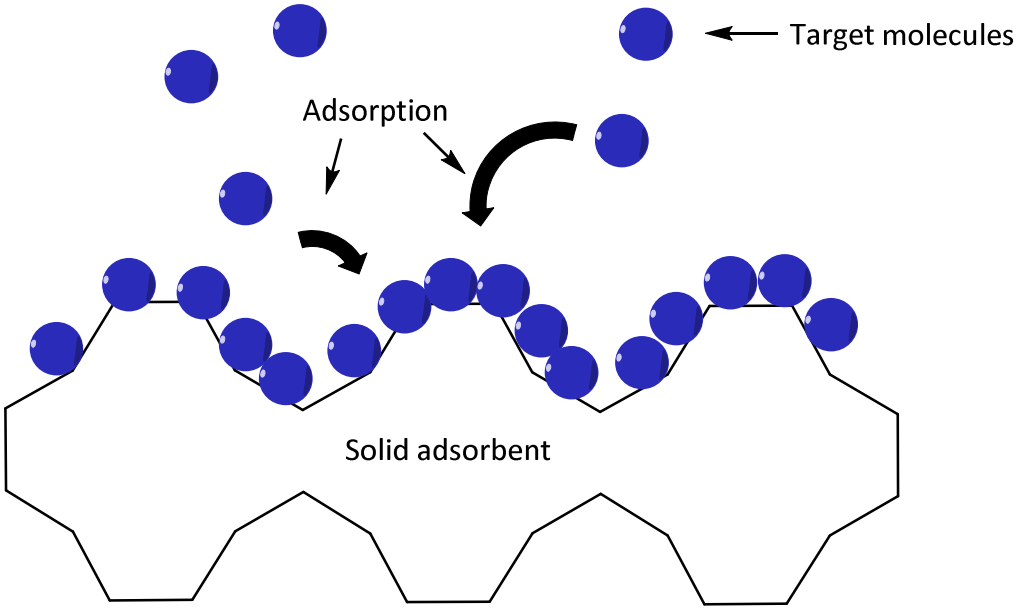


Figure 18: Adsorption mechanism of solid adsorbents
Target molecules adhere to the surface of the solid phase.

Soft-amorphous polymers have arisen as a new class of solid absorbents material denominated "solid solvents" since they extract the target molecule by passive solution and diffusion within the polymer structure itself (Dafoe and Daugulis 2014); differentiating from the classical adsorption mechanism by allowing internal permeation and swelling of the polymer, hence avoiding the competition for site occupation (Figure 19). Additionally, since the entire amount of polymer is used in the extraction process, the overall uptake of the target material is determined by the mass of the polymer, rather than the available surface area (Craig and Daugulis 2013). Moreover, the recovery of the absorbed molecules can be easily achieved by contacting the polymer with a solvent with a greater affinity for the target molecule than water; however, this involves the use of volatile organic solvents. In addition, due to the early stage in the development of this area of research, selection of such polymers has been made *via* trial-and-error (Jain, Khan, and Daugulis 2010; Khan and

Daugulis 2010; Gao and Daugulis 2009), and there is a lack of information of the complex structure-extractant properties relationship of the polymer.

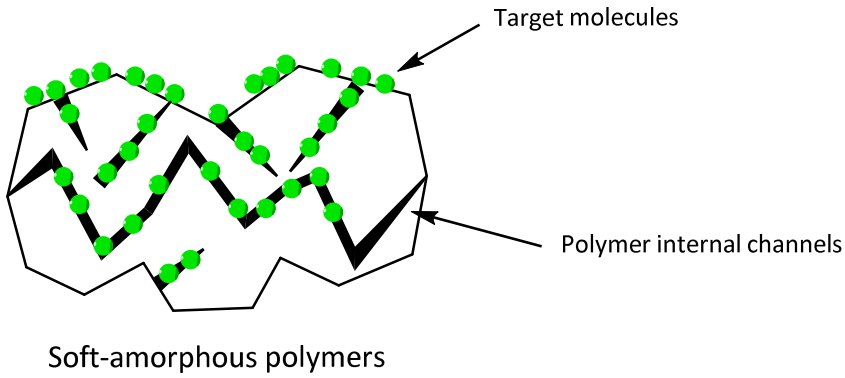


Figure 19: Absorption mechanism of soft-amorphous polymers.
Target molecules are drawn into the internal channels of the polymer.

Gas stripping has been implemented for the *in situ* recovery of organic compounds such as acetone, butanol, ethanol and isopropanol (Figure 20) (Ezeji, Qureshi, and Blaschek, 2004; Ezeji, Qureshi, and Blaschek, 2003; Inokuma, Liao *et al.* 2010; Löser *et al.* 2005; Xue *et al*., 2014); and volatile aromatic compounds such as benzene (Nielsen, Daugulis, and McLellan 2007a; Nielsen, Daugulis, and McLellan 2007b), and styrene (McKenna *et al*. 2014); effectively improving final product concentrations and avoiding end-product inhibition. Even though gas stripping has been shown to be a very effective first extraction step, some drawbacks were pointed out such as the presence of free cells reduced the effectiveness of the gas stripping during the fermentation of butanol, suggesting that the immobilisation of cells may be necessary for the product recovery, adding costs to the process; and, in the case of volatile compounds such as styrene, the continuous air stripping diluted extremely the target product concentration of the bioreactor exhaust gas, generating problems firstly with the analytical sensitivity in the laboratory and, at an industrial scale, increasing the energy requirements for capturing the product vapours, hence reducing the efficiency of the process.

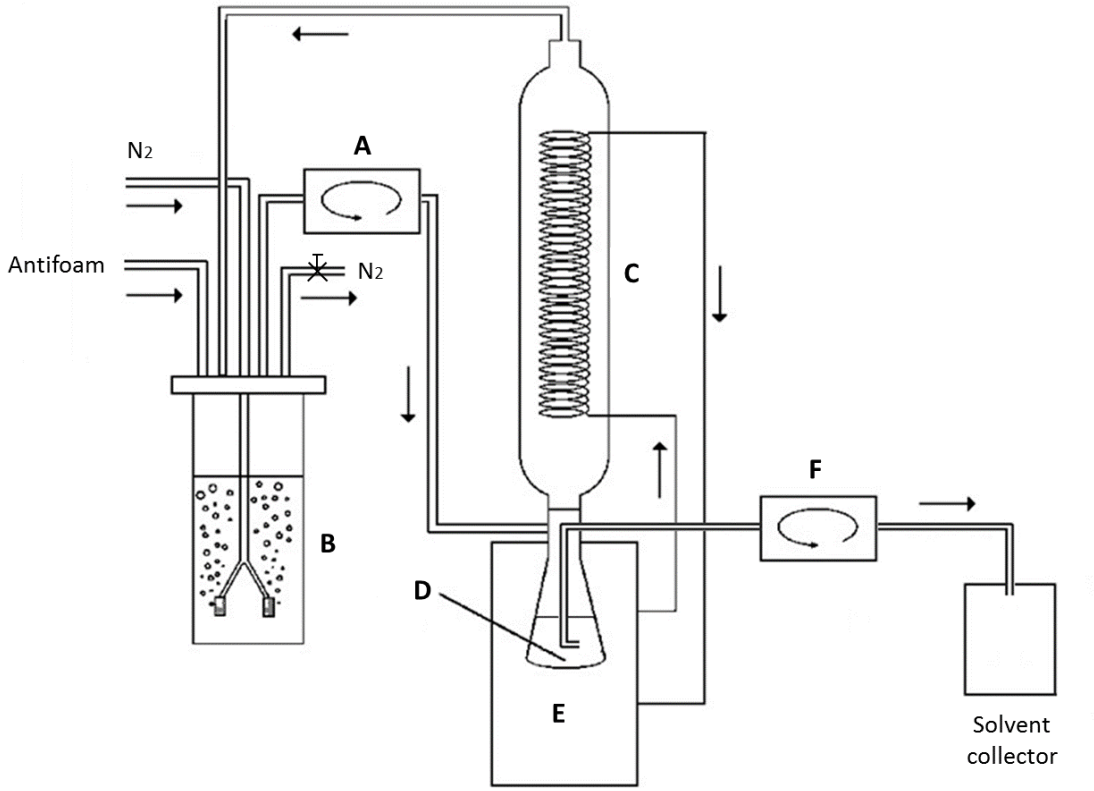


Figure 20: Diagram showing butanol production and in situ recovery by gas stripping.
(A) Gas recycle pump; (B) bioreactor; (C) condenser; (D) condensed ABE vapours; (E) cooling system; (F) condensed solvent removal pump. Modified from T. Ezeji, Qureshi, and Blaschek (2003).

Pervaporation is a process in which a liquid mixture to be separated, containing two or more miscible components, is placed in contact with one side of a selective semi-permeable membrane, and the permeated product (permeate) is removed at the downstream side (or permeate side) of the membrane as a low-pressure vapour, with the permeate side generally kept under vacuum conditions. Following this stage, the desired product can be collected by condensing the permeated vapour (Figure 21) (Feng and Huang 1997; Shao and Huang 2007; Vane 2005). Membrane pervaporation has been implemented in the product recovery of ethanol from fermentation broths for enhanced bioconversion (Ikegami *et al*. 1997; O'Brien and Craig 1996), achieving high

concentrations in the permeate stream, and effectively circumventing product inhibition in the broth. In a coupled fermentation/pervaporation system the fermentation broth is circulated across the feed side of the membrane at a constant flow rate by a peristaltic pump. A vacuum pump maintained a vacuum on the downstream (permeate) side. Primary and total condensers collected the ethanol-rich permeate (Figure 22). The main advantages presented by pervaporation are its compactness, simplicity and versatility for its implementation. Additionally, due to the higher concentrations obtained of the target compound in the downstream side, it is presumed that the energy cost would be lower in the final distillation process. However, the permeated fluxes were generally low, and, since mass transfer is accompanied by heat transfer in pervaporation, its efficiency is influenced by temperature, with a reduction in temperature lowering the permeate flux; therefore, attempts to avoid a temperature drop near the membrane surface would be necessary, which inevitably would increase the energy requirements of the process. Moreover, a decrease in the membrane performance and selectivity were caused by deposition of cells on the membrane, making necessary periodic washing of the membrane if the process was ever to be scaled up. Finally, most of the commercially available membranes are highly selectively for water and not intrinsically selective for organic compounds, therefore, currently there is no a well-established criterion for selecting a membrane for a specific process, and the materials for pervaporation membranes are selected empirically (Feng and Huang 1997).

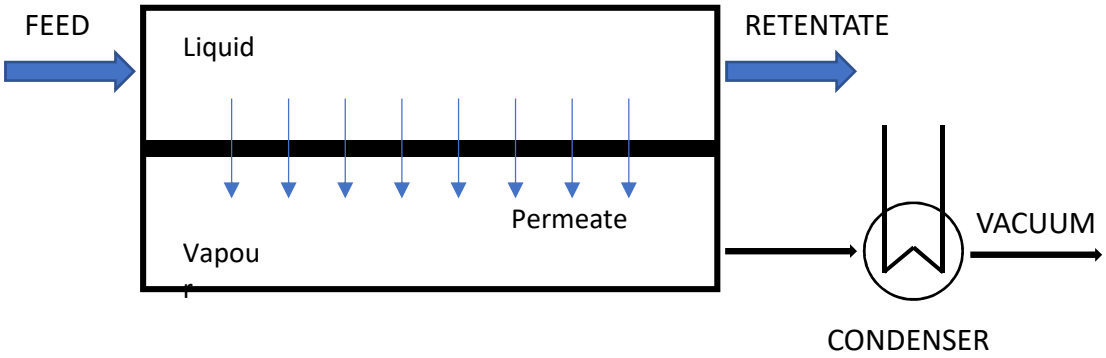


Figure 21: Schematic diagram of the vacuum pervaporation process.

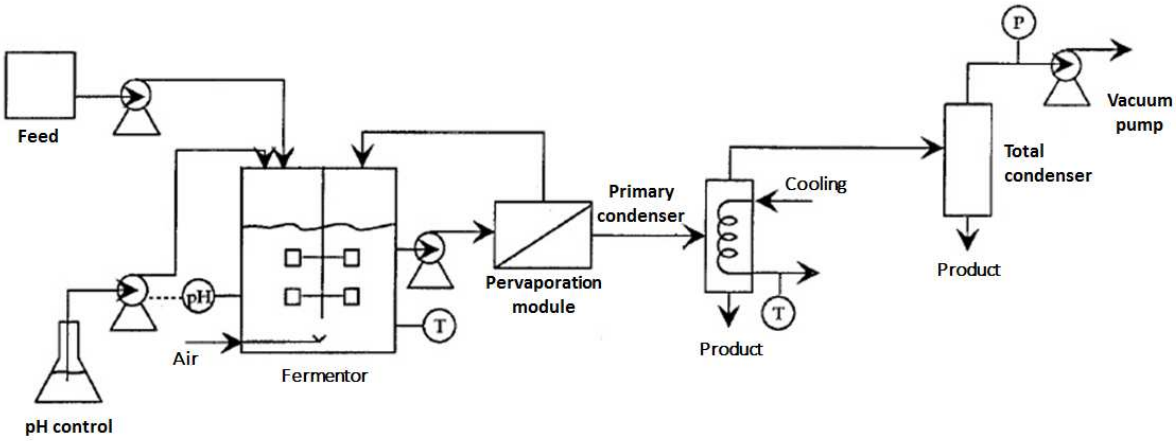


Figure 22: Simplified scheme of a coupled fermentation/pervaporation system.

An attractive and simple technique to remove toxic products from a fermentation system appears to be the design of a biphasic system with an immiscible layer into which the toxic product would be extracted by means of liquid-liquid extraction (Van Hecke *et al.* 2014; Malinowski 2001; Gyamerah and Glover 1996; Weilnhammer and Blass 1994; Collins *et al.* 1995; Straathof 2003; Lilly 1982; van Sonsbeek *et al.* 1993; Cull *et al.* 2000). In particular, this technique has shown very interesting results in increasing the productivity of fermentation of aromatic organic compounds such as phenol (Wierckx *et al*. 2005), *p*-hydroxystyrene (Verhoef *et al*. 2009) and styrene (McKenna *et al*. 2014).

Two liquid-phase biocatalytic reactions have several advantages, such as presenting a high substrate/product ratio in the reactor, reducing product inhibition and facilitating the biocatalyst separation from the product (Dafoe and Daugulis 2014; Weilnhammer and Blass 1994). Additionally, when applied in an industrial-scale process, the economic advantages of the integration of fermentation and a primary product recovery step are mainly due to lower costs in the product recovery and effluent treatment steps as a result of using of a more concentrated feedstock and a reduction in the amount of waste water produced. Nevertheless, even though *in situ* extraction can increase the production of the target product, it presents some limitations (Crabbe, Tse, and Munro 1986). The presence of cells, cell debris, proteins, lipids, and other active components produce emulsions in the culture that reduce the rate of mass transfer in the extraction process in biological systems due to the blockage of the interphase area available for mass transfer. Mass transfer efficiency can lower to one fifth when formation of stable foam by the cells occurs on the phase boundaries.

Regarding the solvent selection for the reaction systems, due to the direct contact between the biocatalyst and the extractant solvent in the broth, the selection of the solvent is restricted largely by its biocompatibility towards the microorganisms (Lilly 1982; Freeman, Woodley, and Lilly 1993; Van Hecke, Kaur, and De Wever 2014; Bechtold and Panke 2009). Further key considerations for the selection of the organic solvent are high capacity (in mass terms) for the target substance in order to reduce the amount of extractant required; high selectivity for the target molecule, low solubility in the broth; and, low tendency to emulsify in the broth. Furthermore, in an industrial context, it is desirable for the separation process to be a generic technique that can be applied to a variety of productions systems with no significant changes (Lye and Woodley 1999; Schmid *et al*. 1998). Additionally, it is essential to consider the toxic and flammable nature of the solvent concerning the safety of the operators and the environment, with its non-flammable qualities of special concern in bioprocesses where air or oxygen has to be supplied to the

reactor. Therefore, the solvent should be non-hazardous in nature and be environmentally friendly, since it also must comply with the rigorous Generally Recognized as Safe (GRAS) and Registration, Evaluation, Authorisation and Restriction of Chemicals (REACh) regulations, from the United States and the European Union, respectively.

Within the area of biotransformation, despite many disadvantages, conventional organic solvents have been commonly used to address toxicity problems in a range of multiphase bioprocess operations. However, they themselves have been shown to be toxic to the microbial cells, causing membrane damage and reducing the biocatalyst's long term operational viability (Osborne *et al*. 1990; Vermuë *et al*. 1993; Wilkinson, Woodley, and Ward 1994). Furthermore, the main concerns for the industrial biotransformation processes are their toxicity towards the process operators and the environment, and the explosion hazard that this kind of solvent presents due to their volatile and flammable nature. Therefore, better alternative solvents are needed to be discovered to overcome the present issues related to conventional organic solvents.

2.5 Ionic liquids

2.5.1 Chemical structure and general physicochemical properties

Room temperature ionic liquids are a class of non-molecular solvents that have arisen as potential alternative solvents for volatile organic compounds (VOCs). They are, by definition, salts with melting temperatures below 100 °C. These organic salts are usually composed of a poorly coordinating, bulky organic cation, and an equally large and weakly coordinating anion; some common ones of which are shown in Figure 23. These asymmetrical and large steric bulky features, combined with the large charge delocalisation across the anion, help to reduce the lattice energies of ionic

liquids, preventing the ions from efficiently forming strong crystal structures and ensuring ILs remain in liquid state at or close to room temperature (Kurnia *et al*. 2014; Wasserscheid and Welton 2008).

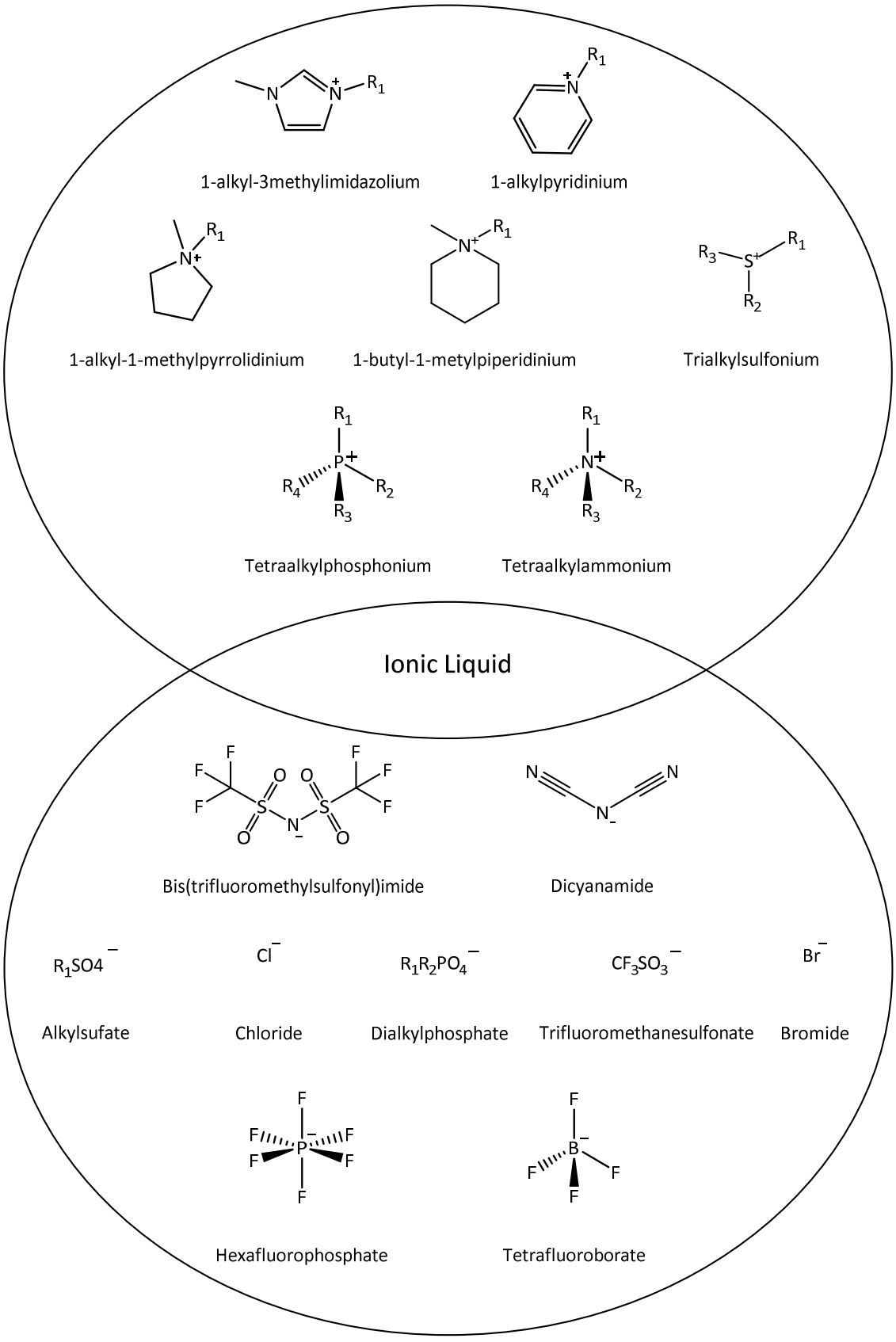


Figure 23. Chemical structures of a selection of common cation and anions used in biotechnology.

Ionic liquids present a number of different attractive physicochemical properties, including wide liquid range, electrical conductivity, high thermal and chemical stability, high solvating capacity and non-coordinating character (Chiappe and Pieraccini 2005). Additionally, they follow a series of Green Chemistry principles which represent a guiding framework for the sustainable design of new chemical products and processes, taking into consideration all aspects of the life-cycle and fate of chemicals throughout the process (Anastas and Eghbali 2010). These principles address the efficiency and safety of chemical transformations, the toxicity and biodegradability of products and reagents used, and the cost-benefits through reduction in the different streams of the whole process. ILs are attractive for their potential use in sustainable chemical processes, and have been considered as appropriate alternatives for a different range of applications in different fields, including chemical industry, energy, pharmaceuticals, food, cellulose processing, waste recycling, chemical and polymer synthesis, among many other (Weuster-Botz 2007).

Among these measures, is the employment of cleaner and safer solvents to replace volatile organic solvents, wherever possible. In this regard, due to their ionic nature, ILs present different levels of electrostatic interaction between the cations and anions, behaving in a different way to conventional organic solvents. The cation and anion mainly interact by strong coulombic interactions that are responsible for the negligible vapour pressures exhibited by ILs, even at high temperatures (Meindersma *et al*. 2007; Mutelet *et al*. 2006). Owing to this property, in addition to their high thermal and chemical stability, ionic liquids cannot evaporate from the reactor vessel and are retained quantitatively, while traditional solvents vaporise, making them suitable and potentially less hazardous substitutes for the highly flammable VOCs (Wasserscheid and Welton 2008; Paulechka *et al*. 2003). The low vapour pressure of ILs give the benefits of eliminating the possibility of air pollution through evaporation of the solvent, and confer higher protection and safer working environment conditions towards the process operators by working with less-toxic and less-flammable solvents, and in the case of volatile products,

it would facilitate solvent and product recovery using techniques as simple as flash distillation or stripping enabling the reuse and recycling of the ILs. However, the simple replacement of VOCs solvents with ILs does not necessarily make a process 'green'. Their low vapour pressure feature also introduces potential challenges to the recovery of non-volatile products and solvent purification due to the accumulation of non-volatile by-products. Compounds such as aldehydes and ketones are remarkably and persistently soluble in ILs, and some dipolar organic compounds are extremely difficult to remove by either extraction or distillation (Holbrey and Rogers 2002), adding the need to search for new separation approaches.

The unique combination of properties of ILs has allowed to accomplish novel chemistry, create new separation and extraction processes, and minimise waste streams. Possibly their most exceptional characteristic is their high manipulability with more than 10^{18} potential useful combinations of ionic liquids (Docherty and Kulpa, Jr. 2005), making it possible to design an IL with a specific set of properties; this special feature has led ILs beings described as "designer solvents" (Freemantle 1998). The possibility to tune their physical and chemical properties with respect to polarity, hydrophobicity, and miscibility by altering functional groups or through selection of the cation and anion, allow ionic liquids to match the specific relevant requirements of a particular application, making possible, for example, to intensify reactions and separations in process technologies (Branco, Crespo, and Afonso 2002; van Rantwijk, Madeira, and Sheldon 2003). Therefore, the possibility of ILs to be tailored to have unique physicochemical properties have triggered considerable interest in their use in various industrial processes, such as organic synthesis, biphasic catalysis, liquid-liquid extraction and biocatalytic extraction (Cantone, Hanefeld, and Basso 2007; Olivier-Bourbigou, Magna, and Morvan 2010; Wasserscheid and Welton 2008; Poole and Poole 2010).

On the other hand, the designer nature of ionic liquids also generates a set of problems which are not encountered when dealing with conventional solvents. The finite number of conventional solvents available, and with the majority of

their properties and applications widely reported in the literature, reduces the pool of potential solvents for a particular process that can be quickly narrowed down to a handful of possibly suitable candidates which can then be considered and tested for suitability. In the case of ILs, the possibility to combine any cation with any anion to produce an entirely unique ionic liquid means that the number of potential solvents is vast, thus making the task of selecting a single solvent which will give optimal results for a given application far more challenging, time consuming and costly than it would be with conventional organic solvents. Additionally, each selected combination should have to be subjected of comprehensive – and expensive – toxicity studies in order to comply with the GRAS and REACh regulations.

Regarding synthesis of ionic liquids, it is usually carried out in two steps: first the formation of the desire cation *via* protonation with free acid, or by quaternisation of an amine, phosphine or sulfide, generally using haloalkanes or dialkylsulfates; followed by an anion exchange reaction to form a specific desired product (Stark and Seddon 2007). With the increasing interest in the use of ionic liquids in different applications and their wider commercial availability, their current synthesis processes have proven unsustainable and not economically viable for a large-scale production in most cases, with, also, their 'green' characteristics being strongly scrutinised; thus, concerns about whether the traditional synthesis routes for ionic liquids are 'green processes' have arisen along with the escalation of their use. In an attempt to give a first insight into how well ionic liquids really perform in the context of the Twelve Principles of Green Chemistry, Kralisch *et al.* (2005) performed an assessment of the energetic, environmental and economic impact of ionic liquids synthesis in the context of a life-cycle assessment, combining computational and experimental methods, taking into account the supply of raw materials, recycling and disposal of chemicals, and the energy and cooling water required for the synthesis process. It was showed that the selection of the cation, anion and reaction solvents have a large impact in the overall energy needed, the toxicity and the economics of the synthesis process, and in some cases such as

for the [BMIM][BF_4] synthesis, almost 50% of the total energy was consumed for the preparation of the cation precursor 1-methylimidazole. Additionally, the use of anion sources such as silver or lithium salts for the synthesis of ILs (Wilkes and Zarawotko 1992; Disley 2016), are noticeably expensive for the economically efficient production of ionic liquids; and even though many of the starting materials are commercially available, some synthetic precursors for the production of task-specific ILs need to be synthesised from first principles, thus adding costs to their production. Furthermore, many ILs synthesis methods that include the metathesis step require a substantial amount of organic solvents (Stark and Seddon 2007; Kralisch *et al.* 2005), with the formation of additional halide salts as by-products with no further use in any other known process at present, turning out to be nothing but waste that need to be discarded. Additionally, the necessity to remove unwanted by-products and other impurities to meet the standards required for each different application increases the number of purification steps needed, with the consequent of both product loss into the washing phase and increase in the manufacturing cost. Therefore, the need to improve the current methods for the sustainable synthesis of ionic liquids is vital for the future production on a large-scale, along with the investigation of the ionic liquids' toxicity and biodegradation, with the latter issues discussed comprehensively later in this chapter. A first a step towards increasing the sustainability of ILs would be the use of cheaper cation and anion options, when technically possible. For instances, ILs based in tetraalkyl ammonium and phosphonium cations should be consider since the production of the tertiary amines and phosphines is much lower in cost (Fraser and MacFarlane 2009); moreover, successful ionic liquid synthesis with alternative cheaper anions has been accomplished by using anions based on the non-nutritive sweeteners saccharinate and acesulfamate paired with a range of cations including a known non-toxic cation, butyryl choline, (Carter *et al.* 2004). Another approach would be the use of biorenewable materials for their synthesis; Handy and co-workers (2003) reported the synthesis of imidazolium ionic liquids derived from fructose, and Fukaya *et al.* (2007) reported the production of ionic liquids using only biomaterials, in particular a

choline cation combined with propionate, tiglate, hydrogen succinate and hydrogen maleate, offering reduced prices in addition to lower toxicity. Further areas for improvement concern the use of elevated temperatures or pressures, use of halogenated reagents, and hazardous reagents. Clearly there are many different factors that need to be addressed in order to improve the efficient synthesis of ionic liquids if large-scale production is meant to be possible; therefore, it is crucial that there is continued effort in the development of improved and more sustainable methodologies for the production of ionic liquids.

ILs represent a class of solvents that may allow both efficient, controlled chemical reactions and selective extractions of products; with their 'tuneable' feature representing a major opportunity to produce optimised solvents for processes and synthesis. However, development of Green processes needs more than only an efficient reaction; clean and efficient methods to recover products or reagents, and easy regeneration of the solvent systems are also fundamental requirements. Additionally, thorough toxicity studies, examination of their chemical and physical properties, and full life-cycle analysis are required in order to demonstrate the credibility of IL-based processes as viable Green technology. Lastly, ILs are not intrinsically 'Green' according to the limited data available, however, they do represent a greener alternative to existing solvents in many applications, offering great opportunity to be used in Green processes and provide novel process solutions.

2.5.2 Application of ILs in biocatalytic processes

The potential application of ILs as solvents and/or catalysts in synthetic chemistry and biocatalytic processes have been widely explored since the synthesis of both less viscous and water sensitive ionic liquids allowed a broader application of these solvents to be studied (Wilkes and Zarawotko

1992; Wilkes *et al*. 1982). In addition to their exceptional physicochemical properties, such as wide liquid range, negligible vapour pressure and the possibility to tailor-make ionic liquids, the increased interest as potential replacements for the VOCs was also enhanced by the immense and clear negative impact produced by the traditional organic solvents and other man-made chemicals on natural ecosystems. This public concern made industry, academia and governments aware that more environmentally friendly alternatives to conventional chemicals were needed and pushed them towards the development of new and more efficient industrial processes, involving the use of less hazardous chemicals, the reduction of waste production and unwanted by-products, and the reduction of the energy demands of the catalytic processes. Additionally, as mentioned earlier, chemical substances and industrial processes began to be under stricter governmental and international regulations, such as the GRAS and REACh guidelines.

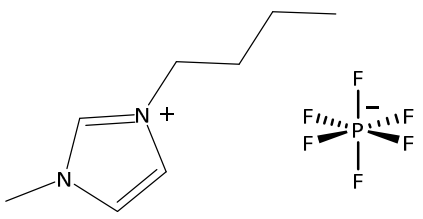
However, ILs are not the only new generation of solvents that have been studied as an alternative secondary phase in biocatalytic processes. Supercritical fluids (SCFs), fluorinated solvents and liquid/soft amorphous polymers have also been screened, although, these alternatives present certain limitations regarding their use in biocatalytic reactions. From the different range of reports that have investigated the SCFs for their use as a solvents in biocatalysis, the vast majority of them have used supercritical CO_2 ($scCO_2$) because it is cheap, readily available and considered the most environmentally benign of the SCFs with the required critical parameters compatible with the necessary conditions for enzymatic reactions (Dzyuba and Bartsch 2003; Hobbs and Thomas 2007). However, CO_2 can potentially reduce or completely inhibit the catalytic activity of an enzyme by the formation of carbamates through the reaction on the surface of the enzyme with lysine residues, or the formation of carbonic acid by reacting with water when present. Additionally, the major disadvantage for its industrial application is the high cost of the equipment for producing and handling $scCO_2$ (Hobbs and Thomas 2007). Regarding fluorinated solvents, their use offers a time- and cost-reduction advantage in the process

due to their facile separation of the biocatalyst from the product. However, there is still uncertainty about the persistence of the fluorous solvents and their by-products in the environment. Additionally, the industrial interest for their used is limited at present due to their high cost (Hobbs and Thomas 2007). Polymers, as pointed out before, have only recently arisen as another alternative to replace organic solvents in two-phase partitioning bioreactors since they generally are not bioavailable (biodegradable) and are biologically inert (non-toxic). Also, the wide diversity of polymer structures available provide vast possibilities of material properties (Dafoe and Daugulis 2014). However, due to a lack of thermodynamic data for solute-polymer mixtures to assess the affinity of a candidate polymer towards the target molecule, in most successful cases polymers have been selected *via* heuristic means since log*P* values of the solute provide minor guidance regarding an ideal polymer sorbent; therefore, the polymer selection requires quantitative measures of the material properties, such as polymer crystallinity, solubility parameter, and glass transition temperature, in order to determine the solute-polymer affinity (Parent *et al*. 2012).

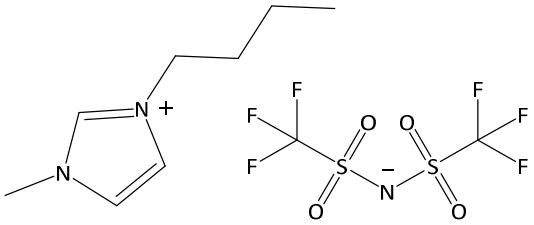
As stated previously, a common issue that limited the biocatalytic production of chemicals using whole-cells is the low solubility of the substrate needed for cell growth and maintenance, and/or of the target product in the aqueous media. Additionally, the accumulation of the product in the media could have inhibitory or toxic effects towards the microorganism, affecting the cell survival and, hence the productivity of the process. In this regard, biphasic systems seem to have a great potential to overcome this difficulty and enhancing the productivity of many biotransformations by using water-immiscible solvents that selectively remove the toxic substance from the reaction media whilst the fermentation is still in progress, hence reducing the cell inhibition during production.

Even though the majority of biocatalysis in ionic liquids studies have been performed using isolated enzymes, the substitution of organic solvents by hydrophobic ionic liquids in whole-cell systems has been reported. The first

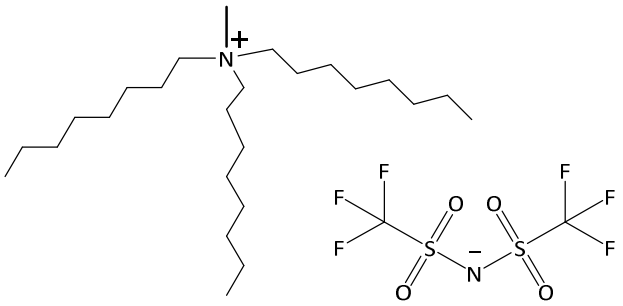
successful report of replacing conventional solvents was performed with whole cell *Rhodococcus* R312 for the bioproduction of 1,3-dicyanobenzene (1,3-DCB) in a two phase IL–water system using 1-butyl-3-methylimidazolium hexafluorophosphate, [BMim][PF_6] (Figure 24) (Cull *et al*. 2000). This work already presumes that ionic liquids have a less damaging effect to the cells membrane compared to conventional organic solvents, such as toluene based on that the specific activity of the biocatalyst in the water-[BMim][PF_6] system was almost an order of magnitude greater than in the water–toluene system. Later, this viability study was extended to *E. coli* and *S. cerevisiae*, showing that water immiscible ionic liquids [BMim][PF_6]; [BMim]-bis(trifluoromethylsulfonyl)imide, [BMim][NTf_2] and methyltrioctylammonium-[NTf_2], [OMA][NTf_2] (Figure 24); have much reduced toxic effect on the cell membrane than most of the organic solvents used (Pfruender, Jones, and Weuster-Botz 2006). Beyond the good biocompatibility demonstrated, an increase of the chemical yield of chiral alcohol synthesis was obtained when using ionic liquids as the solvent phase compared to the typically used organic solvents.



[BMim][PF_6]



[BMim][NTf_2]



[OMA][NTf_2]

Figure 24: Structures of 1-butyl-3-methylimidazolium hexafluorophosphate, [BMim][PF_6]; [BMim]-bis(trifluoromethylsulfonyl)imide, [BMim][NTf_2] and methyltrioctylammonium-[NTf_2], [OMA][NTf_2] ionic liquids.

A very common research area in biocatalytic production processes using whole-cells is the *in situ* extractive fermentation of different organic acids; in particular the production of lactic acid due to its use as the basic material for the production of biodegradable polymers (Matsumoto *et al*. 2004). Regarding the use of ILs as extractive solvents for the fermentation of organic acids, Matsumoto *et al*. (2004) tested a series of imidazolium-based ionic liquids for the feasibility to replace conventional organic solvents for the extraction of lactic acid, and additionally, the solvent toxicity towards *Lactobacillus rhamnosus* was measured. Imidazolium salts showed a very low extraction capacity of lactic acid; therefore, tri-*n*-butylphosphate, a typical extractant used for the extraction of organic acids, was diluted in the ionic liquids, achieving extraction efficiencies similar to those of organic solvents, such as toluene, in

the extractant presence. Furthermore, even though *L. rhamnosus* decreased its activity to half, it still grew, consumed glucose and produced lactate in the presence of imidazolium-based ionic liquids, suggesting they are suitable for an *in situ* extractive fermentation process. Additionally, this study claimed that a change in the alkyl length of the cation had minor influence on the extractability of the organic acids.

Numerous other studies using ILs in a biphasic whole-cell biocatalysis have successfully shown that it is possible to achieve good conversions of up to 80-99% when using ILs as secondary phase in the reaction system (Ernst *et al*. 2005; Pfruender, Jones, and Weuster-Botz 2006; Matsuda *et al*. 2006; Lou, Zong, and Smith 2006; Cornmell *et al*. 2008). However, due to their high cost of production, their industrial use would only be viable if they are able to be recycled and reused as many times as possible. Dennewald *et al*. (2011) investigated the reuse of an ionic liquid in the production of *(R)*-2-octanol by asymmetric reduction of 2-octanone using a recombinant *Escherichia coli* strain in biphasic ionic liquid/water systems (20% v/v 1-hexyl- 1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide, ([HMPyr][NTf_2])). Remarkably, they reported that the ionic liquid could be reused 25 times for the biotransformation with no reduction in the productivity yield or degradation of the solvent, and with an average conversion of 98.5% ± 0.7, and enantiomeric excesses of ≥ 99.5% *(R)*, validating the possibility to recycle and reuse the ILs in biphasic biocatalytic systems.

To conclude, their high solvating abilities and the possibility to manipulate their hydrophobic/hydrophilic nature has increased the interest in using ILs as replacements for VOCs in wide variety of organic reactions as both solvent or extraction medium. Extraction efficiencies in biphasic aqueous systems can be increased by using task-specific ILs designed to enhance the interactions between the target product and the IL, whilst also potentially reducing the toxicity by minimising direct contact with the cells in the aqueous phase. This would also have an impact by increasing the product yield and facilitating downstream processing, therefore making a more economic process. In

addition, their potential capacity to selectively extract polar compounds (van Rantwijk, Madeira, and Sheldon 2003), and the possibility to recycle and reuse them (Dennewald, Pitner, and Weuster-Botz 2011), makes them very promising alternatives. However, although the use of immiscible ILs has shown promising results for the recovery of value-added organic compounds from aqueous phase, even the most hydrophobic ILs present some solubility in water and most of them are non-biodegradable, accumulating in the aquatic ecosystems with potential negative effects towards microorganisms and humans (Earle and Seddon 2000). Hence, the search for optimised selective properties of ILs as extraction solvents for industrial biofermentations processes should be made in parallel with the search of more environmentally friendly solvents.

2.5.3 Toxicity of ionic liquids

Important characteristics of the extraction solvents that are meant to be used in any industrial fermentation process are their biological properties toward the environment. At present ILs have become a practical candidate as unconventional reaction media among chemists due to their unique and flexible properties. However their unusually high stability makes them not easily biodegradable allowing their continuous accumulation in the environment even if released in very small amounts (Pernak, Sobaszkiewicz, and Mirska 2002). Due to the need to implement them into industrial processes, their toxicity and eco-toxicity has become an emerging issue based on the increasing number of studies on the subject (Wood *et al.* 2011; Cascon *et al.* 2011; Gouveia *et al.* 2014; Stolte *et al.* 2007b; Stolte *et al.* 2007a; Matsumoto, Mochiduki, and Kondo 2004; Docherty and Kulpa, Jr. 2005; Ventura *et al.* 2012).

Among all the ionic liquids, the imidazolium-based family it is the most frequently studied regarding synthesis, physico-chemical properties and

ecological impacts. Studies performed by varying the length of the alkyl or alkoxy side chains showed a clear relationship of higher antimicrobial activity with increased length of the side chain (between 1 and 14 carbon atoms) (Pernak, Sobaszkiewicz, and Mirska 2002). However, for very lipophilic compounds, it was observed that their toxic effect was limited after further elongation of the side chains (usually >10), even lowering their antimicrobial activity (Stolte *et al*. 2007a). This is known as the *cut-off* effect, and it is suggested to be caused by steric effects which may obstruct the interaction with the cell membrane. These results also highlight the correlation between the ILs' toxicity and the cation lipophilicity, which may interact with and disrupt the cell surface. This notion was supported by the increased membrane/water partition coefficient and the higher toxic effects that presented with long side-chain ILs (Stolte *et al*. 2007a). Additionally, it was shown that Gram-positive bacteria were more affected than Gram-negative strains, explained on the physico-chemical properties of the Gram-positive cell membrane, which have thicker and more hydrophobic cell walls, and much higher peptoglycan content, increasing the interaction with more lipophilic compounds (Pernak, Goc, and Mirska 2004).

The antibacterial activity of ILs not only involves hampering the growth rate of microbes but also interferes with their productivity. Mentioned previously, Matsumoto *et al.* (2004) studied the tolerance of different *Lactobacillus homohiochii* and *L. fructivorans* bacteria to several organic solvents and imidazolium-based ionic liquids. The tolerance was measured by the variation in the microbial activity based on the concentration of lactic acid produced after adding the extractive solvent compared to a control system with no solvent. Even though all the tested bacteria could produce lactic acid in the presence of ionic liquids, production activities decreased with increasing alkyl chain length in the imidazolium cation moiety. Furthermore, based on their experimental results on ionic solubility (*i.e*. the solubility decreased with an increased in the alkyl chain length), they suggest that the molecular toxicity mechanism of ionic liquids might be different from that of conventional organic solvents, as organic

solvent with log*P* higher than 3 are relatively non-polar molecules suitable for biocatalytic systems due to their low solubility in the aqueous phase, in contrast, the ionic liquids that formed a second phase with water were liquids salts having the polarity similar to acetonitrile or methanol (Aki, Brennecke, and Samanta 2001).

Most studies to date have shown an empirical relationship of the toxicity of ILs with their structure and concentration. However, to date the mechanisms of ILs toxicity is not well understood. Jeong *et al*. (2012), tried to elucidate the mechanism of IL toxicity by studying the effects of three kinds of imidazolium ILs with different lengths of hydrocarbon chains on *Shewanella oneidensis* MR-1 bacteria. They suggested that one of the main reasons for the high level of cell toxicity is that the hydrophobic moiety of the ILs would join into the cell membrane through interactions with the long hydrocarbon tails of ILs, causing the perturbation of the lipid membrane structure, resulting in cell death (Figure 25). Additionally, Jeong and co-workers studied the molecular interactions between lipid membranes and ionic liquids using model cell membranes composed of the amphiphilic biopolymer polydiacetylene (PDA), which in aqueous solution undergoes a self-assembly process to form liposomes. Their results support the penetration of ILs into the cell membrane, and that the lipid bilayer stability is directly affected by them as function of both the ILs concentration and its alkyl chain length.

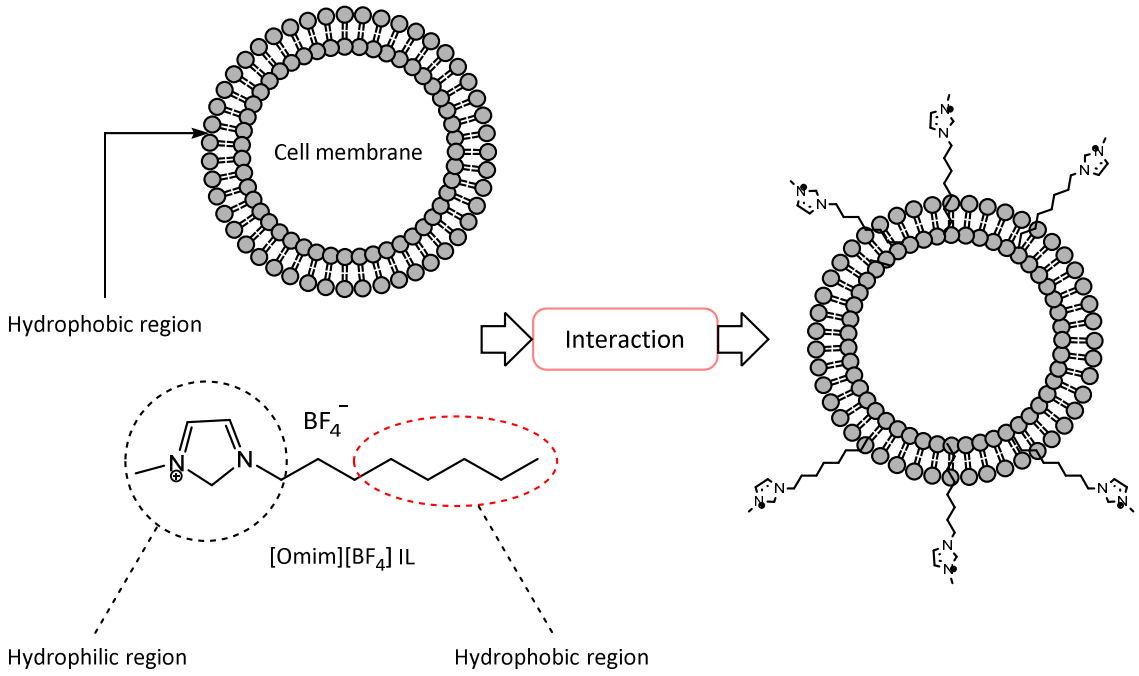


Figure 25: Schematic models of molecular interactions between lipid membranes and ILs.

Concerning the anion contribution to the ionic liquids toxicity, its effect was secondary when compared to the cation influence and less predictable but it becomes more pronounced when combined with less toxic imidazolium cations (Petkovic *et al*. 2011). Nevertheless, data on the anion contribution to the ionic liquids' cytotoxicity is greatly influenced by very lipophilic anions. Additionally, it has been reported that highly fluorinated anions such as $[PF_6]^-$ and $[BF_4]^-$ exhibit much higher toxic effects due to hydrolytic instability (Stolte *et al*. 2007b). Interestingly, Stolte *et al.* also observed that the combination of the $[NTf_2]^-$ anion with different cations could produce either an increased in the toxicity of the IL or lower its antimicrobial activity towards *Vibrio fischeri*. This mixture effect of cation and anion was suggested to be due to the temporary formation of ion pairs on some of the ILs in aqueous solutions, leading to a higher bioavailability and a higher membrane disturbance, resulting therefore in stronger toxic effects. Nonetheless, their direct comparison was difficult because of their wide chemical diversity, not allowing a conclusive analysis on their influence.

Comparison of the effect of the different head groups of the cation moiety on the toxicity of ionic liquids was studied by Stolte *et al.* (2007a) against very different types of organisms (*i.e*. bacteria, plants and algae). The results showed that the aromatic cations (imidazolium and pyridinium rings) displayed higher toxicity than the alicyclic (morpholinium, piperidinium or pyrrolidinium) and quaternary ammonium cations. Salminen *et al.* (2007) studied some physico-chemical properties and toxicities of hydrophobic ionic liquids containing piperidinium and pyrrolidinium cations with selected anions. Cytotoxicity was evaluated toward human breast cancer cell line MCF7, by measuring the half maximal inhibitory concentration (IC_{50}). The tested pyrrolidinium and piperidinium ILs showed an increasing toxicity level with an increase in the alkyl chain length, presenting a similar trend and range to previously reported imidazolium-based ionic liquids. Regarding the anion, even though they suggest its influence is secondary when compared to the cation, replacing $[Br]^-$ anion with $[NTf_2]^-$ increased the toxicity by one order of magnitude.

Extensive research had been made in order to relate the ILs structure and their physico-chemical properties with their toxicity behaviour. Using a large number of ILs, Ventura *et al.* (2013) related the ILs toxicity with their solubility in water indicating that the less soluble non-aromatic ILs also exhibit a lower toxicity when compared to aromatic ILs with higher solubility in water. Additionally, the effect of the isomerism was also assessed aiming to evaluate the possibility of using the isomerism on the cation alkyl chain to design less toxic ILs. It was observed that distributing the cation alkyl chains between the two nitrogen atoms has no effect over neither their solubility in water nor their toxicity. On the other hand, Kurnia *et al.* (2014) studied the effect of the branched alkyl chains in the solubility-toxicity relationship using a series of bis(trifluoromethylsulfonyl)-based ILs, combined with imidazolium, pyridinium, pyrrolidinium, and piperidinium cations with linear or branched alkyl chains. It was observed that increasing from five to six carbon atoms in the central cation ring, regardless of the aromatic/non-aromatic nature of the ILs, leads to a reduction in the solubility in water and to an increase in their toxicity. In

addition, the impact of branched alkyl chains on the toxicity is shown to be not trivial and to depend on the aromatic nature of the ILs. The toxicity of branched alkyl chains attached to the aromatic ring of ILs is lower than that of their linear isomers, whereas the opposite trend was observed for the non-aromatic ring ILs.

Even though some studies have established that the different organisms from the different trophic levels present diverse sensitivity to ionic liquids, it has been demonstrated that the contribution of the cation to the overall toxicity was similar in the majority of the organisms, with higher toxicities increasing with longer substituted alkyl chains, while the anion contribution was unpredictable and varied over the different species (Stolte *et al*. 2007b).

Lastly, despite the increasing interest, there has been a manifest lack of reports using phosphonium-based ionic liquids and hence eco-toxicity studies on them. Nevertheless, some studies of the antimicrobial properties of different phosphonium ILs have been made. $[P_{666n}]^+$ halides (n = 2 to 16) showed that the toxicity of phosphonium chloride towards *E. coli* and *Candida albicans* decreased when increased the alkyl chain (from 8 to 14 carbons) (Cieniecka-Rosłonkiewicz *et al*. 2005), suggesting that the cation structure plays an important role in their cytotoxicity. Interestingly, when the halide anion was swapped with other anions, such as $[NTf_2]^-$, $[OTf]^-$, $[NO_3]^-$, $[N(CN)_2]^-$, $[BF_4]^-$, or $[PF_6]^-$, a lower antimicrobial activity was observed, probably due to the sterically bulky structure and positive charged moiety shielded by the long alkyl chains reduced the interaction with the cell surface, leading to weak inhibition. Specifically, $[P_{6664}]^+$ combined with chloride and $[NTf_2]^-$ led to high and low toxicity against *E. coli*, respectively (Cornmell *et al*. 2008). Ventura *et al.* (2012) compared the toxicity of imidazolium and phosphonium-based ILs towards the luminescent marine bacterium *Vibrio fischeri*. Results suggest that both imidazolium and phosphonium species increased their toxicity with the increase in the alkyl chain length. Moreover, when compared to the analogous imidazolium-based ILs (with the same anion and alkyl chains), the phosphonium family were found to be more toxic towards the studied microorganism.

When a series of phosphonium-based ionic liquids were tested for their anti-microbial properties against selected cocci, rods, bacillus and fungal strains (Cieniecka-Rosłonkiewicz *et al*. 2005), results showed that the anti-microbial activity is selective depending on the type of microorganism they are acting against. Alkyltrihexylphosphonium halides presented strong effects against cocci and bacillus, but minor against rods except *E. coli*. In contrast to their 1-alkyl-3-methylimidazolium analogues, within the phosphonium salts both cation structure and anion type have effects on the biological activity. When the halide was exchanged for other anions, the phosphonium ionic liquids presented a low anti-microbial activity, which is a desirable attribute for an extractive fermentation process.

2.5.4 Biodegradability of ionic liquids

Ionic liquids lack of vapour pressure and good chemical and thermal stability have helped to develop greener, in terms of air quality, and operationally safer processes; however, their impact to the aquatic and terrestrial ecosystems is a rising topic mainly because their effects and fate have not been studied to a great extent and are not predictable for the moment. Hence the importance for an IL to be considered not only not toxic but also biodegradable. The potential biodegradability of a substance is usually assessed under the Organisation for Economic Co-operation and Development (OECD) guidelines, and tests are based on measuring non-specific parameters like dissolved organic carbon, biochemical oxygen demand and CO_2 production. For example, a substance evaluated by the CO_2 headspace test (Organisation for Economic Co-operation and Development 2003) in order to be classified as "readily biodegradable" at least 60% of the theoretical carbon dioxide must be liberated within the first 28 days. However, this battery of tests evaluates the carbon consumption considering the substance as a whole, which in the case of ILs does not provides sufficient information to judge biodegradability, as it is

known that cation and anion will undergo distinct degradation pathways (Deng *et al*. 2015).

Harjani *et al.* (2009) investigated the effect on biodegradation of imidazolium-based ionic liquids by varying the N-substituted side chain and by incorporation of different functional groups and increasing their alkyl chain lengths. Functionalization of the N-substituent seems to impart a degree of biodegradability, and specifically when paired with the alkyl sulfate anion their biodegradability increases considerably (Harjani *et al*. 2009). It was also observed that when the length of the alkyl chain of the alkyl sulfate anion is increased the biodegradability was increased but decreases slightly with an increase in the alkyl chain length on the imidazolium cation. Even with this increase in biodegradability, the majority of these ILs were not able to be classified as "readily biodegradable" under the OECD guidelines. Interestingly, the inclusion of a potential biodegradable anion derived from saccharin (Figure 26), a well-known synthetic non-nutritive sweetener, and an ester linkage in the cation resulted in biodegradation of 61%, classifying as a "readily biodegradable" compound.

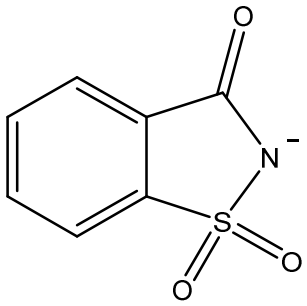


Figure 26: The chemical structure of the biodegradable anion derived from saccharin.

The biodegradability of ionic liquids is a key feature when evaluating their ecological impact and, their environmental fate is complex to predict due to numerous unknown biotic and abiotic factors present in each ecosystem.

Hence the conscious design of ionic liquids based on their structure-activity relationships is essential to enhance their biodegradability. Additionally, anion and cation will go through completely different degradation pathways and, it has been shown that some anions present a faster biodegradation relative to the cations. The latter emphasizes the importance that both the cation and the anion have when selecting them for the successful design of more biodegradable ionic liquids.

2.5.5 General conclusions

Finally, in general, the main conclusions regarding the toxicity of ILs are that is principally influenced by the cation, with non-aromatics exhibiting a lower toxicity when compared to the aromatic equivalent. In addition, higher toxic effects are achieved with increasing length of the side alkyl chain. The introduction of ester linkages, or ether or alcohol groups to the cation alkyl chain, in addition to the use of non-toxic anion precursors such as saccharin and acesulfame, produce less toxic and more biodegradable ILs (Carter *et al*. 2004; Gathergood, Scammells, and Garcia 2006). The anion contribution is usually unpredictable and secondary when compared to the cation influence; however, it becomes more important when coupled with less toxics cations and it is mainly determined by its chemical structure and hydrophilicity, and is generally organism specific (Pernak, Sobaszkiewicz, and Mirska 2002; Biczak *et al*. 2014; Cieniecka-Rosłonkiewicz *et al*. 2005). Finally, even though the wide range of different structures of ILs that have been studied regarding their toxicity have been key to elucidating a structure-toxicity relationship, these only represent a fraction of the potential millions of useful combinations. Therefore, a thorough examination must be done to determine the suitability of any ionic liquid for a particular biocatalytic process involving a specific microorganism.

3. Project Aims

This work forms part of a series of projects carried out in our research group in order to develop a sustainable biosynthetic route to produce chemicals such as *p*-cresol, styrene and toluene from renewable feedstocks; however, all these products are toxic to the producing cells. Therefore, the aim of this project was to solve the issues related with product toxicity during the biocatalytic production of styrene. The first objective was to establish the toxicity threshold of styrene towards the selected biocatalyst *E. coli* in a minimal medium, critical to create a more economical process.

To cope with the toxicity problems of the end-product related with the biocatalytic production of styrene the strategy selected was to develop an *in situ* product removal process *via* liquid-liquid extraction, in efforts to reduce the concentration of styrene in the aqueous phase, thus keeping the toxic material away from the biocatalyst, preventing its inhibition. As there are many problems and disadvantages concerning the sustainability of using conventional organic solvents from an industrial application point of view, the aim was to evaluate the potential of ionic liquids as alternative solvents for the extraction of styrene during its production, thus to create a more sustainable process. The objective was to test a range of ionic liquids regarding their toxicities towards the biocatalyst aiming to identify a number of biocompatible ILs, to then later evaluate their ability to extract styrene from the aqueous phase.

In addition to the toxicity issues, the low bioproduction of styrene has also been related to the low enzymatic activity of the enzymes involved in the already reported pathway using the L-phenylalanine overproducing *E. coli* NST74 strain as a host for the co-expression of PAL2 (phenylalanine ammonia lyase) from *Arabidopsis thaliana* and FDC1 (ferulic acid decarboxylase) from *Saccharomyces cerevisiae*. The enzyme OHBA1, a putative 4-hydroxybenzoic acid decarboxylase from *Aspergillus niger*, has been previously isolated in our

lab by Luca Rossoni; therefore, the objective was to construct and test a novel and alternative biocatalytic pathway for the production of styrene using OHBA1 as a substitute of FDC1 for the decarboxylation of *trans*-cinnamic acid.

4. Materials and Methods

4.1 Chemicals and reagents

All chemical and solvents were purchased from either Sigma-Aldrich, VWR International or Fisher Scientific and used without further purification. Ionic liquids $[P_{4444}][Cl]$, $[P_{8888}][Br]$, $[P_{66614}][Cl]$, $[P_{44414}][Cl]$, $[P_{66614}][NTf_2]$ and $[P_{66614}]$[bis(2-ethylhexyl)phosphate] were purchased from Iolitec (Heilbronn, Germany). $[P_{66614}]$[Saccharinate], $[P_{66614}]$[Cyclamate], $[P_{66614}]$[Acesulfame] and $[P_{66614}]$[Salicylate] were kindly provided by Zoe Disley, University of Nottingham. The remaining ILs $[N_{4444}][AOT]$, $[N_{112(2\text{-}OH)}][AOT]$, $[N_{41(2\text{-}COO\text{-}1)2}][AOT]$, $[P_{66614}][AOT]$, $[P_{5555}]$[Formate], $[P_{66614}]$[Formate], $[P_{8884}]$[Formate], $[P_{8886}]$[Formate], $[P_{8888}]$[Formate], $[P_{88810}]$[Formate], $[P_{88812}]$[Formate], $[P_{88814}]$[Formate], $[BMim][NTf_2]$, $[BMPip][NTf_2]$, $[BMPyrr][NTf_2]$, $[EMim][NTf_2]$, $[N_{8881}][NTf_2]$, $[N_{222(OH)}][NTf_2]$, $[P_{4448}][NTf_2]$, $[N_{24(2\text{-}OH)2}][Br]$, $[N_{1148}][Br]$, $[N_{1148}][Cl]$, $[P_{4448}][Cl]$, $[P_{6667}][Cl]$, $[P_{6668}][Cl]$, $[P_{8884}][Cl]$, $[P_{8885}][Cl]$, $[P_{8886}][Cl]$, $[P_{8887}][Cl]$, $[P_{8888}][Cl]$, $[P_{88810}][Cl]$, $[P_{88812}][Cl]$, $[P_{88814}][Cl]$, $[N_{114(2\text{-}OH)}][DCA]$, $[N_{24(2\text{-}OH)2}][DCA]$, $[N_{112(3\text{-}OH)}][C_2SO_4]$, $[N_{112(2\text{-}OH)}][C_2SO_4]$, $[N_{1(2\text{-}OH)3}][C_1SO_4]$, $[P_{4448}]$[Sal], $[P_{4448}]$[OTf], $[P_{4448}]$[Saccharinate], $[P_{4448}]$[Acesulfame], $[P_{4448}]$[Cyclamate], $[P_{66614}]$[Acetate], $[P_{66614}][C_7COO]$, $[P_{66614}]$[DCA], $[P_{66614}][(^iC_8)_2PO_2]$, $[P_{66614}]$[OTf], $[P_{66614}]$[Dec], $[P_{66614}][HSO_4]$, $[P_{5555}]$[Acetate], $[P_{6666}]$[Acetate], $[P_{6668}]$[Acetate], $[P_{66614}]$[Acetate], $[P_{6888}]$[Acetate], $[P_{8884}]$[Acetate], $[P_{8888}]$[Acetate], $[P_{88810}]$[Acetate] and $[P_{88812}]$[Acetate] were a donation from Prof Kenneth Seddon, Queen's University Belfast.

4.2 Microorganisms and plasmids

The L-phenylalanine overproducing *E. coli* K-12 NST74 strain and both plasmids pSTV28*_pal2* and pTrc99A*_fdc1* were kindly provided by Rebekah McKenna and David R. Nielsen from the Chemical Engineering Department,

Arizona State University, USA. The pET20(+)_*ohbA1* plasmid was kindly prepared by Luca Rossoni, University of Nottingham, UK. *E. coli* K-12 MG1655 and *E. coli* JM107 strains were obtained from Novagen (Madison, WI).

4.3 Microorganisms growth and culture conditions

All the *E. coli* strains were cultured in Luria-Bertani (LB) medium (Sigma Aldrich), MSX (Wahbi *et al*. 1996), MM1 (McKenna and Nielsen 2011) or LUND (Åkesson *et al*. 1999) medium. Media were supplemented with antibiotics when necessary. Antibiotics were prepared as a 1000 times concentrate of the working concentration. Chloramphenicol solutions (34 mg/mL) were prepared in ethanol (100 %) and carbenicillin aqueous solutions (50 µg/mL) were filter sterilised and stored at -20 °C.

4.3.1 Luria Bertani (LB) and agar preparation

Luria Bertani high salt medium (Melford) (25 g/L) was used whenever LB media is referred to throughout this thesis, which is composed of peptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L). Medium was dissolved in distilled water (dH_2O) and then autoclaved (126 °C, 20 min, 15 psi). Liquid LB media was stored in sealed sterile Duran bottles at room temperature until use. LB agar plates were prepared using LB high salt media (25 g/L) and agar (15 g/L). The LB and agar mixture were sterilised by autoclave and allowed to cool in a 50 °C water bath prior to pouring, using aseptic techniques, into sterile petri dishes. Plates were stored at 4 °C.

4.3.2 MSX broth preparation

MSX medium (Wahbi *et al*. 1996) contained sterile MSA (760 mL/L), sterile MSB (200 mL/L), Vishniac's trace elements and sterile 12.5% w/v glucose stock solution (40 mL/L). All solutions were prepared separately and combined as follows.

The Vishniac's trace element solution (Vishniac and Santer 1957) was prepared by dissolving ethylenediaminetetraacetic acid (EDTA) disodium salt (50 g/L) in dH_2O (800 mL/L), this was achieved by adding KOH pellets until EDTA dissolved. To this, $ZnSO_4{\cdot}7H_2O$ (3.9 g/L), $CaCl_2$ (5.54 g/L), $MnCl_2{\cdot}4H_2O$ (5.06 g/L), $FeSO_4{\cdot}7H_2O$ (5 g/L), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1.1 g/L), $CuSO_4{\cdot}H_2O$ (1.57 g/L) and $CoCl_2{\cdot}6H_2O$ (1.61 g/L) were added in that order. The pH was adjusted to 6 with KOH (1 M); dH_2O was then added to a final volume of 1 L. The solution was stored at 4 °C.

MSA contained KH_2PO_4 (7.89 g/L) and Vishniac's trace element solution (2.63 mL/L). The pH was adjusted to 7 with KOH (1M). MSB contained NH_4Cl (15 g/L) and $MgSO_4$ (2.0 g /L). A stock solution of glucose (12.5 % w/v) was prepared by dissolving D-glucose (12.5 g) in dH_2O (100 mL). All solutions were autoclaved separately (126 °C, 20 min, 15 psi) and then mixed aseptically after cooling.

4.3.3 MM1 broth preparation

MM1 medium was adapted from McKenna and Nielsen (2011) and is composed of glucose (nominally 15 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ (4.0 g/L), MOPS (24.7 g/L), KH_2PO_4 (0.3 g/L), K_2HPO_4 (0.7 g/L), and ATCC Trace Mineral Supplement (5 mL/L). ATCC Trace Mineral Supplement contained: EDTA (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (3 g/L), $MnSO_4{\cdot}7H_2O$ (0.5 g/L), NaCl (1 g/L), $FeSO_4{\cdot}7H_2O$ (0.1 g/L), $Co(NO_3)_2{\cdot}6H_2O$ (0.1 g/L), $CaCl_2$ (0.1 g/L), $ZnSO_4{\cdot}7H_2O$ (0.1

g/L), $CuSO_4 \cdot 5H_2O$ (0.01 g/L), $AlK(SO_4)_2$ (0.01 g/L), H_3BO_3 (0.01 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g/L), Na_2SeO_3 (0.001 g/L), $Na_2WO_4 \cdot 2H_2O$ (0.10 g/L), and $NiCl_2 \cdot 6H_2O$ (0.02 g/L). The entire medium was filter sterilized prior to use.

4.3.4 LUND medium preparation

The fermentation medium used for fermentations in a 3 L bioreactor was a modified version of the medium described by Åkesson *et al*. (1999) and contained: K_2HPO_4 (14.6 g/L), glucose (11.9 g/L), yeast extract (5 g/L), $NaH_2PO_4 \cdot 2H_2O$ (3.6 g/L), $(NH_4)_2SO_4$ (2 g/L), ammonium citrate (0.5 g/L), polypropylene glycol (0.1 mL/L), $MgSO_4 \cdot 7H_2O$ (0.49 g/L), carbenicillin solution (0.5 mL/L), chloramphenicol solution (1 mL/L) and fermentation trace elements (2 mL/L). Fermentation trace elements contained: $Na_2EDTA \cdot 2H_2O$ (22.3 g/L), $FeCl_3$ (10.03 g/L), $CaCl_2 \cdot 2H_2O$ (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.18 g/L), $CoCl_2 \cdot 2H_2O$ (0.18 g/L), $CuSO_4 \cdot 2H_2O$ (0.16 g/L), $MnSO_4 \cdot H_2O$ (0.15 g/L). Glucose, $MgSO_4 \cdot 7H_2O$, yeast extract, trace elements, carbenicillin and chloramphenicol solutions were added to the fermentor vessel when cooled. All other components were autoclaved in the fermentation vessel.

4.3.5 Phosphate buffered saline

Phosphate buffered saline (PBS) contained sodium chloride (8 g/L), potassium chloride (0.2 g/L), disodium phosphate (1.44 g/L), monopotassium phosphate (0.24 g/L) in dH_2O. pH 7 was reached by adding HCl (5 M).

4.3.6 Glycerol stock preparation

For long-term storage of all organisms, glycerol stocks were prepared by inoculating fresh LB medium (10 mL) with a single microbial colony and incubated in an orbital shaker (37 °C, 250 rpm) until an optical density (OD_{600}) of ~0.8-1.0 had been reached and were then aliquoted (900 µL) into cryo-vials containing sterile 80% glycerol (100 µL). The vials containing these glycerol cultures were vortexed to create a 16% glycerol stock, which was then stored at -80 °C until use.

4.4 Cultivation of microorganisms in liquid medium

4.4.1 Growth of strains and general handling

For experiments inocula, *E. coli* strains were first grown freshly overnight on agar plates (37 °C) from glycerol stock solutions. Then seed cultures were prepared by transferring single colonies to fresh liquid medium (10 mL) as required using a sterile plastic loop into a sterile Falcon tube (50 mL) and incubated overnight (37 °C, 200 rpm). Later an aliquot of inoculum was removed from the overnight culture using a sterile pipette and transferred to a pre-autoclaved flask in order to inoculate fresh liquid media to an OD_{600} of ~0.05. The volume of medium was 20% of the total volume of the flask to ensure a sufficient supply of oxygen to support growth and each specific bio-transformation. The type of the flask and volumes required at this stage of cultivation was specific to each test and are stated in each relevant section in this chapter. This method was used to prepare the seed cultures used to inoculate all the cultures for fermentation experiments carried out throughout this thesis.

Optical density was measured periodically and OD_{600} values were used to plot a growth curve and to calculate specific growth rates. Specific growth rates (μ) were calculated by selecting two points at time t_1 and t_2 in the exponential growth phase and applying the Equation 1:

$$\mu\ (h^{-1}) = \frac{\ln(OD_2/OD_1)}{t_2 - t_1}$$

Equation 1

Since specific growth rates and *Maximum* OD_{600} varied depending on the medium used and the different tests run, all growth test results were reported as a percentage of the control cultures grown alongside that specific experiment.

4.4.2 Growth inhibition tests

Toxicity tests were carried out using different types of sealed glass vials with polytetrafluoroethylene (PTFE) caps and volumes depending on the solvent or IL tested, as well as the used medium and culture conditions, as described in the text. Styrene and ILs were proven as self-sterile since there was no growth when either were added to sterile uninoculated medium.

Seed cultures of *E. coli* were prepared as already described and used to inoculate fresh liquid medium to a final OD_{600} of ~0.05. Styrene toxicity was evaluated by adding styrene to the flasks with increasing final concentrations. Cultures were incubated (37 °C, 200 rpm, 24 h) and samples were taken only at the end of the assay to measure OD_{600}, avoiding losses of styrene by volatilization. Regarding ILs biocompatibility tests an IL 20% v/v phase ratio was

added and cultures were then incubated for 24 h (37 °C, 200 rpm) and OD_{600} measured periodically. Values were used to plot a growth curve and to calculate specific growth rates. In every toxicity experiment a negative control containing no styrene nor IL was also run. Each test was carried out in triplicate. Growth inhibition was calculated accordingly to the following equation:

$$\% \, Inhibition = \left[\frac{\mu_{control} - \mu_{IL}}{\mu_{control}}\right] \times 100$$

Equation 2

4.4.3 L-phenylalanine production by E. coli K-12 NST74 strain

Seed cultures of *E. coli* NST74 were prepared as described above and used to inoculate fresh MM1 medium (50 mL) to a final OD_{600} of ~0.05. The cultures were incubated for 48 h (32 °C, 200 rpm). Samples (1 mL) were taken from each culture at 24 and 48 h and L-phenylalanine content analysed *via* high-performance liquid chromatography (HPLC), using the method described below.

4.4.4 Co-expression of PAL2, FDC1 and OhbA1 isoenzymes in E. coli NST74 to convert glucose to styrene

Seed cultures of *E. coli* NST74 harbouring either pSTV28_*pal2* and pTrc99A_*fdc1*, or pSTV28_*pal2* and pTrc99A_*ohba1* were prepared as described above and used to inoculate fresh liquid medium (50 mL) to a final OD_{600} of ~0.05. The cultures were grown in 250 mL Quick-fit flasks tightly sealed upon inoculation, in order to avoid loss of volatile product (*i.e.* styrene). A large

headspace was used to preclude the depletion of oxygen from the bottle. Cultures were grown for 10 h (32 °C, 200 rpm) before induction by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM. Culturing continued for 24 h post-induction. Samples (1 mL) were taken from each culture and analysed for relevant metabolite contents *via* HPLC, using the methods described herein.

The amount of vapour in equilibrium with the aqueous phase can be calculated applying Equation 3, and a dimensionless Henry's law constant (H^{CC}) for styrene of 0.113 (Yaws, Yang, and Pan 1991):

$$H^{CC} = \frac{c_a}{c_g}$$

Equation 3

where c_a and c_g correspond to the concentrations under equilibrium condition in the aqueous phase and gas phase, respectively.

In the case of fermentations in the presence of ILs as an extractant phase, seed cultures were grown overnight as described above and used to inoculate sealed glass vials (10 mL) with PTFE caps containing fresh medium with ionic liquid (20% v/v) or without (control); to a final OD_{600} of ~0.05. Cultures were incubated until for 10 h (32 °C, 200 rpm) before IPTG induction at a final concentration of 0.2 mM. Culturing continued for 24 h post-induction. Samples (1 mL) were taken from each culture and analysed for relevant metabolite contents *via* HPLC, using the methods described herein.

4.4.5. Expression of OHBA1 and FDC1 in E. coli BL21 to convert trans-cinnamic acid to styrene

The *E. coli* BL21 (DE3)pLysS strain transformed with the pETohbA1 and pETfdc1 were selected to produce styrene using exogenous *trans*-cinnamic acid. A pET20 of this strain was used as control.

E. coli BL21 (DE3)pLysS was grown overnight (37 °C) on LB agar containing carbenicillin, chloramphenicol and glucose. One colony was used to inoculate LB medium containing carbenicillin, chloramphenicol and glucose (40 mL). The culture was incubated (37 °C, 200 rpm) until an OD_{600} of ~0.6 and cells harvested by centrifugation (6,000 rpm, 20 min, 4 °C). The pellet was resuspended in fresh LB medium containing carbenicillin, chloramphenicol and glucose (400 mL). The culture was incubated (37 °C, 200 rpm) until the OD_{600} reached ~0.6 and protein expression was induced by the addition of IPTG (0.4 mM). Induced culture was incubated (37 °C, 200 rpm) for further 20 h and the cells harvested by centrifugation (10,000 rpm, 20 min, 4 °C). The pellet was washed twice with PBS (pH 7) before being re-suspended in PBS buffer (100 mL). Cells were transferred to sealed glass vials with PTFE caps at a final concentration of OD_{600} ~1.0 (~5 mg/mL wet weight). *trans*-cinnamic acid was then added to a final concentration of 3.75 mM and biotransformation performed for 72 h at 30 °C. Total volume of reaction mixture in vials was 20 mL. Samples (2 mL) were removed at intervals and analysed by HPLC.

4.4.6 Co-expression of PAL2 and FDC1 and OhbA1 isoenzymes in E. coli NST74 to convert exogenous L-phenylalanine to styrene

Seed cultures of *E. coli* NST74 harbouring either pSTV28_*pal2* and pTrc99A_*fdc1*, or pSTV28_*pal2* and pTrc99A_*ohba1* were prepared as already

described and used to inoculate 100 mL LB broth supplemented with carbenicillin (50 µg/mL) and chloramphenicol (34 µg/mL) to an OD_{600} of ~0.05. Cultures were grown at 32 °C while shaking at 250 rpm until an OD_{600} of ~0.6 was achieved, at which time it was induced with 0.2 mM IPTG and then incubated for an additional 8 h. Cells were then centrifuged at 10,000 rpm for 10 min at 4 °C. Each pellet was re-suspended in 100 mL PBS buffer. The OD_{600} was measured in order to dilute every culture to a final OD_{600} of 2 into a 20 mL PBS solution.

Finally, L-phenylalanine was added to fresh PBS buffer and then mixed in sealed glass vials with PTFE caps with every OD_{600} 2 culture to get a 15 mL solution of final OD_{600} ~0.6 and a starting concentration L-phenylalanine of 900 mg/L. The biotransformation was performed for an additional 24 h at 32 °C while shaking at 250 rpm. In the case of biotransformations using ionic liquids as a secondary phase, ionic liquid was added at 0.2 phase ratio (*i.e.* 20% v/v). Samples (1 mL) were taken from each culture, and phase when required, centrifuged and 750 µL of supernatant was taken and analysed for relevant metabolite contents *via* HPLC using the methods described here in.

4.4.7 Fed-batch production of styrene in LUND media by E. coli NST74

Larger scale biotransformations were performed in a New Brunswick™ BioFlo®/CelliGen® 115 3 L bioreactor unit (H_B = 45 cm, D_B = 24 cm), and operated at a working volume of 1 L. To recover the styrene stripped out from the fermentation an absorption column containing 120 mL of dodecane was attached to the condenser/exhaust gas of the bioreactor (H_A = 20 cm, D_A = 3 cm) (Figure 27; Figures 70-74, Appendix 8.8). *E. coli* NST74 harbouring pSTV28*_pal2* and pTrc9A*_fdc1* was grown overnight in 100 mL LUND medium. Cultures were grown overnight at 37 °C while shaking at 200 rpm to prepare seed culture. The OD_{600} was measured in order to dilute the seed culture with

sterile distilled water to an appropriate OD_{600} and a final volume of 50 mL to then inoculate the 950 mL of batch medium to an OD_{600} of ~0.1 into the reactor (1 L). Culture was grown overnight at 37 °C and pH 7.0 controlled by automatic addition of aqueous ammonium hydroxide (28 % v/v) or H_2SO_4 (3 M). Dissolved oxygen (DO) was controlled at a set point of 30% saturation measured by a polarographic oxygen probe, maintained by automatic control of the stirrer speed (between 400-1200 rpm) and air flow (minimum 1 L/min). Foaming was controlled by manual addition of the minimum volume of polypropylene glycol (P2000, 3%v/v) needed to break any foam, as soon as it began to form. Once all of the carbon source was consumed in the batch medium, a glucose feed was used to maintain the pseudo-exponential growth rate at 0.25 h^{-1}. The glucose feed contained: glucose (650 g/L), $MgSO_4 \cdot 7H_2O$ solution (12.32 g/L), fermentation trace elements (11.8 mL/L) and yeast extract (5.9 g/L). Glucose, yeast extract and $MgSO_4 \cdot 7H_2O$ and trace elements solutions were prepared separately and mixed when cooled. The glucose feed was maintained until the culture OD_{600} reached approximately 50, at which point the culture was induced by adding IPTG (0.4 mM). Subsequent glucose feeding is described in the text. Biotransformation was performed for a total of 48 h at 37 °C and 10 mL samples were taken every hour to measure OD, dry weight, glucose and styrene concentrations and any relevant intermedia.

In the case of fermentation in the presence of ILs as an extractant phase, cells were inoculated and grown overnight in the reactor as described above. If the same conditions were met the morning after, then the glucose feed was started, and 10 mL samples were taking every hour to measure OD, dry weight and glucose consumption. Feed rate was constantly increased and culture was grown until an OD_{600} of ~30, at which point $[P_{66614}][NTf_2]$ (250 mL, 20% v/v) was pumped into the bioreactor, so that the final total culture volume was 1.25 L. The OD was monitored to confirm that the cells were growing as expected. In a similar fashion as before, when cell density reached an OD_{600} of ~50, culture was induced with 0.4 mM IPTG. Subsequent glucose feeding is described in the text. Biotransformation was performed for a total 48 h at 37 °C and 10 mL

samples were taken every hour to measure OD, dry weight, glucose and styrene concentrations and any relevant intermedia. It should be unnecessary to remove ionic liquid from the cultures to compensate for sampling, because the culture was completely mixed, and all samples contained both the ionic liquid and aqueous phases in the correct phase ratio. The phases were allowed to separate before chemical analysis of the aqueous phase.

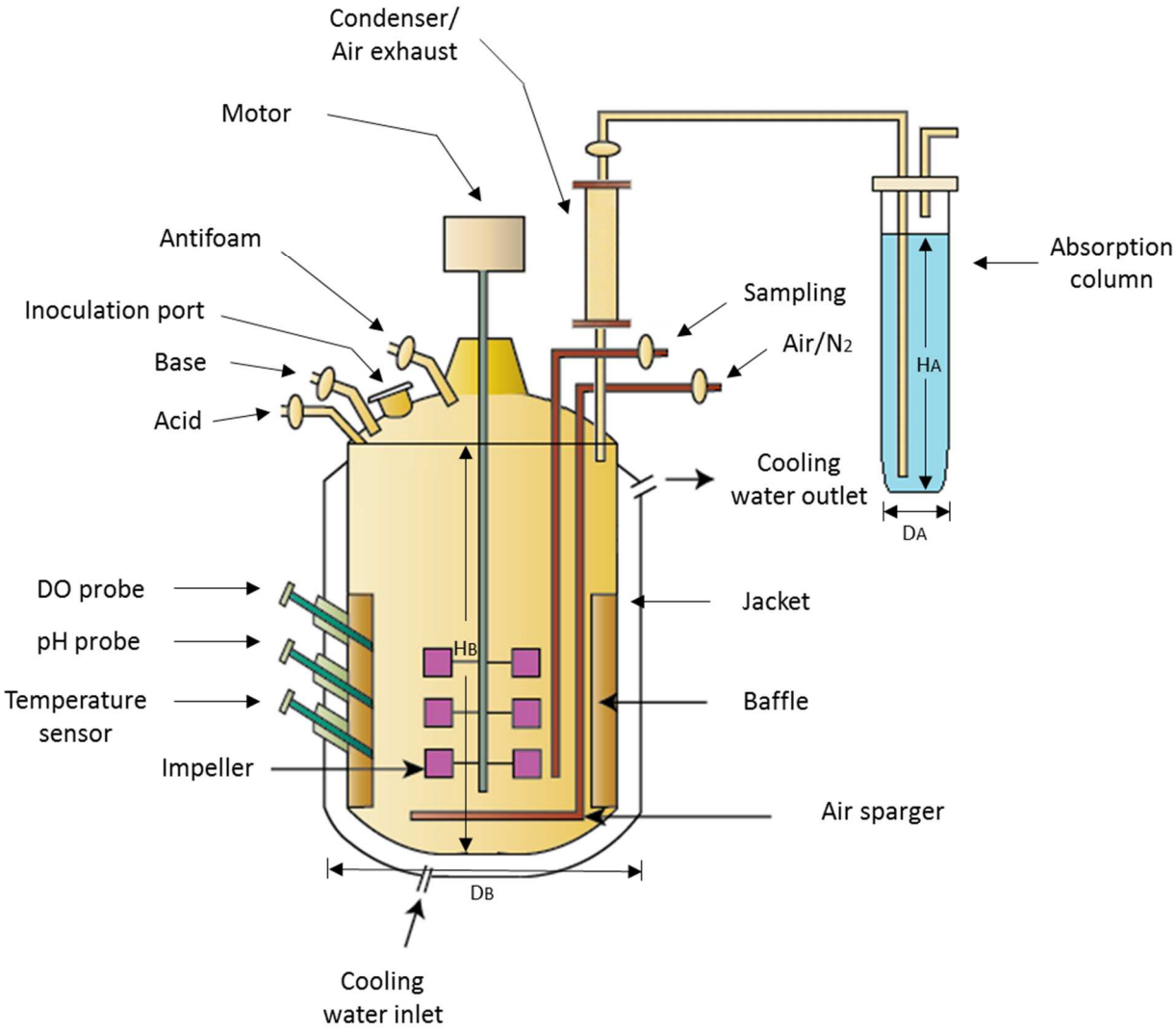


Figure 27: Schematic diagram of the bioreactor and the absorption column.

4.5 Extraction of styrene

Extractions were carried out in sterile 40mL Teflon-sealed glass vials. A stock solution of styrene (2.5 mM) was prepared, and later aliquots (8 mL) were transferred into the sterile 40 mL Teflon-sealed glass vials. An aliquot of IL (2 mL, 20%v/v) was then added in triplicate to the vials, and the lid replaced. The vials were placed in an orbital incubator at 37 °C, 200 rpm for 48 h. The vials were then left to phase separate at the indicated temperature for 1 day to ensure the phases had fully separated and the systems had reached dynamic equilibrium. A sample of the MSX phase (1 mL) was then taken and added directly into a HPLC vial. Samples were taken in triplicate from each test vial and were injected into the HPLC-Ultraviolet (UV) detector, as described in section 4.7.4, in order to determine the concentration of styrene in the MSX phase at equilibrium, using the calibration curve (Appendix 8.6).

4.6 DNA techniques

4.6.1 pJET1.2_ohbA1 construction

The *ohbA1* gene was amplified by Polymerase Chain Reaction (PCR) using pET20(+)_*ohbA1* as a template. The KOD DNA polymerase protocol (Novagen) was used and vials were assembled as described below (Table 1). The forward primer used (5'-CCACAACGGTTTCCCTCTAG-3') was designed to bind to a region upstream to the *ohbA1* gene in order to include the Ribosome Binding Site (RBS) of pET20(+). No RBS would have been present in the final pTrc99A plasmid if this precaution had not been taken. The forward primer also contained an *XbaI* restriction site. The reverse primer used (5'-AAAAAGCTTTTAGTTGCTGAAACCCATC-3') contained a *HindIII* restriction site. PCR was performed at reaction conditions described below (Table 2).

Table 1: PCR reaction mixture.

10X Buffer KOD#1	5 µL
25 mM $MgCl_2$	2 µL
10 mM dNTP Mix	1 µL
100 µM Forward primer	2 µL
100 µM Reverse primer	2 µL
pET20_*ohbA1*	1 µL
KOD polymerase	0.4 µL
Water, nuclease-free	36.6 µL
Total volume	50 µl

Table 2: Reaction conditions.

Step	**Temperature (°C)**	**Time**	**N° of cycles**
Initial denaturation	98	3 min	1
Denaturation	98	15 sec	
Annealing	50	2 sec	25
Extension	72	20 sec	
Final extension	72	5 min	1
Hold (Store)	4	--	--

Amplified *ohbA1* was purified using the GenElute Gel Extraction Kit (Sigma Aldrich) and then ligated into pJET1.2 plasmid using the T4 DNA ligase according to the CloneJET PCR cloning kit (Thermo Scienctific).

4.6.2 Transformation of E. coli JM107 with pJET1.2_ohbA1

Chemically competent *E. coli* JM107 cells were transformed with pJET1.2_*ohbA1* plasmid using the TransformAid Bacterial transformation kit. Transformants were selected by plating on LB medium containing carbenicillin (50 μg/mL). A single colony of transformants was inoculated into LB medium (5 mL) overnight (37 °C, 200 rpm) and the culture was then used for purification of pJET1.2_*ohbA1* plasmid. For plasmid purification the GenElute Plasmid Miniprep Kit (Sigma Aldrich) was used.

4.6.3 pTrc99A_ohbA1 construction

The purified pJET1.2_*ohbA1* was digested with *XbaI* and *HindIII*. The expression vector pTrc99A was similarly digested with *XbaI* and *HindIII*. All digestions were performed for 6 h at 37 °C according to manufacturer's protocols. Digested fragments were gel purified using the GenElute Gel Extraction Kit (Sigma Aldrich) and, subsequently, ligation was performed using the T4 DNA ligase (Thermo Scienctific). *E. coli* JM107 was then transformed with the new plasmid pTrc99A_*ohbA1*, followed by selection on LB plates containing carbenicillin (50 μg/mL). Purification of pTrc99A_*ohbA1* plasmid was performed as described above.

4.6.4 Transformation of E. coli NST74 with combination of plasmids

E. coli NST74 was co-transformed with plasmids pSTV28_*pal2* and pTrc99A_*fdc1*, and with pSTV28_*pal2* and pTrc99A_*ohbA1*, respectively. The TransformAid Bacterial transformation kit was used. *E. coli* NST74 carrying the

plasmids was then isolated by selection on LB medium containing carbenicillin (50 µg/mL) and chloramphenicol (34 µg/mL).

4.7 Analytical methods

4.7.1 Biomass concentration

Optical density measurements were made at 600 nm with a Shimadzu UVmini 1240 UV-Vis spectrophotometer using polystyrene cuvettes (10 mm path length). Samples (1 mL) were taken from the cultures and transferred to the cuvettes and OD was measured. Accuracy of the reading was best when the OD was within the range of 0-0.8, therefore samples were diluted in dH_2O until they were within the specified range.

Cell weight measurements were made in pre-weighed microfuge tubes that had been dried by incubating in the presence of dry silica in a sealed container. Samples (1 mL) were harvested by centrifugation (13,400 rpm; 3 min) and the supernatant discarded. For wet cell weights (WCW), the microfuge tubes were reweighed, and for dry cell weights (DCW), the cell pellets were incubated at 70 °C to constant weight and reweighed. The difference between the empty microfuge tube and that containing the cell pellet was used to calculate the cell weight per L of culture.

4.7.2 Estimation of DNA concentration

Plasmid concentration was determined using a NanoDrop ND1000 Spectrophotometer. PCR fragment concentration was determined using agarose gel electrophoresis and by visually comparing bands with standards at known concentrations.

4.7.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to purify PCR products and digested DNA fragments, to evaluate results of colony PCRs and to estimate DNA concentration, using a Green Range™ Horizontal Gel Electrophoresis Unit (Scie-Plas).

Gel was prepared with Tris-Acetate-EDTA (TAE) buffer (Bio-Rad) containing agarose (1%). TAE buffer (1X) was prepared by diluting 50X TAE buffer stock solution with dH_2O. TAE (50X) was composed of Tris buffer (242 g/L), EDTA disodium salt dehydrate (20.6 g/L) and glacial acetic acid (57.1 mL/L) in dH_2O. Each component was added to the distilled water in the order stated.

Agarose was dissolved in TAE buffer (1X) by microwaving the solution. Then the agarose-TAE solution was allowed to cool down and ethidium bromide (EtBr) (1% w/v) was added before the solution began to set. The solution was thoroughly mixed and then poured in the pre-assembled electrophoresis system. The comb was inserted immediately. When solidification was completed, the gel was submerged in TAE buffer (1X) and the comb was removed, and the DNA samples were loaded in the wells. Before loading, DNA samples were mixed with 6X loading dye in a 5:1 volumetric ratio. Gene Ruler™ 1 kb Plus DNA Ladder was also loaded as standard for each analysis. The agarose gel electrophoresis analyses were performed at 50-80 mV for 40-60 min. DNA was visualised on the gel with a UV transilluminator and pictures of the gels were obtained an uGenius^3 Gel Documentation System (Syngene).

4.7.4 Metabolite analysis by High Performance Liquid Chromatography (HPLC)

Samples (1 mL) were taken from the aqueous phase and centrifuged (13,400 rpm; 3 min; room temperature) to remove cells. The supernatant (750

µL) was then filtered (45 µm filter) into HPLC analysis vials (Agilent) sealed with a Teflon-lined cap.

Tyrosine, L-phenylalanine, *p*-coumaric, *trans*-cinnamic acid and styrene detection was carried out using an Agilent 1200 Series HPLC system equipped with an autosampler, UV detector, and reverse-phase Agilent Eclipse XDB – C18 column (5 µm, 4.6 mm × 150 mm). Samples (5 µL) were injected for analysis at a total constant flow rate of 1.0 mL/min and constant column temperature of 45 °C. Elution was performed with 'solvent A' (consisting of 0.22 µm filtered water with addition of 0.1% v/v trifluoroacetic acid) and 'solvent B' (consisting of methanol with addition of 0.1% v/v trifluoroacetic acid) mixed at different ratios. The eluent began as a mixture of 90% solvent A and 10% solvent B before a linear gradient was applied over 25 min to then reach a mixture of 20% solvent A and 80% solvent B. Subsequently a second linear gradient was applied over the course of 5 min to achieve a final mixture of 90% solvent A and 10% solvent B. The eluent was monitored at each of 210 nm for tyrosine and L-phenylalanine, and 258 nm for *p*-coumaric acid, *trans*-cinnamic acid and styrene. Concentrations were estimated from calibration curves prepared in the respective minimal media for each compound and examples are given in Appendix 8.6.

Organic acids were detected, and concentrations measured using an Agilent 1200 series HPLC system equipped with a UV detector (210 nm) and refractive index detector. Organic acids were separated using a Rezex column ROA organic acid H+ (8%) from Phenomenex LC column. The column (300 x 7.8 mm) was equipped with a guard column and operated at 55 °C using a 5 mM aqueous H_2SO_4 solution at 0.5 mL/min as the mobile phase. Standard curves were produced at every HPLC analysis for each compound and examples are given in Appendix 8.6.

4.7.5 Gas Chromatography – Mass Spectrometry (GC-MS)

Styrene present in the aqueous phase was also detected and quantified using an Agilent 7890 A 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. 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.

Samples (10 mL) were taken from the aqueous phase and centrifuged in a bench top centrifuge (12,000 rpm; 2 min; 4 °C) in order to separate any remaining trace of cells. Then the supernatant (*i.e.* 5, 3 and 1 mL) was taken and mixed in a 1:1 ratio with solution of a selected organic solvent (*i.e.* hexane, ethyl acetate, dichloromethane (DCM), toluene and diethyl ether), containing *t*-butyl benzene (10 mM) used as internal standard (IS). Solutions were thoroughly mixed by vortex and samples from the organic phase (750 µL) were then transferred to a glass GC vial and sealed with a Teflon-lined cap.

For styrene detection samples (1 µL) were injected using an autosampler with a split ratio of 100:1. The inlet temperature was 250 °C and the oven temperature was initially kept at 45 °C for 5 min then increased at 20 °C/min until 250 °C followed by 10 °C/min to 300 °C at which point this temperature was held for 5 minutes. The injector and detector temperatures were 300 °C and 230 °C, respectively. Concentrations were estimated from a linear calibration using authentic styrene in a concentration range of 0.5 – 5 mM using *t*-butyl benzene (10 mM) as internal standard (Appendix 8.3).

4.7.6 Analysis by Gas Chromatography – Flame Ionisation Detector (GC-FID)

An HP-5 column (Agilent, USA), 30 m x 320 µm x 0.25 µm, was used in conjunction with a 10 m deactivated fused silica guard column (Agilent, USA), used to trap non-volatile ILs, on an Agilent 7820 GC. For the measurement of styrene, the temperature program consisted of 70 - 300 °C at 20 °C/min, held for 1 min at 70 °C, 230 °C and 300 °C, the detector was held at 280 °C. Split injection (1 µl, 10:1) was used.

Ionic liquid samples were prepared by first completely removing the IL phase from the biotransformation culture. Afterwards centrifugation of the IL phase was carried out in a bench top centrifuge (12,000 rpm; 2 min; 4 °C) in order to separate any remaining trace of aqueous phase. Then an IL sample (1 mL) was taken and mixed in a 1:1 ratio with solution of ethyl acetate with *t*-butyl benzene (10 mM) used as internal standard. Solution was thoroughly mix by vortex and samples (750 µL) were then transferred to a glass GC vial and sealed with a Teflon-lined cap. Concentrations were estimated from calibration curves prepared in the respective IL using authentic styrene at known concentrations using *t*-butyl benzene (10 mM) as internal standard (Appendix 8.5).

Dodecane samples (250 µL) were taken from the dodecane absorption column and directly transferred to a glass GC vial with insert and sealed with a Teflon-lined cap. Concentrations were estimated from calibration curves prepared in dodecane using authentic styrene at known concentrations (Appendix 8.5).

4.7.7 Nuclear Magnetic Resonance (NMR)

The analysis was performed by Shaz Aslam (School of Chemistry, University of Nottingham). Samples (1 mL) were taken from the previously centrifuged IL phase and transferred into 1.5 mL Eppendorf tubes. Tubes were centrifuged (13,400 rpm, 2 min); part of the IL phase (200 µL) transferred into

a clean 1.5 mL tube and mixed with 800 µL of deuterated chloroform as solvent. The mixture (600 µL) was transferred into a NMR tube. The ^{1}H NMR spectrum was recorded at 400 MHz with 64 scans on a Bruker AV(III) 400 NMR Spectrometer. Data were processed and analysed using ACD/NMR Processor version 12.01. Authentic standards of styrene, prepared at a concentration of 100 mM, were analysed in the same way (Appendix 8.2).

5. Results

5.1 Ionic liquids as alternative solvents for the *in situ* extraction of styrene

5.1.1 Styrene toxicity

One of the principal constraints in the bioproduction of chemicals is that the end product can have inhibitory or toxic effects towards the biocatalysts (Prpich and Daugulis 2007; Verhoef *et al*. 2009; Wierckx *et al*. 2005; Van Hecke, Kaur, and De Wever 2014). Its accumulation hampers the microorganisms' productivity, limiting the product titres and the overall viability of the production process. Therefore, it is of great importance to understand the interaction of the target product with the biocatalyst in order to design a suitable extraction strategy to overcome the toxicity hindrance and enhance the productivity of the biocatalytic process. The biosynthetic production of styrene has been reported successfully (McKenna and Nielsen 2011), however at very low concentrations possibly due to the highly toxic nature of styrene. It is for this reason that an *in situ* product recovery method is of interest due to its great potential in enhancing the productivity of many bioprocesses (Lye and Woodley 1999; Daugulis 2001; Malinowski 2001).

Therefore, the toxicity of styrene towards *E. coli* K-12 MG1655 was investigated. Growth tests were carried out as described in detail in the Materials and Methods section (4.4.2). Briefly, *E. coli* MG1655 was grown in MSX medium with styrene added at different concentrations. A negative control with no styrene was also grown. OD_{600} was measured and toxicity of styrene evaluated on the basis of the maximum OD reached. Maximum OD_{600} values were taken as the highest OD_{600} after 24 h during stationary phase (Figure 28).

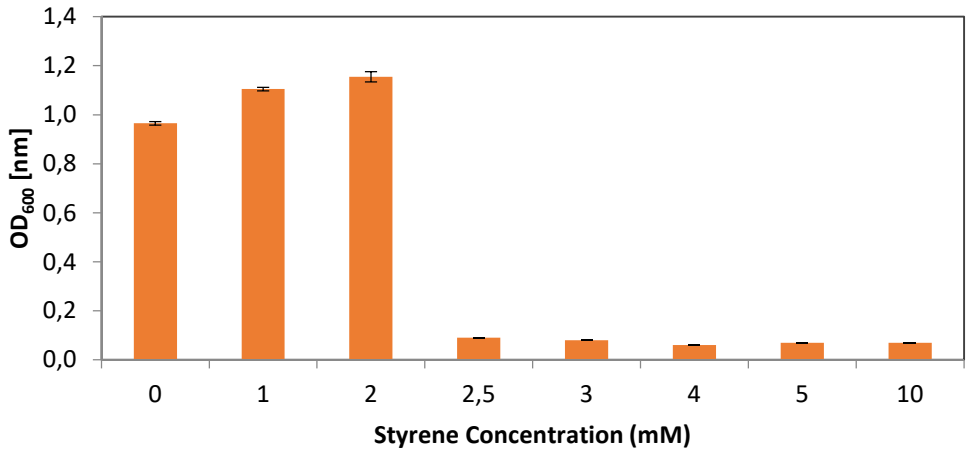


Figure 28: Styrene toxicity towards *E. coli* MG1655.

A seed culture (20 mL) of *E. coli* MG1655 was grown in MSX medium and then used to inoculate sealed glass vials (40 mL) with PTFE caps. At the same point, styrene was added at different concentrations and cells were grown at 37 °C for 24 h. OD_{600} was then measured and toxicity of styrene evaluated on the basis of the Maximum OD_{600} reached. Maximum OD_{600} values were taken as the highest OD_{600} after 24 h during stationary phase. Data and standard deviations are the average of triplicate experiments.

The results show that *E. coli* grows in the presence of styrene up to a 2 mM concentration. Cell growth was completely inhibited at 2.5 mM (*i.e.* 260 mg/L), suggesting a toxicity threshold slightly lower than its solubility limit (*i.e.* 310 mg/L at 25 °C (U.S. Environmental Protection Agency 1994)), when experiments are carried out in minimal medium.

5.1.2 Toxicity of ionic liquids towards E. coli

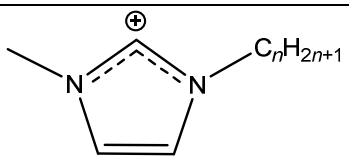
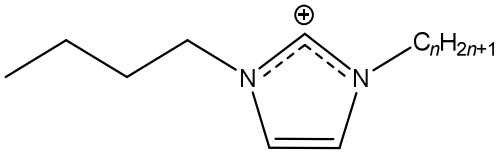
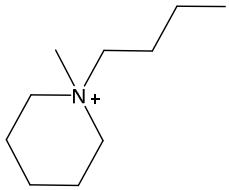
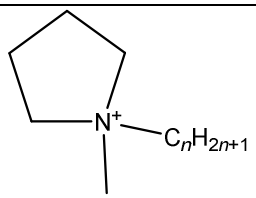
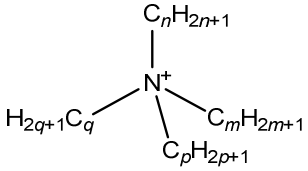
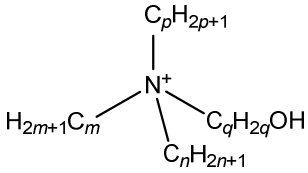
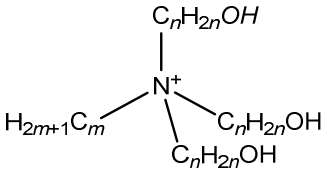
The possibility to tune the physico-chemical properties of ionic liquids by combining different cations and anions, allows ionic liquids to suit the specific requirements of a particular process and has increased the interest in using ionic liquids as potential solvents in two-phase fermentation processes to replace the classic volatile organic compounds (Chen *et al*. 2013; Garcia *et al*. 2004; Han and Row, 2010; Pfruender, *et al*. 2004). Due to their unique properties such as negligible vapour pressure, high thermal and chemical

stability and flexible nature, allowing them to be used in a diverse range of applications, ionic liquids have been usually referred as “green solvents” (Olivier-Bourbigou, Magna, and Morvan 2010). However, they are neither inherently non-toxic, nor non-hazardous for the environment when disposed irresponsibly or accidentally (Deng *et al*. 2015; Viboud *et al*. 2012). Therefore, the potential harmful properties of a specific ionic liquid must be evaluated before it can be considered as a green alternative to organic solvents.

Their high manipulability, allowing the different combinations of anion and cation to generate an entirely new and unique ionic liquid with its own set of physico-chemical properties, also generates a vast number of potential solvents. Hence, with such a large number of possible solvents to choose from, the task of selecting an ionic liquid suitable for a particular application in biocatalysis is much more challenging and time-consuming that with the conventional organic solvents.

In order to evaluate the use of ILs as alternative solvents for this process, the selection of the ionic liquids was based on certain specific characteristics to suit a biocatalytic process, such as their solvent properties, which are largely affected by its ability to form hydrogen bonds and the degree of localization of the charges on the anion (Dupont, Consorti, and Spencer 2000), and their toxicity features already reported in the literature. In addition, their hydrophobic nature was taken into account in order to procure the necessary biphasic systems for the *in situ* extraction process. Therefore, different commonly used ILs were chosen to study their application in the whole cell biocatalytic production of styrene and *in situ* extraction, with the structures and abbreviations of the cation and anion moieties found in the ionic liquids used in this study listed in Tables 3 and 4, respectively.

Table 3: Cation structures and abbreviations of the ionic liquids used in this study

Name	Abbreviation	Structure
1-alkyl-3-methylimidazolium	[C_nMim]	
1-alkyl-3-butylimidazolium	[C_nBim]	
1-butyl-1-metylpiperidinium	[BMPip]	
1-alkyl-1-methylpyrrolidinium	[C_nPyrr]	
Tetraalkylammonium (hydrocarbon chains)	[$N_{m\ n\ p\ q}$]	
Tetraalkylammonium (singly hydroxylated)	[$N_{m\ n\ p\ q\text{-OH}}$]	
Tetraalkylammonium (tris hydroxylated)	[$N_{m\ (n\text{-OH})3}$]	

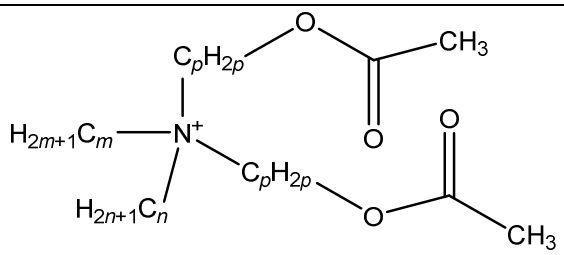
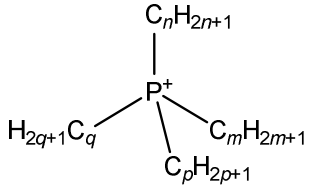
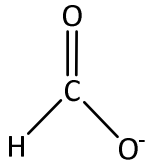
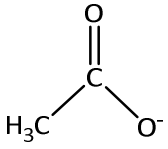
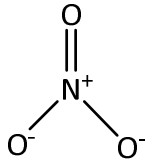
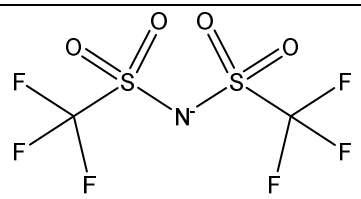
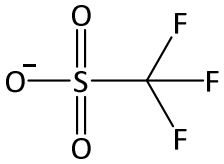
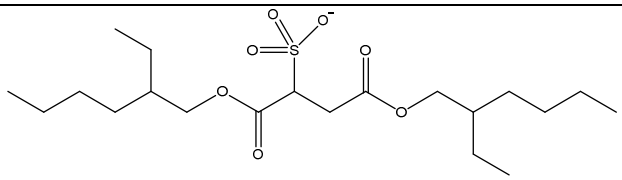
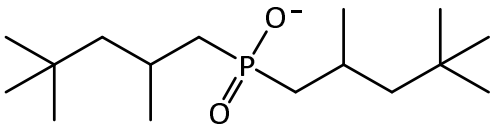
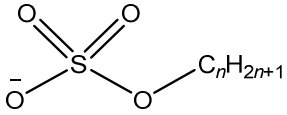
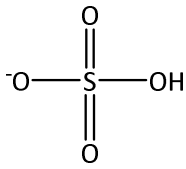
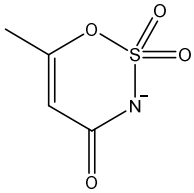
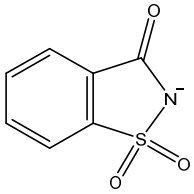
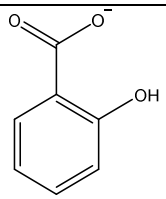
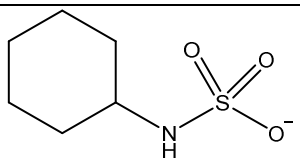
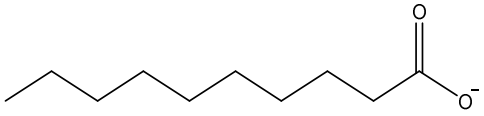
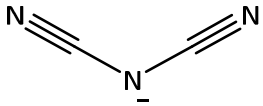
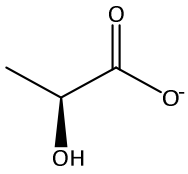
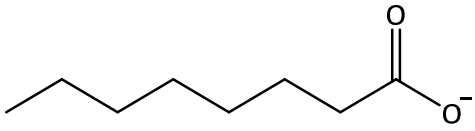
Tetraalkylammonium (di caboxylated)	$[N_{m\ n\ (p\text{-}COO\text{-}1)2}]$	
Tetraalkylphosphonium (hydrocarbon chains)	$[P_{m\ n\ p\ q}]$	

Table 4: Anion structures and abbreviations of the ionic liquids used in this study.

Name	Abbreviation	Structure
Chloride	[Cl]	Cl^-
Bromide	[Br]	Br^-
Formate	[HCOO]	
Acetate	$[CH_3COO]$	
Nitrate	$[NO_3]$	

bis(trifluoromethyl sulfonyl)imide	[NTf_2]	
Trifluoromethanesulfonate	[OTf]	
Docusate	[AOT]	
Bis(2,4,4-trimethylpentyl) phosphinate	[$(^{i}C_8)_2PO_2$]	
Alkyl sulfate	[C_nSO_4]	
Hydrogen sulfate	[HSO_4]	
Acesulfame	[Ace]	
Saccharinate	[Sacc]	
Salicylate	[Sal]	

Cyclamate	[Cyc]	
Decanoate	[Dec]	
Dicyanamide	[DCA]	
L-Lactate	[$C_3H_5O_3$]	
Octanoate	[C_7COO]	

At present, two different methods have been successfully used to assess the biocompatibility of ionic liquids: the 'High throughput agar diffusion test' and 'Microcultures in a Bioscreen C incubator/plate reader' (Wood *et al*. 2011; Cornmell *et al*. 2008; Rebros *et al*. 2009); however both methods present certain limitations, in particular when used to test water-immiscible ionic liquids. The agar diffusion test is a simple, rapid and inexpensive screening method widely used to measure the susceptibility of bacteria to antibiotics (Gavin 1957; Davis and Stout 1971; Vedel *et al*. 1996); and is based on the principle that water-miscible compounds will diffuse though the agar with an even concentration gradient around the impregnated disk. However, it has been shown that immiscible ionic liquids are unable to diffuse through the media and instead spread over the agar surface forming a film (Wood *et al*. 2011), therefore the size of the inhibition zone would be much more associated to the viscosity of the ionic liquid rather than to its real toxic attributes. Regarding the microcultures in a Bioscreen C incubator, its use presents a rapid

and easy way to obtain growth data without the necessity of actually being present to manually acquire the OD values since the Bioscreen C microplate reader automatically takes measurements of turbidity of the cultures in real time which are collected with the 'Bioscreen software™' to generate growth curves. However, when used with some very viscous or water-immiscible ionic liquids, the OD readings from these cultures were much higher than the control cultures or blanks, suggesting that the ILs themselves are absorbing or scattering light (Wood *et al*. 2011). In addition, some ILs formed a paste/gel-like substance when mixed with the culture media, causing a high variability in the OD measurements. Therefore, due to its higher reliability regarding OD readings, it was decided to test the ionic liquids biocompatibility in flask cultures since it is possible to avoid the IL phase to measure the OD of the culture when working with immiscible ionic liquids. In addition, to minimise the amount of IL used, it was decided to scale down the cultures to small 4 mL vials.

Hence, the biocompatibility of the selected ionic liquids towards *E. coli* was investigated as described in the Materials and Methods section (4.4.2). Briefly, *E. coli* cells were incubated in sterile vials (4 mL) with PTFE liners in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and were reported as the Maximum OD_{600}. These were taken as the highest OD_{600} value after 24 h, during stationary phase, and are also reported as a percentage of the maximum OD_{600} reached by the control culture with no ionic liquids. Additionally, the formation of either a single or biphasic system was recorded, and its behaviour was documented in order to compare the major number of properties to select the most suitable ionic liquid to the current bioprocess (Tables 5-11).

Results show that the majority of the Maximum OD_{600} readings present a small standard deviation (Tables 5-11), which supports the selection of flask cultures to measure the biocompatibility of ionic liquids. Nevertheless, certain ionic liquids were found to be problematic to work with, since, due to their high viscosity, it was very difficult to accurately pipette them from their container into the culture flasks. Ammonium-based ionic liquids presented a higher

viscosity when compared to the phosphonium-based ionic liquids. This trend in viscosity was opposite to the trend expected, since the substitution for a heavier atom usually increases the density and viscosity of a compound. Therefore, it was suggested that the substitution of the cation central atom with a heavier and bigger atom, results in weaker interionic interactions due to the increase in the IL volume (Tsunashima and Sugiya 2007; Carvalho *et al*. 2014; Blundell and Licence 2014). Since it is known that the viscosity of ionic liquids is highly influenced by temperature (Baker *et al*. 2001), the ionic liquids that presented this characteristic were warmed up in the incubator at 37 °C and 200 rpm for 3 h before transferring them into the culture flasks; however, even after this treatment, the ionic liquids still showed a high viscosity. Perhaps a much higher increase in the temperature would have reduced the viscosity of the ionic liquids to easier handling levels pipetting-wise; however, the selected temperature and rpm were chosen to resemble the fermentation conditions.

Additionally, it was difficult to analyse the toxicity of some ionic liquids that contained the docusate anion $[AOT]^-$ (Table 5). Even though the ILs comprising this anion were able to form a biphasic system at the beginning of the culture, gradually some of the ionic liquid mixed with the broth and created a gel/paste mixture, therefore affecting the OD readings. This was confirmed by the high Maximum OD_{600} values obtained by the ionic liquids $[N_{112(2\text{-}OH)}][AOT]$ (OD_{600} 10.77 ± 0.81) and $[N_{41(2\text{-}COO\text{-}1)2}][AOT]$ (OD_{600} 31.23 ± 12.20) when compared to the control, and the high variability in the Maximum OD_{600} (%) showed by the majority of the selected ILs (*i.e.* $[P_{66614}][AOT]$ 76% ± 13.1, $[N_{112(2\text{-}OH)}][AOT]$ 182% ± 13.7 and $[N_{41(2\text{-}COO\text{-}1)2}][AOT]$ 529% ± 206.9).

Table 5: Effect of ammonium and phosphonium cations when paired with the docusate anion on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[N_{4444}][AOT]$	0.11 ± 0.09	2 ± 1.6	6.75 ± 0.11	2 phases
$[N_{112(2\text{-}OH)}][AOT]$	10.77 ± 0.81	182 ± 13.7	7.00 ± 0.05	2 phases
$[N_{41(2\text{-}COO\text{-}1)2}][AOT]$	31.23 ± 12.20	529 ± 206.9	6.45 ± 0.04	2 phases
$[P_{66614}][AOT]$	4.28 ± 0.73	76 ± 13.1	7.34 ± 0.69	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

Finally, when working with some ILs that contained the anion formate (Table 6), such as $[P_{5555}]$[Formate], cultures turned to a dark yellow colour right after inoculation, affecting immediately the initial OD of the broth. Moreover, after 24 h the culture formed a copper-like colour mixture with the ionic liquid, making difficult to accurately take samples from the culture, hence affecting the OD measurements. This complication was later reflected in the high values of $[P_{5555}]$[Formate] Maximum OD_{600} and Maximum OD_{600} (%), 6.16 ± 1.77 and 109%± 31.2, respectively, with both presenting large standard deviations. Additionally, despite the fact that *E.coli* has been documented to survive in a wide range of pH values (5-9) (Small *et al*. 1994), the addition of formate-based ILs combined with tetraalkylphosphonium cations was observed to shift the global pH in the medium towards the threshold of survival growth rate, thus decreasing the viability of the strain/population over time.

Table 6: Effect of alkyl phosphonium cations when paired with the formate anion IL moiety on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[P_{5555}]$[Formate]	6.16 ± 1.77	109 ± 31.2	7.87 ± 0.08	1 phase
$[P_{66614}]$[Formate]	0.08 ± 0.02	1 ± 0.4	9.16 ± 0.24	2 phases
$[P_{8884}]$[Formate]	0.01 ± 0.01	0 ± 0.2	8.81 ± 0.00	2 phases
$[P_{8886}]$[Formate]	0.16 ± 0.03	3 ± 0.5	8.67 ± 0.10	2 phases
$[P_{8888}]$[Formate]	0.24 ± 0.05	4 ± 0.9	5.51 ± 0.01	2 phases
$[P_{88810}]$[Formate]	0.21 ± 0.20	4 ± 3.6	9.20 ± 0.19	2 phases
$[P_{88812}]$[Formate]	1.18 ± 0.61	21 ± 10.8	9.12 ± 0.65	2 phases
$[P_{88814}]$[Formate]	2.09 ± 0.71	37 ± 12.5	8.95 ± 0.22	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

All these observed difficulties make tetraalkylphosphonium formate ionic liquids unsuitable for an integrated fermentation/extraction process since the downstream process will be more complicated due to the possible inconveniences to separate the IL from the culture.

Regarding the heterocyclic-based ILs such as the N-alkyl-methyl imidazolium and the N-alkyl-N-methyl pyrrolidinium, reported publications have stated that the biocompatibility of these kinds of ILs is directly dependent on the length of the alkyl chain, with a well-known increase in toxicity with increasing alkyl side chain length (Docherty and Kulpa, Jr. 2005; Demberelnyamba *et al*. 2004). Therefore, the selected imidazolium, piperidinium and pyrrolidinium ILs listed in Table 7 presented a side chain length limited to 4 carbon atoms, aiming to prevent the absorption of the lipophilic cations into the membrane and avoiding its disruption. When comparing the results obtained for the maximal growth in the presence of the various ILs (Table 7), it is firstly observed that

ionic liquids containing either aromatic head groups such as imidazolium, or non-aromatic heterocycles, such as piperidinium and pyrrolidinium were all highly toxic towards *E. coli* when paired with the bistriflimide anion since they completely inhibited growth when *E coli* was cultured in complex media. This behaviour is consistent with the intrinsic cytotoxicity of the bistriflimide anion against rat cells and different aquatic organisms when paired with heterocyclic aromatic and non-aromatic cations with different alkyl chain lengths, explained by the temporary formation of ion pairs in the aqueous solution of these specific combinations of cations and anions, leading to a higher bioavailability and higher membrane disturbance (Stolte *et al.* 2007b; Stolte *et al.* 2007a). However, when the $[NTf_2]^-$ anion was paired with quaternary ammonium and phosphonium cations (Table 7), the ILs became less toxic towards *E. coli*. This trend is in agreement with Wood *et al*. (2011), who showed the biocompatibility of different long alkyl chain quaternary ammonium-based ILs when paired with the bistriflimide anion. Moreover, recent work performed in our lab has shown the biocompatibility of these bistriflimide ionic liquids towards other microorganisms such the *Trachydiscus minutus* algae (Aidan Grimsley, personal communication) and *Saccharomyces cerevisiae* (Disley 2016). It is proposed that the electronegative oxygen and fluorine atoms within the bistriflimide anion are able to form dipole interactions with the hydrogen atoms of the long alkyl chains, resulting in the bistriflimide anion being enwrapped by the alkyl chains, hence preventing the insertion of the alkyl chains into the cytoplasmic membrane. This provides a rationale for the apparent lack of toxicity of cations with long alkyl chains paired with bistriflimide. On the other hand, Wood *et al*. suggested that the concentration in the aqueous phase of a hydrophobic anion would depend on the hydrophilicity of the cation, which could “pull” the anion into the media. In this case, coordination of bistriflimide with the more highly hydrophobic ammonium and phosphonium cations would probably have the contrary effect, thus avoiding further addition of the bistriflimide anion in the liquid media. Regarding the pH values of the culture media, despite the addition of ILs to the media, pH was kept within the range of survival of *E. coli*, suggesting that the

absence of growth was due to the toxic effects of the ILs towards the microorganisms rather than any a chemical reaction with the media.

Since it seems to be able to exhibit both completely toxic and entirely biocompatible ILs containing the $[NTf2]^-$ anion, this indicates that the choice of cation can have an important effect with regards to altering the toxicity of an IL. Table 7 clearly shows that the combination of both cation and anion have significant effects on the expressed toxicities of these ILs as $[P_{66614}][NTf_2]$, $[P_{4448}][NTf_2]$ and $[N_{8881}][NTf_2]$ all showed to be biocompatible ILs with *E. coli*, whereas $[BMim][NTf_2]$, $[BMPyrr][NTf_2]$, $[BMPip][NTf_2]$ and $[EMim][NTf_2]$, completely inhibited growth, demonstrating the cationic influence on $[NTf_2]$ toxicity.

Table 7: Effect of varied cation IL moieties when paired with the bistriflimide anion on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[BMim][NTf_2]$	0.04 ± 0.00	1 ± 0.0	--	2 phases
$[BMPip][NTf_2]$	0.05 ± 0.02	1 ±0.5	6.80 ± 0.00	2 phases
$[BMPyrr][NTf_2]$	0.04 ± 0.00	1 ±0.1	6.75 ± 0.05	2 phases
$[EMim][NTf_2]$	0.05 ± 0.00	1 ± 0.0	6.98 ± 0.05	2 phases
$[N_{8881}][NTf_2]$	3.82 ± 0.06	91 ± 1.0	6.80 ± 0.07	2 phases
$[N_{222(OH)}][NTf_2]$	0.00 ± 0.00	0 ± 0.0	5.86 ± 0.06	2 phases
$[P_{4448}][NTf_2]$	3.93 ± 0.13	94 ± 3.1	7.09 ± 0.64	2 phases
$[P_{66614}][NTf_2]$	3.02 ± 0.14	72 ± 3.3	8.84 ± 0.19	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

ILs containing halide anion such as $[Cl]^-$ and $[Br]^-$, and either a quaternary ammonium or phosphonium cation were highly toxic ionic liquids (Table 8). This behaviour is in agreement with reported studies working with

alkyltrihexylphosphonium chlorides and bromides, with alkyl chains from 6 to 14 carbons length, that showed different levels of antimicrobial activity against *E. coli*, *B. subtilis* and 4 different types of cocci (Cieniecka-Rosłonkiewicz *et al*. 2005; Wood *et al*. 2011). Nonetheless, Cieniecka-Rosłonkiewicz *et al*. also showed that the toxicity of phosphonium chloride towards *E. coli* and *Candida* decreased when the alkyl chain was increased (from 8 to 14 carbons). This could explain the only exception observed for the $[P_{88812}][Cl]$ ionic liquid, which allowed a final maximal OD_{600} equivalent to 54% of the maximal growth of the control. On the other hand, this trend was not confirmed with $[P_{88814}][Cl]$, and a high toxicity was observed with this IL. This result could also suggest that the presence of impurities in the ILs with similar alkyl chain length, such as $[P_{8888}][Cl]$, $[P_{88810}][Cl]$ and $[P_{88814}][Cl]$, could have affected the cells and the pH of the medium, thus inhibiting their growth. Therefore, further analysis of the purity of these ILs by NMR would have been desirable in order to verify this hypothesis. However, unfortunately the ILs used herein were donated by Prof Kenneth Seddon, from the Queen's University Belfast, UK, and there were no ILs available after this set of experiments to run additional tests. In conclusion, it is difficult to ascertain whether the toxicity was due to the intrinsic influence of the ILs, or to impurities present in IL samples. Moreover, it can be noticed that the addition of ionic liquids containing a halide anion has a great effect on the overall pH, changing the pH of the medium below the acid limit for growth (about 4.5) (Small *et al*. 1994), thus repressing the growth of the *E. coli* cells. In the case of the $[P_{88812}][Cl]$ the pH was maintained near an optimal value of 6.97, thus allowing the cells to grow. These results suggest a relationship between the ionic liquid structure and its capacity to shift the pH of the medium towards toxic limits.

Table 8: Effect of quaternary ammonium and phosphonium cation IL moieties when paired to halide anions on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[N_{24(2\text{-}OH)2}][Br]$	0.01 ± 0.00	0 ± 0.1	6.68 ± 0.02	1 phase
$[N_{1148}][Br]$	0.12 ± 0.00	2 ± 0.1	5.21 ± 0.05	1 phase
$[N_{1148}][Cl]$	0.21 ± 0.08	4 ± 1.4	6.73 ± 0.01	1 phase
$[P_{4448}][Cl]$	0.09 ± 0.01	2 ± 0.3	3.95 ± 0.02	1 phase
$[P_{6667}][Cl]$	0.00 ± 0.00	0 ± 0.0	2.64 ± 0.34	2 phases
$[P_{6668}][Cl]$	0.03 ± 0.05	1 ± 0.8	3.04 ± 0.30	2 phases
$[P_{66614}][Cl]$	0.09 ± 0.02	2 ± 0.4	5.12 ± 0.14	2 phases
$[P_{8884}][Cl]$	0.00 ± 0.00	0 ± 0.0	4.18 ± 0.01	2 phases
$[P_{8885}][Cl]$	0.04 ± 0.03	1 ± 0.5	4.13 ± 0.14	2 phases
$[P_{8886}][Cl]$	0.09 ± 0.06	2 ± 1.1	5.06 ± 0.04	2 phases
$[P_{8887}][Cl]$	0.06 ± 0.04	1 ± 0.8	4.21 ± 0.12	2 phases
$[P_{8888}][Cl]$	0.07 ± 0.03	1 ± 0.5	4.62 ± 0.43	2 phases
$[P_{88810}][Cl]$	0.14 ± 0.09	2 ± 1.7	5.61 ± 0.22	2 phases
$[P_{88812}][Cl]$	3.03 ± 0.89	54 ± 15.8	6.97 ± 0.29	2 phases
$[P_{88814}][Cl]$	0.09 ± 0.07	2 ± 1.3	4.30 ± 0.15	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

Substitution of one or more of the alkyl chains for an alcohol group in various alkyl ammonium ionic liquids have been showed to decrease the ionic liquid toxic activity towards *E. coli* (Wood *et al*. 2011). Additionally, quaternary ammonium-based ILs paired with alkyl sulfate anions were shown to be relatively non-toxic towards this kind of bacterium. When working with quaternary ammonium-based ILs, neither the introduction of a hydroxyl group

in the cation alkyl chain nor the exchange for an alkyl sulfate anion of an ammonium-based ILs resulted in a biocompatible ionic liquid (Table 9), which instead turned out to be highly toxic, inhibiting growth completely. The discrepancy in trends with previous reports could be explained by the different methodology used for the toxicity tests in each work; whilst in the present work a 20% v/v IL phase was used, in their previous work Wood *et al*. (2011) used only a 2% v/v IL phase; a 10-fold higher IL concentration in this work suggests that the total amount of the IL phase also influences the toxic activity of the ILs. Additionally, in previous work minimal MSX medium was used, which has a higher buffer capacity compared to the complex LB media used in the present case, which could have helped counteract the increase in the pH towards the alkaline threshold of *E. coli* previously reported to be pH 9 (Small *et al*. 1994) (Table 9).

Table 9: Effect of varied anion IL moieties when paired with alkyl ammonium hydroxylated cations on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[N_{222(OH)}][NTf_2]$	0.00 ± 0.00	0 ± 0.0	5.86 ± 0.06	2 phases
$[N_{114(2\text{-}OH)}][DCA]$	0.01 ± 0.00	0 ± 0.0	8.95 ± 0.31	1 phase
$[N_{24(2\text{-}OH)2}][DCA]$	0.02 ± 0.00	0 ± 0.1	9.14 ± 0.09	1 phase
$[N_{112(3\text{-}OH)}][C_2SO_4]$	0.06 ± 0.01	1 ± 0.1	9.38 ± 0.04	1 phase
$[N_{112(2\text{-}OH)}][C_2SO_4]$	0.00 ± 0.00	0 ± 0.0	8.01 ± 0.05	1 phase
$[N_{1(2\text{-}OH)3}][C_1SO_4]$	0.00 ± 0.00	0 ± 0.0	6.75 ± 0.02	1 phase

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

Comparison of the toxicities of ILs containing a phosphonium cationic core suggests specific toxic effects due to both the cationic and anionic components, individually (Table 10). The majority of $[P_{66614}]^+$ ILs appear to be biocompatible, reaching Maximum OD_{600} % values even greater than 90% of the maximum OD of the control. Exceptions include the coordination of $[P_{66614}]^+$ with chloride, formate and hydrogen sulfate anions, all of which completely inhibited *E.coli* growth and changed the pH of the culture towards either the acidic or basic survival limit, thus supporting the earlier assumption that toxicity of some ILs is due to their capacity to shift the pH of the culture medium towards toxic thresholds. Another exception is when $[P_{66614}]^+$ is paired with the acetate anion (Table 11), which, on the other hand, inhibits growth even though the pH was maintained close to neutral values. This behaviour was observed when the acetate anion was paired with phosphonium cations of different alkyl chain lengths, suggesting that *E. coli* is sensitive to the acetate anion, specifically, rather than to the cationic component.

Cost and biodegradability of ionic liquids have also been a main concern in the evaluation of ILs as alternative solvents for any catalytic process, and particularly in a process leading to a moderately added-value product like styrene. The high cost of the reagents for the synthesis of the ionic liquids have constraint their general use at an industrial level; and additionally, because of the relatively high stability of ionic liquids, their persistence in the environment becomes feasible and therefore their biodegradability has also become an important issue when evaluating their environmental impact (Fukumoto, Yoshizawa, and Ohno 2005; Fukaya *et al*. 2007). The use of saccharin $[Sacc]^-$, acesulfame $[Ace]^-$, salicylate $[Sal]^-$ and cyclamate $[Cyc]^-$, as alternative anions offering reduced prices, have been suggested as a good starting point to form less toxic, more readily biodegradable and cheaper options than ILs not derived from bioresources (Carter *et al*. 2004). Therefore, a range of quaternary phosphonium cations paired with either of these anions were tested for toxicity (Table 10).

Of the shorter chain phosphonium ILs $[P_{4448}][Sacch]$, $[P_{4448}][Sal]$, $[P_{4448}][Ace]$ and $[P_{4448}][Cyc]$, all inhibited growth completely. Conversely, $[P_{4448}][NTf_2]$ and $[P_{4448}][OTf]$ showed a higher degree of biocompatibility, achieving Maximum OD of 3.93 and 2.33, respectively, which would indicate a specific anion effect on the toxicity when coordinated with the $[P_{4448}]^+$ cation. This observed trend in toxicity could be attributed to an increased solubility of the IL in the aqueous phase, allowing increased interactions of both that cation and anion with the cell surface. This is further supported by the observation that $[P_{4448}][NTf_2]$ demonstrated complete biocompatibility with *E. coli*. As the $[NTf_2]^-$ anion is highly fluorous and hydrophobic in nature, it maintains a much lower water solubility than chaotropic anions such as salicylate and saccharinate, which are highly water soluble, and can therefore interrupt the hydrogen bonding network between water molecules. On the other hand, combination of the same anions with the more highly hydrophobic cation $[P_{66614}]^+$, resulted in a less significant inhibition over the cell growth by the presence of the IL, indicating that the toxicity had been reduced significantly on extension of the alkyl chains within the cation. This outcome suggests that it is the combination of the $[P_{4448}]^+$ cation with the chaotropic anions which creates the inhibitive effects, and supports the statement that an IL is only as toxic as its most toxic component. However, there is a strong indication that chaotropicity of the anion and hydrophilicity of both the cation and anion plays a large role in the resulting toxicity of the IL. Moreover, since the majority of the $[P_{66614}]^+$ ILs showed an increased biocompatibility, it appears that an increase in the alkyl chain length decreases the toxicity of this class of ILs, which may be unexpected as generally the mechanism of toxicity of the cation is due to insertion of the alkyl chains in the plasma membrane of cells. This observed trend related to the toxicity of the phosphonium ILs, challenges previous reports that have stated that there is an increased toxicity of IL with increasing alkyl chain length (Demberelnyamba *et al*. 2004; Docherty and Kulpa, Jr. 2005; Matsumoto *et al*. 2004). The reduced toxicity shown by this kind of IL is believed to be due to the high charge density phosphonium core being shielded by the long lipophilic chains, essentially preventing attraction of the positively charged atom to the

negatively charged phospholipid cell membrane within microorganisms. Additionally, these long, bulky, alkyl chains also ensure that the IL is hydrophobic in character allowing for the enhancement of a biphasic system. Lastly, it was observed that each of the ionic liquids that allowed *E. coli* cell growth kept the pH of the medium within its optimal growth range; and on the contrary, the ILs that inhibited cell growth changed the pH of the cultures close to the toxic limits, indicating an interaction with the complex medium. This is particularly noticeable with the ILs holding the $[P_{4441}]^+$ cation, supporting the idea that an increase in solubility of the IL would allow a higher interaction of both cation and anion moieties with the cell membrane.

Regarding the anion contribution to ionic liquid toxicity, it has been suggested that the anion exerts almost no effect in the case of a range of imidazolium ILs (Docherty and Kulpa, Jr., 2005; Garcia, Gathergood, and Scammells, 2005; Pernak *et al*., 2002). However, this is not the case for the quaternary phosphonium and ammonium salts (Tables 5-11), since both cation structure and anion type have an influence on their antimicrobial activity in this kind of ionic liquid. In this regard, a small number of publications have reported on the contribution of anion specific effects towards the toxicity of ILs (Biczak *et al*., 2014; Blundell and Licence, 2014; Garcia *et al*., 2005), although the mechanism in which they act upon cells is much more poorly understood than for cationic effects.

Table 10: Effect of varied IL anion moieties when paired with tetraalkylphosphonium cations on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[P_{4448}][Cl]$	0.09 ± 0.01	2 ± 0.3	3.95 ± 0.02	1 phase
$[P_{4448}][NTf_2]$	3.93 ± 0.13	94 ± 3.1	7.09 ± 0.64	2 phases
$[P_{4448}][Sal]$	0.03 ± 0.01	1 ± 0.3	5.40 ± 0.03	2 phases
$[P_{4448}][OTf]$	2.33 ± 0.25	47 ± 5.0	6.66 ± 0.01	2 phases
$[P_{4448}][Sacc]$	0.03 ± 0.00	1 ± 0.1	4.38 ± 0.06	2 phases
$[P_{4448}][Ace]$	0.04 ± 0.00	1 ± 0.0	3.80 ± 0.00	2 phases
$[P_{4448}][Cyc]$	0.04 ± 0.04	1 ± 1.2	6.81 ± 0.08	2 phases
$[P_{66614}][Cl]$	0.09 ± 0.02	2 ± 0.4	5.12 ± 0.14	2 phases
$[P_{66614}][Formate]$	0.08 ± 0.02	1 ± 0.4	9.16 ± 0.24	2 phases
$[P_{66614}][Acetate]$	0.14 ± 0.01	2 ± 0.2	6.34 ± 0.04	2 phases
$[P_{66614}][NTf_2]$	3.02 ± 0.14	72 ± 3.3	8.84 ± 0.19	2 phases
$[P_{66614}][C_7COO]$	0.44 ± 0.06	10 ± 1.4	--[a]	2 phases
$[P_{66614}][DCA]$	3.00 ± 0.11	72 ± 2.6	8.17 ± 0.92	2 phases
$[P_{66614}][(^iC_8)_2PO_2]$	1.42 ± 0.13	34 ± 3.2	8.12 ± 0.42	2 phases
$[P_{66614}][Cyc]$	1.42 ± 0.48	38 ± 12.9	--[a]	2 phases
$[P_{66614}][Sal]$	0.41 ± 0.07	11 ± 1.8	5.97 ± 0.01	2 phases
$[P_{66614}][Sacc]$	3.95 ± 0.46	95 ± 9.2	6.55 ± 0.07	2 phases
$[P_{66614}][OTf]$	3.85 ± 0.15	93 ± 4.1	8.45 ± 0.34	2 phases
$[P_{66614}][Ace]$	2.96 ± 0.08	80 ± 2.1	7.34 ± 0.86	2 phases
$[P_{66614}][Dec]$	0.02 ± 0.01	0 ± 0.3	7.69 ± 0.24	2 phases
$[P_{66614}][HSO_4]$	0.01 ± 0.01	0 ± 0.3	3.39 ± 0.55	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

[a] Cultures were lost due to broken vials after measuring the OD_{600}, hence pH measurements were not possible.

Table 11: Effect of phosphonium cations when paired with the acetate anion on the growth of *E. coli* in LB medium at 37 °C.

IL	Maximum OD_{600}	Maximum OD_{600} (%)	pH	System phases
$[P_{5555}]$[Acetate]	0.20 ± 0.04	4 ± 0.8	5.55 ± 0.10	2 phases
$[P_{6666}]$[Acetate]	0.00 ± 0.00	0 ± 0.0	6.29 ± 0.07	2 phases
$[P_{6668}]$[Acetate]	0.02 ± 0.00	0 ± 0.1	6.61 ± 0.00	2 phases
$[P_{66614}]$[Acetate]	0.14 ± 0.01	2 ± 0.2	6.34 ± 0.04	2 phases
$[P_{6888}]$[Acetate]	0.18 ± 0.13	3 ± 2.2	6.44 ± 0.04	2 phases
$[P_{8884}]$[Acetate]	0.01 ± 0.01	0 ± 0.2	6.57 ± 0.04	2 phases
$[P_{8888}]$[Acetate]	0.18 ± 0.13	3 ± 2.4	7.13 ± 0.04	2 phases
$[P_{88810}]$[Acetate]	0.14 ± 0.13	3 ± 2.4	6.70 ± 0.15	2 phases
$[P_{88812}]$[Acetate]	0.13 ± 0.04	2 ± 0.8	6.70 ± 0.04	2 phases

The growth of *E. coli* in LB media in the presence of ILs – A seed culture (20 mL) of *E. coli* MG1655 was grown in LB medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in LB media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} values were measured at 24 h and are reported as the Maximum OD_{600}. Results and standard deviations are the average of triplicate experiments and are also displayed as a percentage of the control containing no IL.

The ILs which presented the highest biocompatibility towards *E. coli* in complex media, based mainly on the Maximum OD_{600} values reached after 24 h growth, were $[P_{4448}][NTf_2]$, $[P_{4448}][OTf]$, $[P_{66614}][NTf_2]$, $[P_{66614}][DCA]$, $[P_{66614}][Sacch]$, $[P_{66614}][OTf]$, $[P_{66614}][Ace]$ and $[N_{8881}][NTf_2]$; however, not only their non-toxic nature was of importance when selecting an ionic liquid for further applicability in the present bioprocess. Therefore, since some of the tested ionic liquids were either miscible or very gelatinous and viscous making them not easy to work with, only the ILs that presented characteristics such as low viscosity, immiscibility and biocompatibility towards *E. coli* were used in further stages of this project.

The viscosity of an IL has a critical influence on stirring, mixing, mass transport properties, such as diffusion, and pumping operations (Deive, Rivas, and Rodríguez 2013), thus an ionic liquid with a low viscosity must be selected to make sure the mass transfer is not limited and to facilitate the mixing and the

downstream stages of this process. It was observed that ammonium-based ionic liquids presented a higher viscosity compared to the phosphonium-based ionic liquids. This unexpected trend in viscosity was rationalised by Carvalho *et al*. (2014) due to the substitution of the cation central atom with a heavier and bigger atom resulting in weaker interionic interactions due to the increase in the IL volume.

Additionally, it is known that the polarity of an ionic liquid, and hence its solubility in water, it is influenced by the length of the cation alkyl chains. In general, it has been established that longer alkyl chains attached to the cation core increase the hydrophobic nature of an ionic liquid, and therefore a decrease in the mutual solubility between water and ILs (Freire *et al.* 2007; Wasserscheid and Welton 2008). Regarding quaternary ammonium and phosphonium-based ILs, it has been reported that tetraalkylammonium and tetraalkylphosphonium cations with short alkyl chains presented a higher water sorption compared to their longer chain congeners (Carvalho *et al*. 2014). It was also indicated that the ILs containing an ammonium core have an enhanced polarity when compared with the respective phosphonium analogues due to a more noticeable charge delocalization occurring in the tetraakylammonium cation thus making this cation moiety more polarised, suggesting that they would have an improved attraction towards polar compounds. Therefore, taking all these results together, it can be speculated that, if the hydrophobicity of the IL is a key factor for styrene extraction, tetraalkylphosphonium ILs with longer chains could potentially have a higher recovery capacity for styrene from an aqueous phase.

In efforts to predict the interaction of different ammonium and phosphonium-based ILs with polar molecules, water uptake levels of ionic liquids have been related, to a very reasonable approximation, to the polarity of the ILs (Disley 2016). It was observed that ILs comprising of the anions $[NTf_2]^-$ and $[OTf]^-$ presented very low uptake ratios of water, demonstrating a hydrophobic character. This behaviour was attributed to the presence of the hydrophobic and electronegative fluorine atoms which produce a noticeable charge

delocalization around the molecule. Therefore, based on the low degree of miscibility with water presented by ILs containing these anions, and the common principle described as “like dissolves like”, it would be expected that ILs comprising these kinds of anions would be more successful at extracting styrene.

The biodegradability of ionic liquids should be also taken into account for the selection of an ionic liquid. Additionally, since cost is a critical concern in the development of any industrial process, synthesis of cost-effective solvents is of great importance to improve the economics of a production process. These two important concerns can be met within the conscious design of ILs using materials derived from bio-renewable feedstocks or using non-toxic low-cost biomaterials. The use of saccharin and acesulfame for the formation of new ILs, both extensively used as non-nutritive sweeteners, have been previously reported (Carter *et al*. 2004), and in the present work we have demonstrated their biocompatibility when they are paired with quaternary phosphonium cations, which suggest their possible use in biocatalytic processes as cheaper alternatives in order to enhance the global sustainability of the process.

Lastly, in addition to the higher biocompatibility observed throughout this work in comparison to the imidazolium and ammonium ILs, alkyl phosphonium-based ionic liquids present a higher thermal stability than the corresponding ammonium and imidazolium salts (Fraser and MacFarlane 2009), an important feature that makes them a more interesting choice for industrial purposes, since it would ease further downstream processes, such as separation of the target product by distillation, which usually operates at high temperatures, and the subsequent recycle of the ILs allowing its reuse. Therefore, according to the above-mentioned characteristics, only the following ILs: $[P_{66614}][NTf_2]$, $[P_{66614}]$[Sacch], $[P_{66614}]$[OTf] and $[P_{66614}]$[Ace], were taken forward for further testing of their styrene extraction capacities in the next stages of the project.

5.1.3 Extraction of styrene with biocompatible ionic liquids

Despite the advantages that organic solvents offer to the economics of biocatalytic processes due to their low cost, recyclability and low viscosities; alternatives to these traditional solvents are being investigated due to reported issues when used in industrial fermentation processes, such as their high volatile and flammable nature represented an additional hazard for the operators and the environment (Bar 1988; Cull *et al*. 2000; Rodriguez *et al*. 2008). Therefore, it was of great interest to evaluate the styrene extraction capacities of the previously selected biocompatible ILs. In order to be considered viable options for an integrated styrene production system, they must also present high distribution ratios and extraction capacity for styrene.

The aim of this experiment was to measure the capacities of particular biocompatible ionic liquids identified in the previous toxicity tests to extract styrene and determine their distribution coefficient in order to compare their efficiencies to extract styrene and, also, to elucidate a relationship, if any, between the styrene extraction and the structure of ionic liquids, which would help to future design of a more suitable ionic liquid for an integrated process of bioproduction of styrene or other compounds with similar structure. Besides being biocompatible, the considered ILs were purposely selected to be water immiscible, hence a two-phase system would be obtained with the aqueous media and distribution ratios and extraction efficiencies could be determined for each IL.

First, the affinity of each ionic liquid towards styrene must be estimated. This affinity is represented by the partition coefficient (K_P) parameter, which refers to the ratio of styrene concentrations (or any compound), specifically for the un-ionised molecules, in an immiscible two-phase system at dynamic equilibrium. K_P is a simple but robust parameter used to determine the extraction efficiency (EE) of the process (Cascon *et al*. 2011). A large K_P value

would indicate that the extraction of styrene into the IL-rich phase is favourable, and it is calculated according to Equation 4:

$$K_P = \frac{[\text{Styrene}_{\text{IL}}]}{[\text{Styrene}_{\text{aq}}]}$$

Equation 4

where [$\text{Styrene}_{\text{IL}}$] and [$\text{Styrene}_{\text{aq}}$] are styrene concentrations at equilibrium in the ionic liquid (IL) and aqueous (aq) phase, respectively.

However, to evaluate the efficiency of the extraction process, the total concentration of styrene in each phase must be considered. This is quantified by the distribution coefficient (D), which is a parameter that considers all forms of the compound—ionised plus unionised, at equilibrium (Equation 5). In this particular two-phase system, as styrene is only present in the unionised form, K_P and D will have the same value. The extraction efficiency was then calculated by dividing the amount of styrene extracted into the IL phase over the initial amount of styrene present in the aqueous phase (Equation 6).

$$D = \frac{[\text{Styrene}_{\text{IL}}]_{\text{total}}}{[\text{Styrene}_{\text{aq}}]_{\text{total}}}$$

Equation 5

$$Percentage\ removal = \frac{C_0 - C_t}{C_0} * 100\ \%$$

Equation 6

where, C_0 is the initial concentration of styrene in the media and C_t is the concentration of styrene in the aqueous phase after a time between 0 and t.

Extraction tests were carried out as described in the Materials and Methods section (4.5). Briefly, a styrene stock solution (2.5 mM) was prepared in MSX medium and then aliquots (8 mL) were transferred into 40 mL sealed glass vials with PTFE liners. Later a specific volume of IL (2 mL, 20% v/v) was added to the vials. Vials were placed in the incubator at 37 °C and 200 rpm for 48 hours. Samples from the aqueous phase were taken regularly. Final samples at 48 hours were left 1 day leading to phase separation at equilibrium. The concentration of styrene which remained in the aqueous phase was then determined by HPLC. Distribution coefficients and percentage removal of styrene for each ionic liquid were then calculated using the Equations 5 and 6 (Tables 12-13).

First, it was important to establish the kinetics of the extraction process in order to determine how long it would take to reduce the styrene concentration from toxic levels. Therefore, extraction of styrene was monitored by taking samples at defined time intervals of 15 min during the first hour and extraction efficiencies were calculated (Table 12).

Table 12: Efficiency of styrene extraction into different biocompatible ILs at various time intervals.

	IL - % Extraction (avg)			
t(h)	**$[P_{66614}][Sacc]$**	**$[P_{66614}][Ace]$**	**$[P_{66614}][NTf_2]$**	**$[P_{66614}][OTf]$**
0.25	92.1 ± 1.05	93.6 ± 0.26	88.7 ± 2.06	91.5 ± 0.37
0.5	93.9 ± 0.11	93.9 ± 0.02	91.3 ± 0.72	94.4 ± 0.09
0.75	94.0 ± 0.02	94.0 ± 0.06	93.2 ± 0.91	94.5 ± 0.03
1	94.0 ± 0.07	93.9 ± 0.08	93.9 ± 0.49	94.5 ± 0.04
2	95.0 ± 0.01	95.0 ± 0.02	94.8 ± 0.09	94.9 ± 0.07
3	95.0 ±0.06	94.8 ± 0.09	94.9 ± 0,06	94.9 ± 0.11
6	95.0 ± 0.06	94.8 ± 0.05	94.9 ± 0.10	95.0 ± 0.03
24	95.0 ± 0.03	94.8 ± 0.02	94.9 ± 0.07	94.9 ± 0.09

Extraction efficiencies of biocompatible ILs of styrene in MSX at 37 °C – ILs extractions were carried out in 40 mL Teflon vials containing a stock solution of 2.5 mM styrene in MSX and IL (20% v/v). The IL systems were incubated at 37 °C for 48 h with constant agitation (200 rpm). Styrene in the aqueous phase was detected by HPLC-UV (258 nm) and concentrations were calculated from calibrations curves prepared in MSX using authentic styrene. Results and standard deviations are the average of triplicate experiments.

The results indicated that the extraction was occurring almost immediately, since the difference in concentration of styrene in each IL after the first 30 min was negligible, showing a difference of only 1-2% in the extraction efficiency. This suggested that the equilibrium was reached fairly quickly and that the mass transfer of the majority of the styrene from the aqueous phase to the ionic liquid has ceased, and therefore the concentrations of styrene in each phase would remain constant. Even though the maximum extraction by the ILs seems to occur immediately, as the extraction process to be achieved is dependent upon the styrene first being produced from within the cell and then to come into contact with the ionic liquid phase, it would be expected that in the presence of the biocatalyst it would take a longer time for the equilibrium to be reached due to, firstly, the difficulty of styrene escaping from the cells into the bulk medium, and, also, the obstruction to styrene to diffuse across the

medium imposed by the presence of cells (Crabbe, Tse, and Munro 1986), thereby also affecting the extraction efficiency.

Table 13: Distribution ratio and extraction efficiency of biocompatible ionic liquids towards styrene and 37 °C after 48 h.

IL	Distribution ratio (D)	Extraction efficiency (EE) (%)
$[P_{66614}][Sacc]$	18.3 ± 0.00	94.8 ± 0.00
$[P_{66614}][Ace]$	18.4 ± 0.02	94.8 ± 0.01
$[P_{66614}][NTf_2]$	18.5 ± 0.04	94.9 ± 0.01
$[P_{66614}][OTf]$	18.4 ± 0.02	94.8 ± 0.00

Extraction efficiencies of biocompatible ILs of styrene in MSX at 37 °C – ILs extractions were carried out in 40 mL Teflon vials containing a stock solution of 2.5 mM styrene in MSX and IL (20% v/v). The IL systems were incubated at 37 °C for 48 h with constant agitation (200 rpm). Styrene in the aqueous phase was detected by HPLC-UV (258 nm) and concentrations were calculated from calibrations curves prepared in MSX using authentic styrene. Results and standard deviations are the average of triplicate experiments.

As indicated earlier, a high distribution ratio for the organic phase it would be desirable in order to reduce the used volume of the extractant compound, and thus improve the economics of the process regarding cost, recovery and recycling of the ionic liquids. It is observed that all the investigated ionic liquids extracted close to 95% of the styrene present in the aqueous phase and achieved high distribution coefficients after 48 h (Table 13), which denote their huge potential to be used for the *in situ* extraction of styrene in the bioproduction process. Analysis of the IL structures and their capacity to dissolve styrene suggest a relationship between the polarity of the anion and cation moieties and the obtained extraction efficiencies. The high extraction of the ILs paired with less polar and highly hydrophobic anions, such as $[NTf_2]^-$ and $[OTf]^-$, could be explained since these anions can delocalise their charge across the molecule due to the electronegative fluorine atoms part of their structure, enhancing the induced ion-dipole interactions with the styrene molecule. Particularly, the anion $[NTf_2]^-$ is a large, non-coordinating charge diffuse anion,

in which negative charge is delocalised among the sulfur and nitrogen atoms, in addition to its negative charge being shielded by the oxygen atoms and the CF_3 groups (Fang, Smith, and Qi 2014), thus reducing further the coulombic ion—ion interactions with polar molecules, hence increasing the level of the van der Waals interaction with apolar compounds. On the other hand, the $[Sacc]^-$ and $[Ace]^-$ anions were previously indicated as more hydrophilic and chaotropic anions, suggesting a more polar character; however, the presence of aromatic rings and double bonds in both anions could also offer the potential for π—π interactions with the aromatic ring and unsaturated double bond of styrene. These kind of interactions were also observed by Disley (2016) when extracting methacrylic acid with [Sal] and [Sacch]-based ILs, suggesting that the π—π interactions with the unsaturated double bond of the methacrylic acid molecules enhanced the extraction abilities of these ILs towards this compound. The influence of the cation in the extraction of styrene would be linked to the decrease in the polarity with an increase of the alkyl chains, which is believed to be due to the high charge density phosphonium core being shielded by the long lipophilic chains.

5.1.4 Styrene toxic threshold in the presence of biocompatible ionic liquids

In the previous experiments the use of ILs as an extracting phase to reduce the concentration of styrene in the aqueous phase from toxic levels has been successfully demonstrated; however, the maximal capacity of ILs to retain styrene remain undetermined due to the complete miscibility between styrene and the selected ILs, thus making it difficult to clearly estimate the saturation point by optical visualisation of the IL with styrene. Therefore, the aim of this experiment was to develop an indirect method to measure the maximum extraction capacity of the selected ILs by assessing the possibility of an increase to the toxicity threshold of styrene towards *E. coli* by using biocompatible ionic liquids as a secondary phase in the culture medium, thus the impact of the

exogenous addition of styrene at a higher final concentration to growing cultures was investigated in the presence of ILs. To minimise the amount of IL used, it was decided to scale down the cultures to small 4 mL vials.

Seed cultures of *E. coli* MG1655 were prepared in minimal MSX medium and grown overnight then used to inoculate sealed glass vials (4 mL) with PTFE caps containing MSX medium with ionic liquid (20% v/v) or without (control); to a final OD_{600} of ~0.05. Styrene was added to the flasks reaching a 25 mM final concentration. Control cultures were also grown with no ionic liquid nor styrene present in the medium. Cultures were incubated (37 °C, 200 rpm, 24 h) and samples were taken only at the end of the assay to measure OD_{600}, in order to avoid styrene loses by volatilization (Figure 29).

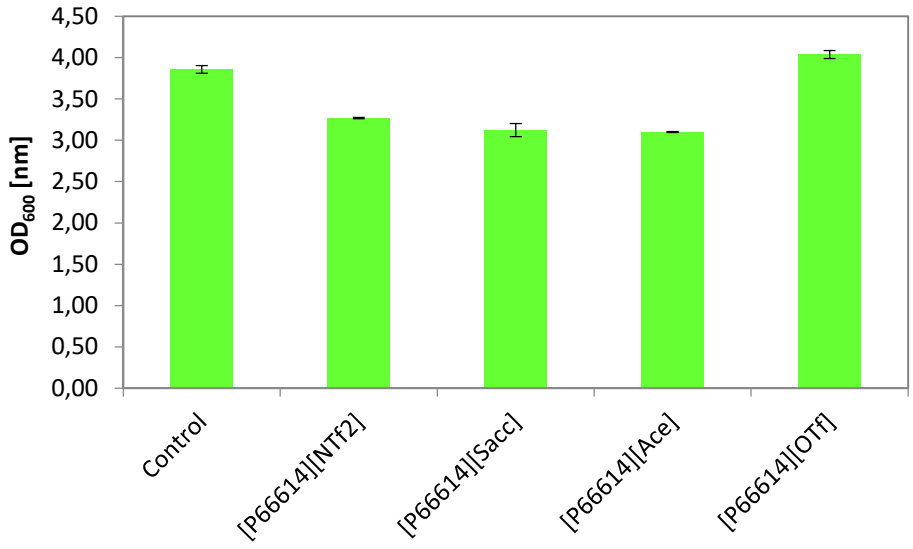


Figure 29: Growth of *E. coli* in the presence of styrene 25 mM and ionic liquid 20% v/v.

A seed culture (20 mL) of *E. coli* MG1655 was grown in MSX medium and then used to inoculate sealed glass vials (4 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in MSX (1 mL) medium. Styrene (25 mM) and IL (20% v/v) were added. A negative control containing no IL nor styrene was also run. OD_{600} was then measured and toxicity of styrene evaluated on the basis of the Maximum OD_{600} reached. Maximum OD_{600} values were taken as the highest OD_{600} after 24 h during stationary phase. Results and standard deviations are the average of triplicate experiments.

E. coli grew normally in all of the ionic liquids tested in the presence of 25 mM of styrene (Figure 29), which represent a 10-fold increase in the toxicity threshold when compared to the toxicity limit established earlier in the absence of ionic liquid, with the majority of ILs achieving Maximum OD_{600} up to 90% of that of the control, or similar values such as in the particular case of $[P_{66614}][OTf]$. Interesting results were obtained by the ILs paired with the anions saccharinate and acesulfame, which, despite their more polar character, achieved Maximum OD_{600} of 80% and 81% with respect to the control, supporting the previous suggestion that their high solubility of styrene could be due to strong π—π stacking interactions between the aromatic rings and the unsaturated double bonds present in styrene and the above-mentioned anions.

Since the purpose of this project is to develop an integrated fermentation process in a higher scale than the volumes previously used in this chapter, first using culture flasks with a working volume of 10 and 50 mL of media, and later using a fed-batch bioreactor operated at a working volume of 1 L, this extraction experiment was scaled up to similar volumes that will be used later on for the bioproduction of styrene in the presence of ionic liquid. The selected biocompatible ILs have shown so far similar extraction efficiencies at different concentrations of styrene, hence it was anticipated that ILs with similar structures would have a similar extraction behaviour at higher ILs volumes, and higher concentrations of styrene. Therefore, only the ionic liquid $[P_{66614}][NTf_2]$ was selected for the following experiments of bioproduction and extraction of styrene at a larger scale, mainly because its commercial availability makes its acquisition easier, compared to the other selected ILs that need to be synthesised in the lab.

First, the suitability of using this particular ionic liquid in a bioprocess needed to be verified, thus *E. coli* growth rates were measured in the presence of the ionic liquid. Seed cultures of *E. coli* MG1655 were prepared in minimal MSX medium and grown overnight then used to inoculate sealed glass vials (10 mL) with PTFE caps containing MSX medium with ionic liquid (20% v/v) or without (control); to a final OD_{600} of ~0.05. Cultures were incubated for further 25 h (37

°C, 200 rpm) and samples were taken periodically to measure OD_{600} and calculate growth rates. Growth inhibition was calculated accordingly to Equation 2 from the Materials and Methods section (4.4.2) (Table 14).

The presence of the ionic liquid prevented maximal growth of *E. coli* by 13%, however it was still able to grow in its presence achieving a final OD_{600} of 2.0 after 25 h (Figure 30). Additionally, growth rate in the aqueous media containing $[P_{66614}][NTf_2]$ was inhibited by only about 20% compared to the control without ionic liquid (Table 14), what it is in agreement with previously published work which found $[P_{66614}][NTf_2]$ inhibited growth of *E. coli* by 23% (Cornmell *et al*. 2008). Even though Cornmell and co-workers also performed the experiments in minimal MSX medium, growth rate measurements were made by using a using a Bioscreen C incubator/plate reader, which could explain the observed difference, since the use of the Bioscreen incubator has been reported to produce a higher variance when working with immiscible ILs (Wood *et al*. 2011).

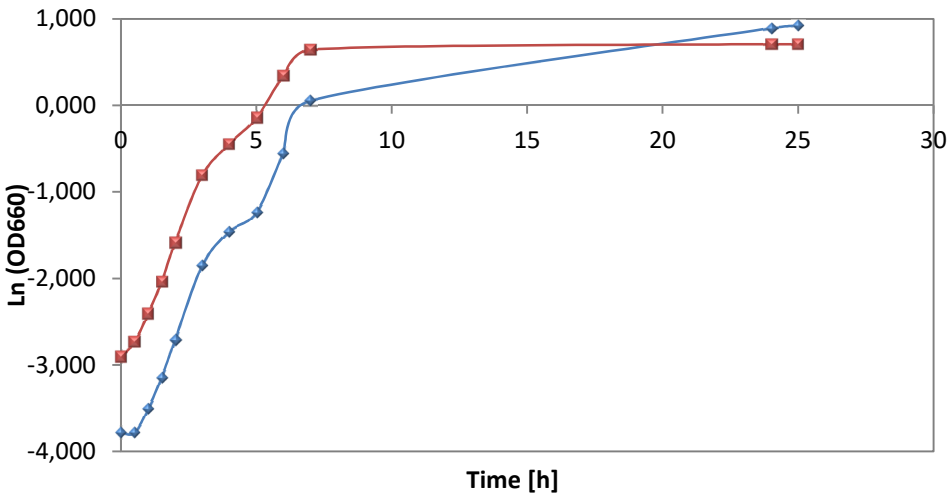


Figure 30: Growth curve of *E. coli* MG1655 in MSX in presence of $[P_{66614}][NTf_2]$ (blue diamond) and in absence of ionic liquid (red square).

A seed culture (20 mL) of *E. coli* MG1655 was grown in MSX medium and then used to inoculate sealed glass vials (40 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in MSX (10 mL) media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} was then measured periodically. Values were used to plot a growth curve and to calculate specific growth rates. Results and standard deviations are the average of triplicate experiments.

Table 14: Effect of $[P_{66614}][NTf_2]$ on growth of *E. coli* in MSX medium. Growth rates were determined in the absence (control) and presence of $[P_{66614}][NTf_2]$ (phase ratio 20% v/v).

Strain	Medium	μ_{max} (h^{-1})
MG1655	(Control)	0.623 ± 0.004
	$[P_{66614}][NTf_2]$	0.499 ± 0.009

Growth of *E. coli* in the presence of ionic liquid 20% v/v – A seed culture (20 mL) of *E. coli* MG1655 was grown in MSX medium and then used to inoculate sealed glass vials (40 mL) with PTFE caps. The cells were incubated for 24 h at 37 °C in MSX (10 mL) media and IL (20% v/v). A negative control containing no IL was also run. OD_{600} was then measured periodically. Values were used to plot a growth curve and to calculate specific growth rates. Growth curves are shown in Appendix 8.1. Results and standard deviations are the average of triplicate experiments.

Later, the impact of increasing concentrations of styrene towards the activity of the biocatalyst in the presence of the selected ionic liquid was investigated. Seed cultures of *E. coli* MG1655 were prepared in MSX broth and grown overnight. The seed culture was used to inoculate MSX medium (10 mL) with or without $[P_{66614}][NTf_2]$ (20% v/v); to a final OD_{600} of ~0.05 in 40 mL sealed glass flasks with PTFE caps. Styrene was then added to the flasks at different final concentrations. Cultures were incubated (37 °C, 200 rpm, 24 h) and samples were taken only at the end of the assay to measure OD_{600}, avoiding losses of styrene by volatilization (Figure 31).

After 24 h *E. coli* grew normally up to 50 mM styrene and from 100 to 300 mM was still able to reach an OD_{600} of roughly 50% of the OD in the control culture (Figure 31). A toxicity threshold of 300 mM is more than a 100-fold improvement compared to the toxicity limit in the absence of ionic liquids. Taking into consideration that the quantities of styrene added to cells cultures were beyond the solubility of styrene in water, this result suggests that, up to 300 mM, styrene is almost completely extracted into the ionic liquid phase and the concentration in medium remains below the toxicity level.

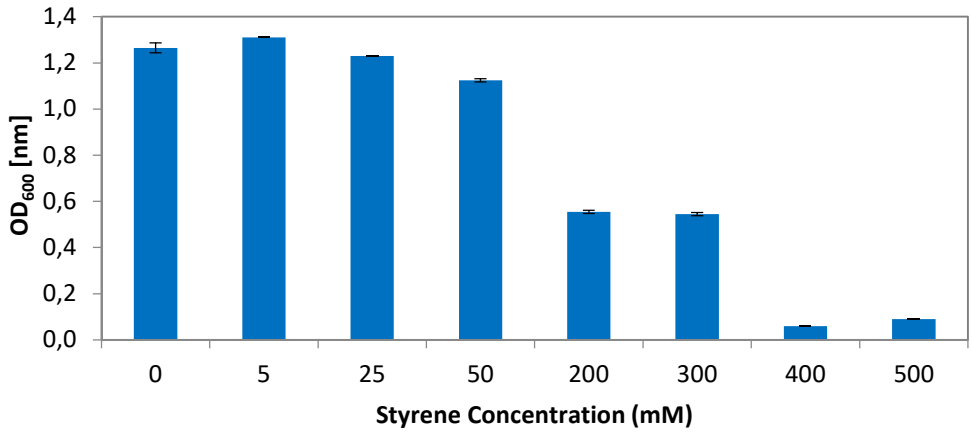


Figure 31: Growth of *E. coli* in the presence of styrene and $[P_{66614}][NTf_2]$.
A seed culture (20 mL) of *E. coli* MG1655 was grown in MSX medium and then used to inoculate sealed glass vials (40 mL) with PTFE caps containing MSX medium (10 mL) and $[P_{66614}][NTf_2]$ (20% v/v). At the same point, styrene was added at different concentrations and the cells were incubated for 24 h at 37 °C and 200 rpm. A negative control containing no IL nor styrene was also run. OD_{600} was then measured and toxicity of styrene evaluated on the basis of the Maximum OD_{600} reached. Maximum OD_{600} values were taken as the highest OD_{600} after 24 h during stationary phase. Results and standard deviations are the average of triplicate experiments.

Therefore, the ability to allow the growth of *E. coli* in the presence of high toxic concentrations of styrene, confirm the high efficiency and capacity of this particular phosphonium-based ionic liquid for *in situ* extraction of styrene, and together with the previous results, it would suggest that structurally similar ILs could have the same extraction capacity. Additionally, these results illustrate the possibility to use the measure of the biological growth of a microorganism in the presence of a biocompatible IL and toxic concentrations of styrene as an indirect method to estimate the solvent capacity. However, to determine an exact and accurate value of the extraction capacity of these solvents the analysis of both the IL and aqueous phase would be required.

5.1.5 Conclusions and discussion

Styrene toxicity towards *E. coli* MG1655 was investigated, and the preliminary tests under the studied conditions have shown that cell growth was completely inhibited at 2.5 mM. The established toxic threshold was slightly lower than the previously reported 3 mM limit (McKenna and Nielsen, 2011); however, the present experiments were carried out in minimal medium, crucial in the economic perspective of an industrial scale process, and this might have affected cells resistance, whereas McKenna and Nielsen (2011) used complex LB medium, rich in essential amino acids to the growing bacteria, in addition to vitamins, trace elements and minerals that enhance bacterial growth. Furthermore, in their previous work McKenna and Nielsen (2011) measured the impact of exogenous styrene by adding increasing concentration to cultures with an initial cell density of OD_{600} ~0.6, when the bacterial cells were in an established exponential growth phase, which represents the period when cells are most metabolically active, and perhaps, more resistant to the shock of toxic compounds. Additionally, it is worth noting that the toxic effects of styrene vary depending on the *E. coli* strain and the culture media used. It has been reported that 110 mg/L styrene decreased the growth of *E. coli* K12 by half its final OD when cultured in LB after only 2 h of exposure (Mingardon *et al*. 2015), while *E. coli* NST74 slowed its growth rate when exposed to 200 mg/L of styrene in the same medium (McKenna and Nielsen 2011), and growth of *E. coli* JM101 was completely inhibited at a concentration of 1.8 mM (~190 mg/L) when cultured in M9* medium, which contains three times more phosphate salts than the M9 medium previously developed by Miller (1972) (Park *et al*. 2006).

Styrene has been estimated to have a log*P* value of 2.95, making it toxic to microorganisms (Hansch and Leo 1979). Toluene, a compound with a similar chemical structure to styrene, has been widely studied regarding its toxic effects towards different microorganisms, and its log*P* value has been estimated to be 2.5. When measuring its toxic effect towards *E. coli* it was determined that a 0.1% v/v toluene shock inhibited its growth, representing an

almost 3-fold higher concentration when compared to styrene (Wood 2011). The higher toxicity of styrene towards *E. coli*, despite the similarity in structure to toluene, could be explained due to its greater partition coefficient towards lipophilic compounds therefore accumulating more in the phospholipid membrane. It has been suggested that this greater lipophilicity of styrene compared to toluene might be due to the extra C-atom (Campo *et al*. 1999).

Regarding the toxicity mechanism of styrene, it is generally accepted that, given its hydrophobic nature, its toxicity is due to damage of the cell membrane. However, membrane integrity of *E. coli* was evaluated by Lian *et al*. (2016) after 30 min exposure to 200 mg/L (~2mM) exogenous styrene and little damage was found in membrane integrity, with less than 10% of the cells presenting membrane leakage. In contrast, when the styrene was produced by the cell, the membrane damage was directly dependent on the styrene concentration, increasing the percent of total cells with damaged membranes as the total styrene produced by the cell increases, with more than 50% of the cell population presenting membrane leakage by the end of the fermentation. These results highlight the importance of the difference in the inhibition effects of target compounds when they are supplied exogenously and when they are produced intracellularly.

As it has been seen, different levels and/or types of responses to structurally similar compounds it is shown by different microorganisms. These different behaviours highlight that, even though there are certain empirical rules very useful to correlate a compound's toxicity with its structure or properties, in order to have an idea of its toxic level, it is always necessary to experimentally study the toxic effects of the target products and chosen solvents towards the selected biocatalyst to develop a more efficient biocatalytic process and production strategy. The determined toxic threshold results confirmed that the styrene's toxic nature would be an important problem during its bioproduction; therefore, the *in situ* removal of styrene from the aqueous phase whilst it is being produced could represent a viable approach to reduce the direct contact with the biocatalyst, thus preventing inhibition.

Therefore, the potential use of ILs as alternative solvents for the *in situ* extraction of styrene was evaluated. A wide range of ILs were selected based of certain specific properties to match its biocatalytic production such as their hydrophobic nature and solvent features. Then the toxicity of each IL was determined towards *E. coli* MG1655 as a potential biocatalytic host. Finally, the styrene extraction efficiencies of the aqueous phase/IL systems were determined using the biocompatible ILs that were previously identified.

First, the method developed to test the toxicity/biocompatibility of ionic liquid using culture flasks on a small scale provides a simple and economic screening method to test a large number of different ionic liquids structures since it can be performed using cheap microbiological media and a small amount of ionic liquid. Additionally, it can be used regardless of their miscible or immiscible behaviour in the aqueous phase and, based on the small standard deviation obtained in the majority of the replicates tested, it offers a reliable and straightforward screening system. Moreover, the major advantage of the developed method in comparison to other high-throughput tests, such as the agar diffusion test and the Bioscreen incubator/plate reader, is the possibility to measure the changes in the pH of the culture due to the addition of the solvent, providing critical information to explain, in more depth, the toxicity of the studied compounds. Nevertheless, care should be taken when working with ionic liquids presenting high viscosities due to the difficulties of handling the small volumes with accuracy and, furthermore, attention must be paid when assessing information of ionic liquids for which precipitate formation or gel/paste mixture are observed as this could indicate a physico-chemical interaction with the medium affecting the OD readings. Despite the latter limitations, the results obtained show that the method allows for an easy identification of biocompatible ionic liquids suitable for their further study in a particular biocatalytic processes.

Regarding the interaction of the studied ILs with the culture media, the information acquired shows that the addition of ionic liquid to the culture medium does appear to affect the overall pH of the flask culture, and that the

degree to which the pH of the cultures is influenced by the addition of ionic liquids seems to be very much dependent on the particular ionic liquid. However, from the results collected it is difficult to ascertain if the fluctuations in the pH of the culture medium, which inhibit the cells' growth, were due to the extraction by the ionic liquids of essential components out of the growth medium, or if the fluctuations in pH are indicative of other biochemical reactions occurring in the culture medium as the result of the addition of these ionic liquids. Analysis of both the IL and the aqueous phases of the system *via* ion chromatography, could help elucidate if some components were transferred from the aqueous phase into the IL layer or if any reaction took place. Additionally, the use of a strong buffered medium would help to clarify if the growth inhibition is mainly due to the capacity of the IL to change the pH of the culture or rather due to the interaction of the IL with the cell membrane.

It has been shown that the toxicity of ILs is greatly influenced by their chemical structure, *e.g.* the alkyl chain length, cation family and anionic moiety. Regarding the cation family and alkyl chain length, the ionic liquids comprised of aromatic or non-aromatic heterocycles containing nitrogen cations are highly toxic, completely inhibiting the growth of *E. coli*. Most of the quaternary ammonium-based ILs inhibited the growth of the cells, irrespective of the alkyl length chain; however, the majority of these ILs were miscible with water, increasing the possibility of interaction with the cell membrane. Interestingly, phosphonium ionic liquids do not follow the trend of increasing toxicity with an increase in the alkyl chain, since a decrease in their inhibition impact was shown in the phosphonium-based ILs with longer alkyl chains; and $[P_{66614}]^+$ ILs in particular, regardless of the anions that were attached, shown low inhibition towards *E. coli*. Concerning the anion contribution to the toxicity of the ionic liquids, its influence is more noticeable when they are coordinate to less toxic cations. Mainly, anions of greater chaotropicity play a major role in the toxicity of ILs; however, generally, only when paired with shorter chain phosphonium ILs. Particular influence of the anion can be observed in the $[P_{4448}]^+$ ILs, which presented a varied toxicity depending on the specific coordinated anion.

Concerning the extraction process, the selected phosphonium ILs showed a high efficiency and capacity for the extraction of styrene supported by the high distribution coefficients obtained, hence reducing the toxic concentrations of styrene in the aqueous phase. The high affinity towards the styrene molecule is thought to be mainly due to the strong van der Waals and π—π stacking interactions between the aromatic rings of styrene and the different anions moieties of the selected ILs, which would be in accordance with previous work developed by Disley (2016). Regarding the cation influence in the extraction process, it seems that a reduction in the polar character of the cation by increasing the alkyl chain would favour the extraction of styrene. This non-polar behaviour towards apolar substances seems to be in agreement with Armstrong, He, and Liu (1999), who investigated the interaction of a variety of solutes with ILs, using ILs as stationary phases in gas chromatography. They found that ILs act as low-polarity phases to separate non-polar compounds and increasing solubility with an increase in the alkyl chain. Moreover, based on the phenomenon of "like dissolves like", it would be expected that less polar and highly hydrophobic ILs would have a higher level of interaction with other non-polar molecules such as styrene, hence increasing the rate of extraction. Therefore, the understanding of the intramolecular interactions of ILs and the target compounds is crucial for the selection of an IL as an extractive solvent. Furthermore, the elevated cost of the starting materials for the synthesis of ionic liquids has hindered their use at industrial scale and cheaper alternatives are desired. Thus, very interesting and promising results have been obtained with the quaternary phosphonium cations coordinated with the saccharinate and acesulfame anions, two artificial non-nutritive sweeteners extensively used in food and drinks as a substitute of sugars. Their use would reduce the synthetic cost of ionic liquids whilst having an improved extraction efficiency for styrene.

Finally, an indirect method to measure the styrene extraction capacity of biocompatible ionic liquids and their possible use to increase the effective toxic threshold of styrene was developed by means of measuring the growth of *E.*

coli in the presence of high toxic concentrations of styrene. Using the $[P_{66614}][NTf_2]$ ionic liquid a 100-fold improvement was achieved compared to the toxicity limit in the absence of ionic liquids, supporting the high capacity of this type of ionic liquids for the extraction of styrene.

5.2 Improving the method for styrene analysis

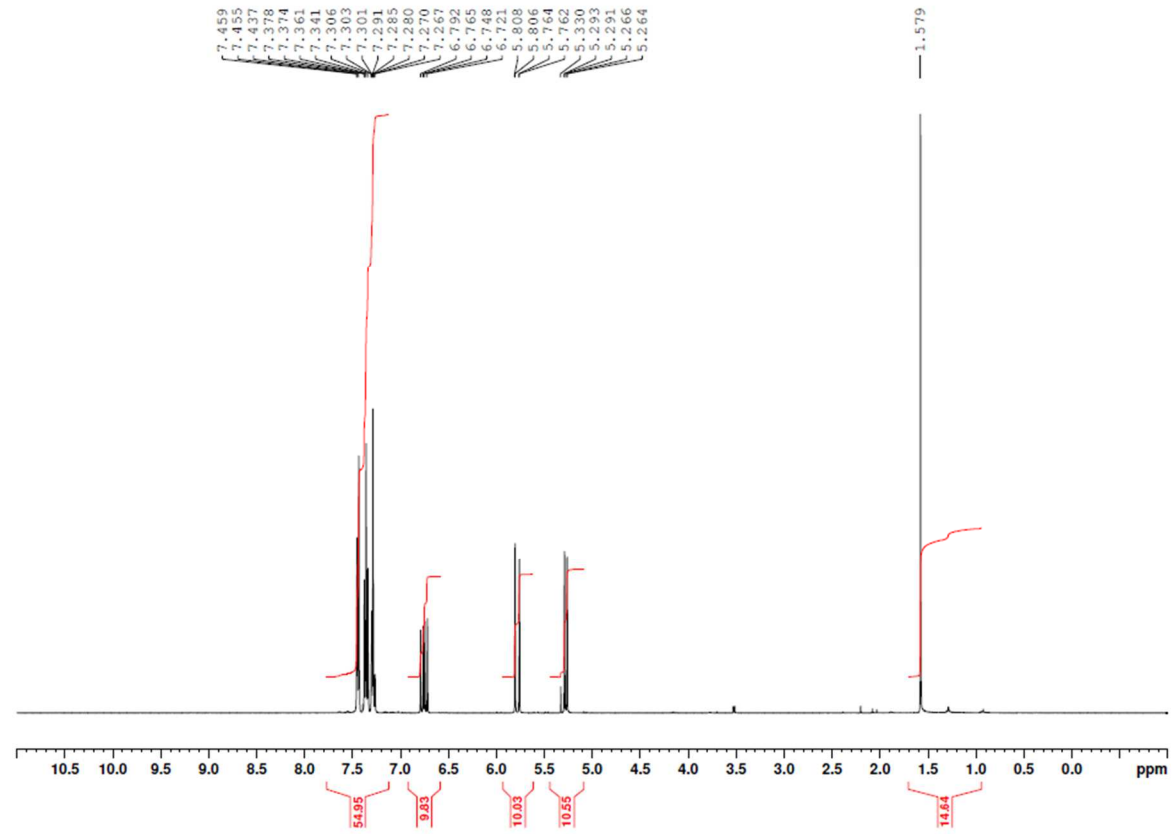
5.2.1 Analysis of the ionic liquid phase

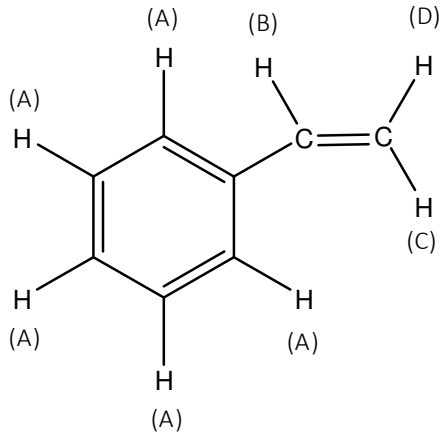
A crucial target is to develop a robust analytical method in order to accurately measure the production of styrene in the presence of ionic liquids. Therefore, different analytical methods were assessed in an attempt to detect and quantify styrene in the ionic liquid phase within the range of concentrations generated by the biocatalyst. First, GC analysis it is believed it would be the best approach to address this objective. However, it is not possible to directly inject IL/styrene samples into the GC to be analysed, since ionic liquids are non-volatile (Huddleston *et al*. 1998) and hence might damage the column by accumulating. Thus, Nuclear Magnetic Resonance spectroscopy was investigated as a possible option.

NMR spectroscopy exploits the magnetic properties of certain atomic nuclei. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus giving access to details of the electronic structure of a molecule. In organic chemistry practice, NMR analysis is used to determine the content and purity of a sample as well as its molecular structure. Different functional groups are clearly distinguishable, and identical functional groups with differing neighbouring substituents still give distinguishable signals. Based upon their chemical structures and according to the characteristic chemical shift values of different proton NMR spectra, both styrene and $[P_{66614}][NTf_2]$ should not present absorption at similar frequencies. Thus, NMR spectra of pure samples of both styrene and $[P_{66614}][NTf_2]$ were obtained (Figures 32-33).

The styrene spectrum (Figure 32) presents its characteristic absorption peaks at 7.5 ppm due to the proton resonance of the aromatic ring, and at 6.75, 5.81 and 5.28 ppm due to the proton resonance on the C nuclei from the double bond, which are in agreement with the values found in the literature (Lide and Milne 1993; National Institute of AIST 1999). In the case of the ionic liquid (Figure 33), its characteristic absorption peaks are only due to the alkyl phosphonium cation since the bistriflimide anion has no protons. In the vicinity of 2.2 ppm the absorption peaks are due to the proton resonance on the CH_2 groups attached to the P nuclei, at 1.3 ppm the resonance signals are due to all the hydrogens on the remaining CH_2 groups that form part of the long alkyl chains, and in the vicinity of 0.9 ppm the signals are due to the proton resonance on the CH_3 groups at the end of each alkyl chain; with all these values in accordance to the literature (Deyko *et al*. 2012). As expected, no peaks from either set of characteristic chemical shift values should overlap when a mixed sample is analysed by this technique.

Therefore, the minimal concentration of styrene in the ionic liquid solution that can be detected by NMR was investigated. Ionic liquid solutions were prepared with a range of styrene concentrations set up by the expected production titres from our biotransformation process. Stock solutions of 1 to 100 mM styrene concentrations in $[P_{66614}][NTf_2]$ were prepared as controls. Samples (200 µL) were taken from each solution and then diluted with deuterated chloroform (800 µL) as solvent. Aliquots (600 µL) were taken and submitted for NMR analysis (Figures 51-53, Appendix 8.2). Styrene characteristic peaks could only be detected in the 100 mM styrene solution (Figure 34); although this titre is within the range of styrene toxicity threshold towards *E. coli* when grown with $[P_{66614}][NTf_2]$ in the culture achieved in previous chapters (*i.e.* 300 mM), it is still a concentration too high when compared with our bioproduction titres, thus better analysis techniques for the IL phase are needed.

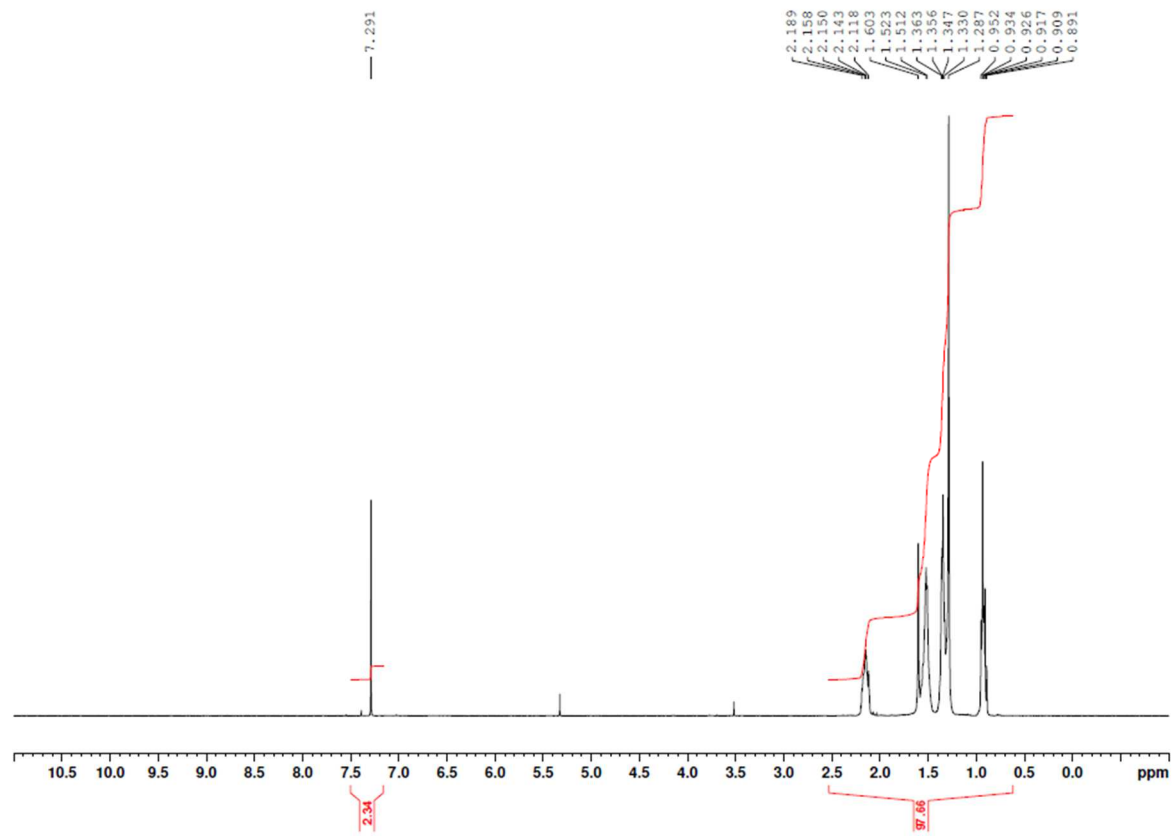


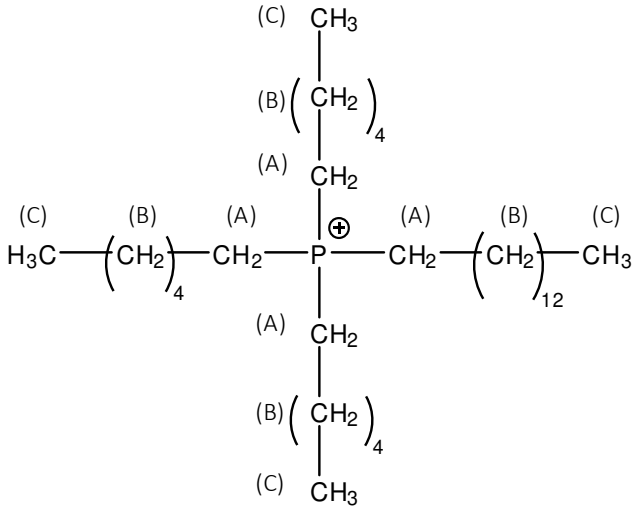


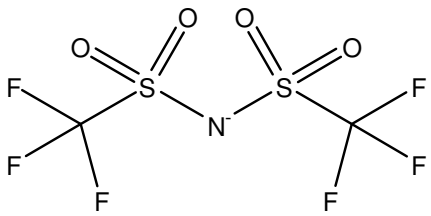
Assign.	Shift (ppm)
A	7.50 to 7.10
B	6.75
C	5.81
D	5.28

Figure 32: NMR spectrum of authentic styrene.

A styrene solution (1M) was prepared in deuterated chloroform, and samples (600 µL) were analysed by the Bruker AV(III) 400 NMR Spectrometer. Data were processed and analysed using ACD/NMR Processor version 12.01.







Assign.	Shift (ppm)
A	2.20
B	1.30
C	0.90

Figure 33: NMR spectrum of authentic [P_{66614}][NTf_2].

A [P_{66614}][NTf_2] solution (1M) was prepared in deuterated chloroform, and samples (600 µL) of were analysed by the Bruker AV(III) 400 NMR Spectrometer. Data were processed and analysed using ACD/NMR Processor version 12.01.

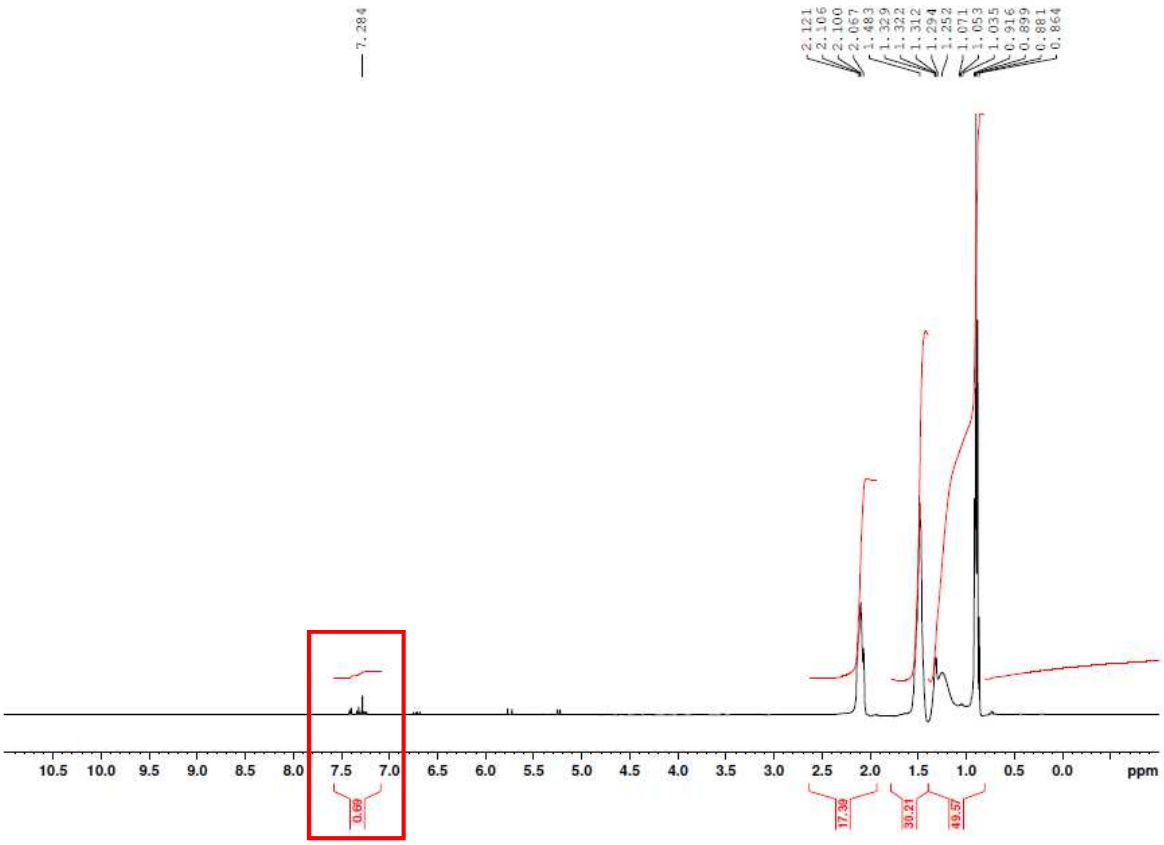


Figure 34: NMR spectrum of the 100 mM styrene solution in [P_{66614}][NTf_2].
Stock solutions of 1 to 100 mM styrene concentrations in [P_{66614}][NTf_2] were prepared. Samples (200 µL) were taken from each solution and then diluted with deuterated chloroform (800 µL) as solvent. Aliquots of the mixture (600 µL) were transferred into a NMR tube. The ^{1}H NMR spectrum was recorded at 400 MHz with 64 scans on a Bruker AV(III) 400 NMR Spectrometer. Data were processed and analysed using ACD/NMR Processor version 12.01.

Styrene extraction from the IL phase was also attempted by using different non-polar organic solvents (*i.e.* hexane, ethyl acetate, dichloromethane, toluene, diethyl ether), which can be injected into the GC. Solutions with concentrations from 50 mM to 1 M of styrene dissolved in IL were prepared. Samples (1 mL) were taken and then mixed in a 1:1 ratio with each organic solvent. However, all these solvents turned out to be miscible with the [P_{66614}][NTf_2]–styrene solution, forming a homogeneous solution which should not be directly injected into the GC column, since, as stated earlier, accumulation of ionic liquids might damage the column. Since styrene could not be directly extracted from the IL using organic solvents, an alternative extraction method using a

silica gel column to capture the ionic liquid from this homogeneous solution was examined. In chemistry, silica gel is used in column chromatography as a stationary phase, which is most often composed of silica gel particles of 40–63 μm, though different particle sizes are used for achieving a desired separation of certain molecular sizes. In this application, due to the silica gel's polarity, non-polar components tend to elute before more polar ones, hence it is hypothesised that this would be the case for the styrene molecule due to its apolar nature. Taking this specific characteristic into consideration, a silica gel column (Figure 57, Appendix 8.4) was used to try to separate the phosphonium bistriflimide ionic liquid from the homogenous solution generated by the mixing with each organic solvent and the IL–styrene solution. Samples (1 mL) were passed through the column and then the column was eluted repeatedly with the same organic solvent present in the solution. Later, a sample (1 mL) of the flow-through was taken and analysed by GC-FID.

Since the results and trends observed with all the organic solvents used are similar, gas chromatograms of only the solutions using hexane and DCM as organic solvents, both the control with no ionic liquid and the samples after having been passed through the silica gel column, are shown. Styrene was detected at 3.54 min in both flow-through and control samples (Figures 35-36). No detectable peak of styrene in any of the gas chromatograms corresponding to the samples taken from the flow-through of the silica column was observed (Figure 35). These results indicate that the styrene was also retained in the silica column within the ionic liquid, suggesting that the interaction between the styrene and the ionic liquid is stronger than the affinity/solubility in any of the organic solvents tested.

Styrene detection after passing the solution through the silica column was only possible at an initial concentration of 1M in the ionic liquid when DCM was used as mixing solvent (Figure 36). Although styrene detection is achievable by this technique, the initial concentration needed is much higher than the titres obtained by the biotransformation process with our engineered strain.

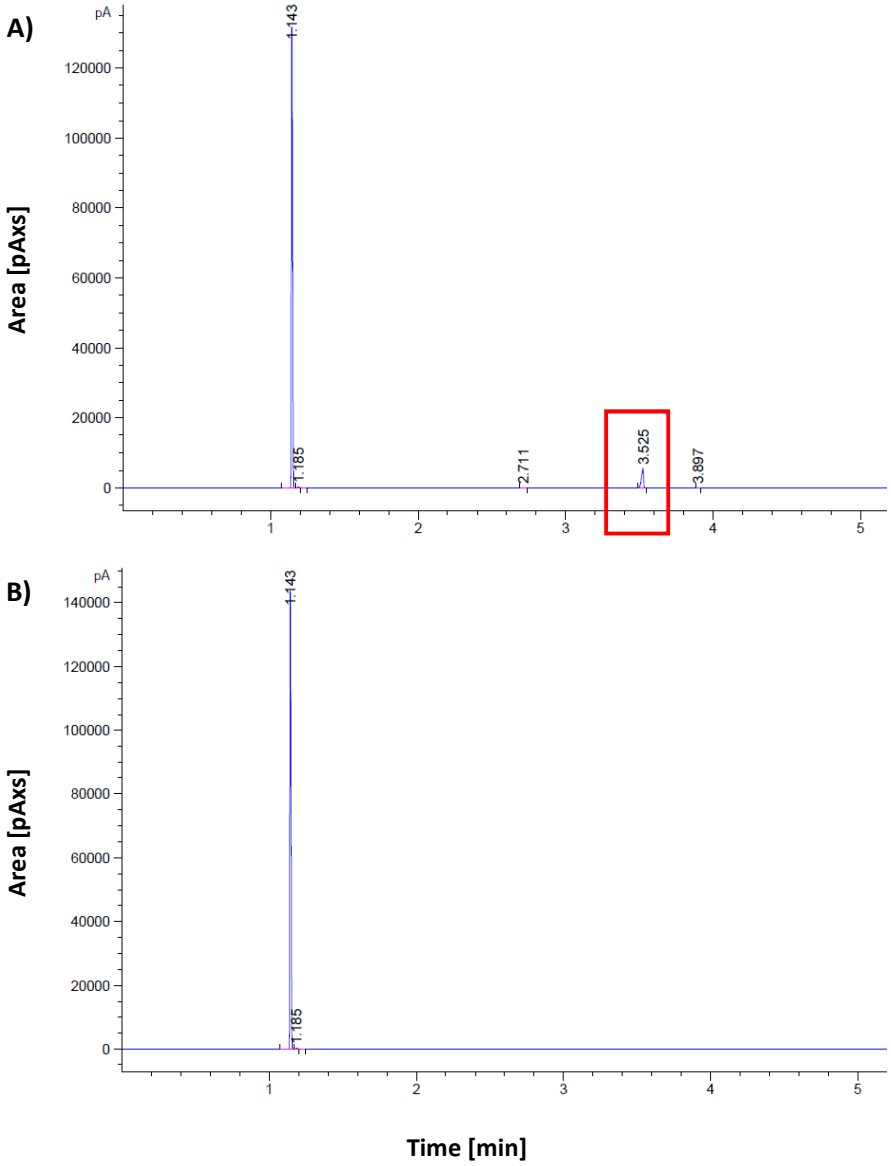


Figure 35: Gas chromatograms of the hexane organic solvent/IL–styrene solutions. (A) Styrene (500 mM) in hexane control solution. Styrene peak highlighted in red square. (B) Styrene (500 mM) in hexane solution after passing through the silica gel column. No detectable peak of styrene is observed.

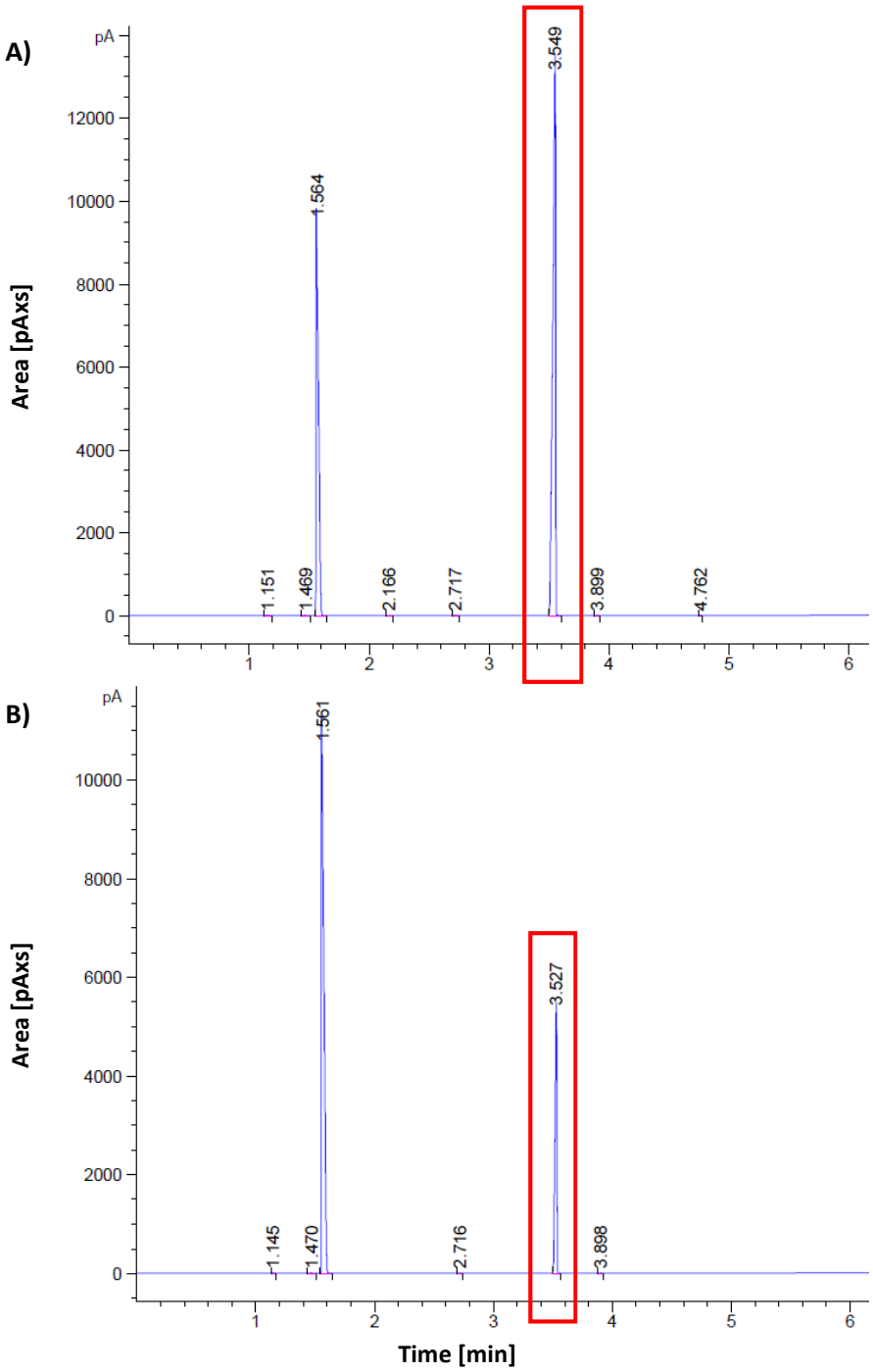


Figure 36: Gas chromatograms of the DCM organic solvent/IL–styrene solutions. (A) Styrene (1 M) in DCM control solution. Styrene peak highlighted in red square. (B) Styrene (1 M) in DCM solution after passing through the silica gel column. Styrene peak highlighted in red square.

At a late stage of this project, a deactivated fused silica guard column (Agilent, USA), used to trap non-volatile ILs, was acquired by our laboratory, thus direct injection of the IL samples was possible. Therefore, styrene solutions in ionic liquid in the range of 1 to 10 mM were prepared, to which *t*-butyl benzene was directly added to the GC vials as internal standard (10 mM). The solutions were vigorously vortexed and analysed by GC-FID (Figure 37; Table 18, Appendix 8.7). Even though styrene could be detected at very low concentrations, the peak areas of both the styrene and the internal standard were randomly varying for the different analysed concentrations, thus the ratios of the areas resulted in inconsistent values that did not follow a linear trend (Figure 27). It is believed that these variabilities in the measurements could be that, giving the high viscosity of the ionic liquid, despite the vigorous mixing, styrene and the *t*-butyl benzene do not form a homogeneous solution in the ionic liquid. Instead form liquid clathrate structures, trapping the molecules in a lattice or "cages" within the ionic liquid (Atwood 1984). Atwood reported that the high solubility of aromatic chemicals in ionic liquids can be attributed to clathrate formation, and Holbrey *et al*. (2003), working with 1-alkyl-3-methylimidazolium-based ionic liquids showed liquid clathrate formation in an ionic liquid-aromatic mixture, reporting that aromatic hydrocarbons could be trapped in the ionic liquid forming a crystalline 2:1 inclusion compound.

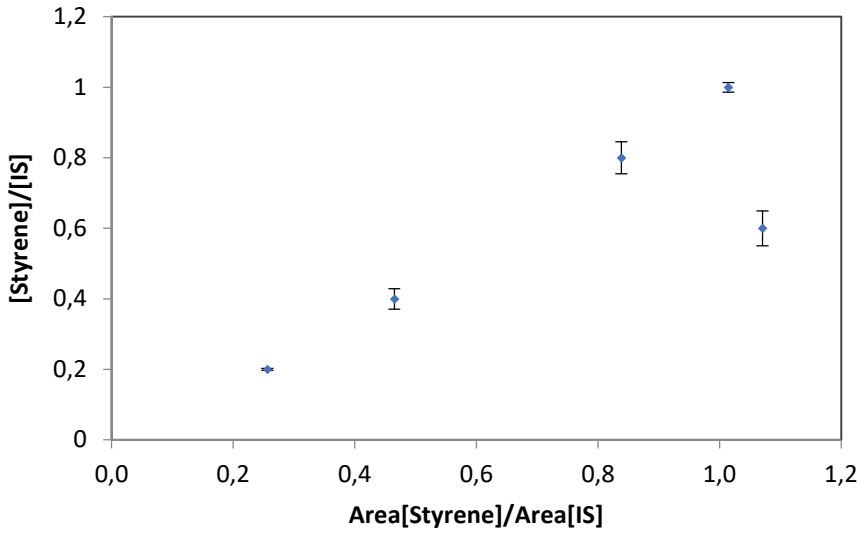


Figure 37: Styrene calibration curve in $[P_{66614}][NTf_2]$ without using an organic solvent.

Styrene solutions in ionic liquid were prepared directly in the GC vials, to which *t*-butyl benzene was added as internal standard (10 mM). Solutions were vigorously vortexed and analysed by GC-FID.

Therefore, taking into consideration the previous results of the miscibility of different organic solvents with the ionic liquid–styrene solutions, in an attempt to obtain a less viscous and more homogeneous solution, the IL phase was mixed with organic solvent in the presence of an internal standard. Thus, the IL–styrene solutions were mixed in a 1:1 ratio with a solution of ethyl acetate containing *t*-butyl benzene (10 mM). Solutions were thoroughly mixed by vortex and samples (750 µL) were then transferred to a glass GC vial and sealed with a Teflon-lined cap and analysed by GC-FID (Figure 38; Table 19, Appendix 8.7). Consistent values of the peak areas for the all range of concentrations of styrene and *t*-butyl benzene were obtained when solutions were mixed with ethyl acetate, validating the premise of creating a homogeneous solution when mixed with organic solvents. Additionally, a linear trend was achieved (Figure 28), hence calibration curves were prepared in the respective IL using authentic styrene at known concentrations and *t*-butyl benzene (10 mM) as internal standard (Appendix 8.5).

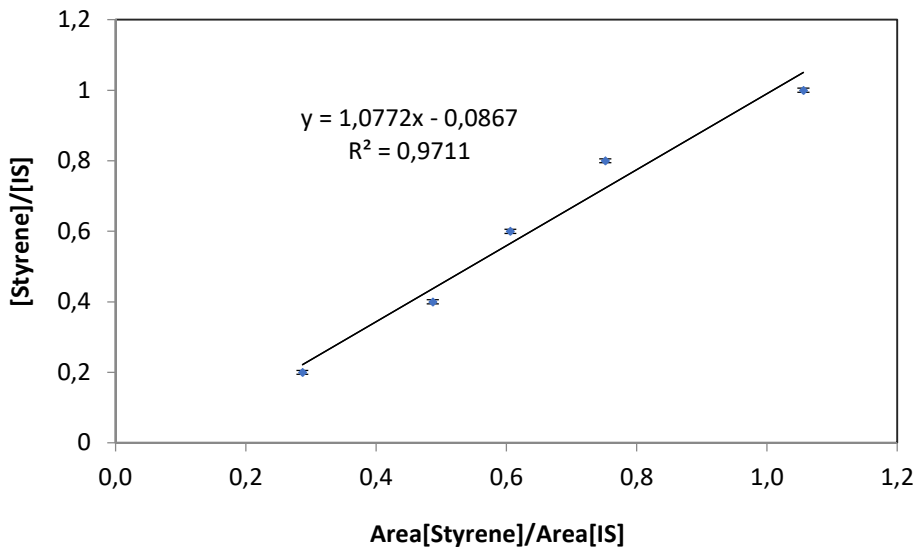


Figure 38: Styrene calibration curve in $[P_{66614}][NTf_2]$ using an organic solvent. The IL–styrene solutions were first prepared separately and later mixed in a 1:1 ratio with a solution of ethyl acetate containing *t*-butyl benzene as internal standard (10 mM). Solutions were thoroughly mixed by vortex and samples (750 µL) were then transferred to a glass GC vial and sealed with a Teflon-lined cap and analysed by GC-FID.

5.2.3 Conclusions

Styrene in ionic liquids could be detected by NMR spectroscopy; however, the detection limit was not sufficiently low to yet determine the concentrations obtained by the biosynthetic production process. Following attempts to extract styrene from $[P_{66614}][NTf_2]$ ionic liquid with various organic solvents for further analysis by GC-FID was not possible due to their miscibility with the IL. Finally, with the later acquisition of a deactivated fused silica guard column, direct injection of the ionic liquid solutions into the GC-FID was possible and an analytical method for the styrene analysis in ionic liquid was successfully developed. Due to the possible formation of liquid clathrates of styrene within the ionic liquid structure, solutions were mixed with organic solvents that previously displayed miscibility with the ionic liquid in order to get a homogeneous solution and an internal standard was added. Even though this method has only been proved useful for this particular ionic liquid and a non-polar compound, perhaps with a similar approach, taking in consideration the

chemical structures and properties of the compounds that want to be analyse, this analytical method could be of use for other ionic liquid solutions.

5.3 Biocatalytic production of styrene

5.3.1 Synthesis of styrene from glucose by E. coli NST74 expressing PAL2 and FDC1 enzymes

The medium composition, from an industrial scale perspective, is crucial in the economics of a fermentation process. Therefore, two different minimal media, MSX and MM1, were tested for the production of styrene following the previously reported fermentation designed by McKenna and Nielsen (2011). First, plasmids pSTV28_*pal2* and pTrc99_*fdc1*, kindly provided by Rebekah McKenna and David R. Nielsen from the Chemical Engineering Department, Arizona State University, USA., were first separately transformed into the JM107 (Novagen) cloning strain to create a cell bank for each plasmid. The L-phenylalanine overproducing *E. coli* NST74 strain (Choi 1981) was then transformed with pSTV28_*pal2* and pTrc99A_*fdc1* to allow the conversion of L-phenylalanine to styrene.

Styrene synthesis from glucose was investigated as described in the Materials and Methods section (4.4.4). Briefly, seed cultures of *E. coli* NST74_pSTV28_*pal2*_pTrc99A_*fdc1*, were prepared in MSX and MM1 broth and grown overnight, then used to inoculate Quickfit flasks (250 mL), containing fresh media (50 mL). Flasks were tightly sealed upon inoculation, in order to avoid loss of volatile product. Cultures were grown for 10 h (32 °C, 200 rpm) before induction by addition of IPTG. Culturing continued for 24 h post-induction.

Final production titres of styrene reached in MSX and MM1 media were 0.26 and 0.49 mM, respectively. However, the headspace volume available in the flasks also allows the accumulation of styrene vapour, which in the case of scaling-up this system must be considered in the total production. Under dilute

and near-ambient conditions, the amount of vapour in equilibrium with the aqueous phase can be calculated by applying Henry's Law (Equation 3, section 4.4.4), predicting that the styrene contained in the headspace in equilibrium with styrene present in the aqueous phase of each medium are 0.03 and 0.05 mM, respectively. It was observed that titres of styrene obtained in either MSX or MM1 did not reached high levels of production. It was believed that the late induction of the cultures was detrimental for the expression of the enzymes and therefore for the production of styrene since the cells could have already reached the stationary phase. Additionally, it was suggested that most of the glucose was used for increasing the biomass rather than for styrene production.

In order to confirm this hypothesis, growth curves of *E. coli* NST74_pSTV28_*pal2*_pTrc99A_fdc1 on both minimal media, MSX and MM1, were built in order to establish the time range of the exponential growth phase of the strain (Figure 39). It is observed that the exponential phase take place between the 4 and 10 hours of culturing, thus by the moment of the induction at 10 h the cells have reached the stationary phase. Therefore, the styrene production was reassessed only in MM1 medium by reducing the time of induction to when an OD_{600} ~0.6 was reached, and then cultures were again incubated for 24 h after IPTG induction. The final styrene concentration in the aqueous phase was increased to 0.69 mM, which represents a 1.4-fold increase when compared to the previous production in MM1. This increment could be associated with the earlier IPTG induction triggering the expression of the enzymes at a younger and stronger age of the culture, and moreover permitted the use of more glucose for styrene production instead of cell growth. Therefore, in all future experiments IPTG induction will be performed when OD_{600} ~0.6 is achieved in the culture.

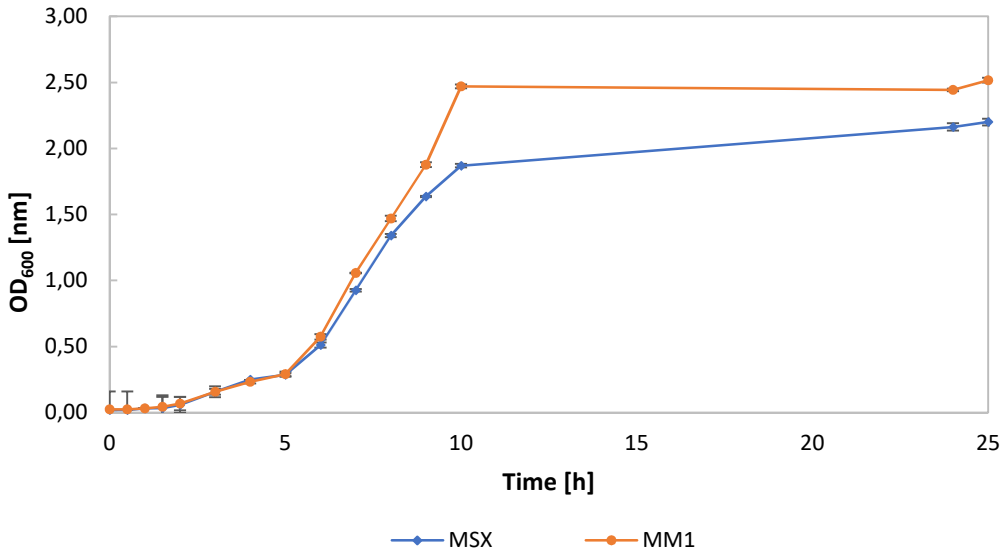


Figure 39: *E. coli* NST74 growth curve in minimal media.

Seed cultures (20 mL) of *E. coli* NST74_pSTV28_pal2_pTrc99A_fdc1 were grown overnight and used to inoculate fresh minimal media, either MSX (blue) or MM1 (red), in Erlenmeyer flasks (250 mL). Cultures were grown for 25 h (32 °C, 200 rpm). Optical density was measured periodically and OD_{600} values were used to plot a growth curve and to calculate specific growth rates. Data and standard deviations are the mean of three replicates.

5.3.2 Styrene production with different glucose initial concentration

Despite the fact of the late induction of the cells, higher concentrations of styrene were obtained when using MM1 medium rather than MSX. It is observed from the analysis of their composition (Table 15) that MM1 has a 3-times higher glucose initial concentration (*i.e.* 15 g/L in MM1 and 5 g/L in MSX). This result could suggest that a higher initial concentration of glucose would improve the production of styrene. In order to confirm this hypothesis, production of styrene with increasing initial concentration of glucose was investigated in MSX medium.

Briefly, seed cultures of *E. coli* NST74_pSTV28_*pal2*_pTrc99A_*fdc1*, were prepared in MSX medium and grown overnight, then used to inoculate Quickfit flasks (250 mL), containing fresh MSX medium (50 mL) with initial glucose concentration of 5, 10, 15 and 20 g/L. Flasks were tightly sealed upon

inoculation, in order to avoid loss of volatile product. Cultures were incubated (32 °C, 200 rpm) until the OD_{600} reached ~0.6 and protein expression was induced by addition of IPTG. Culturing continued for 24 h post-induction and final concentrations of styrene were measured (Figure 40).

Final styrene concentrations of 0.38, 0.37, 0.35, 0.33 mM were achieved when 5, 10, 15 and 20 g/L initial glucose concentration were added to MSX medium, respectively. It is observed that, despite the increase in the initial concentration of glucose, styrene productions in all of the experiments were still lower than the styrene concentration achieved in MM1, even with late induction, disproving our initial assumption. Particularly, the styrene production obtained in MSX with an initial glucose concentration equal to the concentration present in MM1 medium (*i.e.* 15 g/L), was two-fold lower than the production obtained with MM1 under the same conditions (*i.e.* induction at OD_{600} ~0.6).

It is believed that the lower styrene productions at increasing concentrations of glucose might be due to higher amounts of acidic by-products that could have been produced by *E. coli* due to an excess in glucose availability in the media, of which acetate is the most predominant and known to be an inhibitor for the fermentation process, (Luli and Strohl 1990). This glucose-mediated aerobic acidogenesis is known as the bacterial Crabtree effect. The formation of acetate has been suggested to be caused by an imbalance between glucose metabolism and a limited respiration capacity in which the excess of glucose that cannot be oxidised completely is metabolised into acetate to generate additional ATP and $NADH_2$ (Reiling, Laurila, and Fiechter 1985). Another explanation that has been suggested is that the presence of excess $NADH_2$ switches the carbon flow toward acetic acid (Doelle, Ewings, and Hollywood 1982). When compared the chemical composition of both minimal media (Table 15), it is presumed that MM1 has a greater buffering capacity than MSX conferred by the presence of high concentrations of MOPS, and the mixture of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate at the specified concentrations, hence making MM1 a better medium for styrene production than MSX under the same culturing conditions.

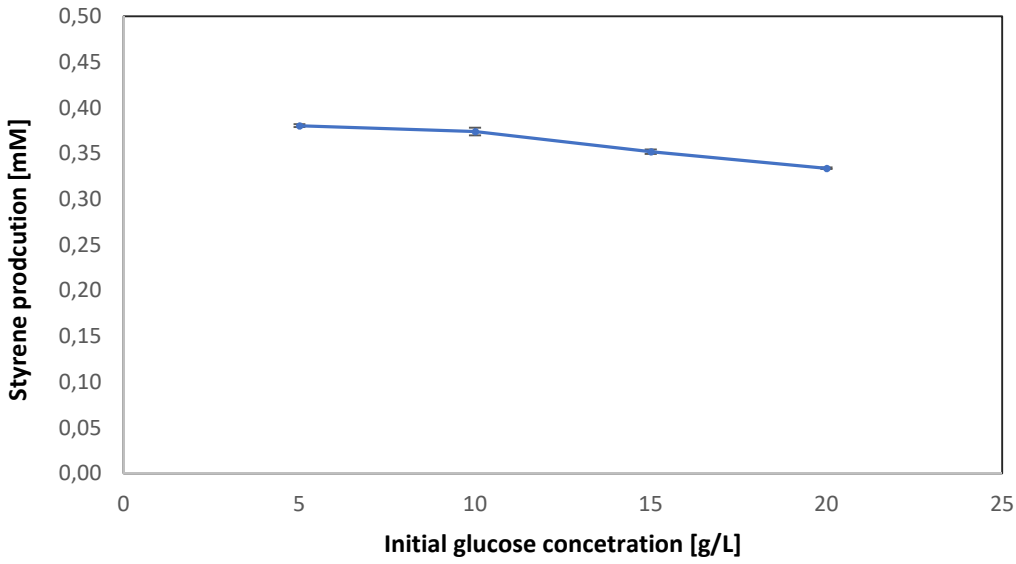


Figure 40: Styrene production by *E. coli* NST74 pSTV28_pal2 pTrc99A_fdc1 in MSX increasing initial concentration of glucose.

Seed cultures (20 mL) of *E. coli* NST74 pSTV28_pal2 pTrc99A_fdc1 were grown overnight and used to inoculate fresh MSX media with different initial glucose concentration, in sealed Quick-fit flasks (250 mL) in order to avoid losses of product by volatilization. Cultures were grown until OD_{600} ~0.6 was reached (32 °C, 200 rpm) before being induced with IPTG (0.2 mM). Culturing continued for 24 h post-induction. Samples (1 mL) were taken from each culture and analysed for relevant metabolite contents via HPLC. Data and standard deviations are the mean of three replicates.

Table 15: Chemical composition of both minimal media MM1 and MSX.

Material	MSX	MM1
KH_2PO_4	6 (g/L)	0.3 (g/L)
K_2HPO_4		0.7 (g/L)
NH_4Cl	3 (g/L)	
$MgSO_4.7H_2O$	0.4 (g/L)	0.5 (g/L)
$(NH_4)_2SO_4$		4.0 (g/L)
Glucose	5 (g/L)	15 (g/L)
MOPS		24.7 (g/L)
Vishniac's trace element	2 mL/L	
ATCC Trace Mineral Supplement		5 mL/L

Trace Elements	Vishniac's trace element (g/L)	ATCC Trace Mineral Supplement (g/L)
EDTA disodium salt	50	0.5
$ZnSO_4 \cdot 7H_2O$	3.9	0.1
$CaCl_2$	5.54	0.1
$MnCl_2.4H_2O$	5.06	
$FeSO_4.7H_2O$	5.0	0.1
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10	
$CuSO_4 \cdot 5H_2O$	1.57	0.01
$CoCl_2.6H_2O$	1.61	
$MgSO_4 \cdot 7H_2O$		3
$MnSO_4 \cdot 7H_2O$		0.5
NaCl		1
$Co(NO_3)_2 \cdot 6H_2O$		0.1
$AlK(SO_4)_2$		0.01
H_3BO_3		0.01
$Na_2MoO_4 \cdot 2H_2O$		0.01
Na_2SeO_3		0.001
$Na_2WO_4 \cdot 2H_2O$		0.1
$NiCl_2 \cdot 6H_2O$		0.02

5.3.3 An alternative route for the biosynthetic production of styrene: expression of OHBA1 enzyme

Up to this point, irrespective of the medium that was used, very low concentrations of styrene had been achieved. McKenna and Nielsen (2011) suggested that the enzymatic efficiency of the enzymes involved in the pathway is still a limitation that needs to be improved in order to produce an enhancement to the current styrene bioproduction process. Therefore, alternative metabolic routes for its synthesis were investigated. In the search for a more efficient enzyme for the conversion of *trans*-cinnamic acid to styrene, OHBA1, a putative 4-hydroxybenzoic acid decarboxylase required for the decarboxylation of sorbic acid to 1,3-pentadiene by *Aspergillus niger* (Plumridge *et al*. 2010; Stratford *et al*. 2012); was tested for the decarboxylation of *trans*-cinnamic acid and compared to FDC1.

Briefly, *E. coli* BL21 (DE3)pLysS strain was transformed with either pETohbA1 and pETfdc1 plasmids, kindly provided by Luca Rossoni, University of Nottingham, UK. Seed cultures of each of the modified *E. coli* BL21 (DE3)pLysS strains were grown overnight in LB medium and used to inoculate fresh LB medium (400 mL). Cultures were incubated (37 °C, 200 rpm) until an OD_{600} of ~0.6 and induced with IPTG. Induced cultures were incubated for a further 20 h and the cells harvested by centrifugation. Pellets were resuspended in fresh PBS solution and the cells were transferred to sealed glass vials (20 mL) with PTFE caps at a final concentration of OD_{600} ~1.0. *trans*-cinnamic acid was added (3.75 mM) and biotransformation performed for 24 h at 30 °C (Figure 41).

Activity was observed confirming, for the first time to the best of our knowledge, that OHBA1 is a valid alternative enzyme for *trans*-cinnamic acid decarboxylation. Moreover, *trans*-cinnamic acid consumption with OHBA1 was two-fold higher than with FDC1 (after 5 and 24 h) suggesting a higher efficiency. Styrene production rates in the first 5 h were estimated to be 0.068 and 0.037 mmol/L/h for OHBA1 and FDC1, respectively, confirming what had been already observed with *trans*-cinnamic acid consumption.

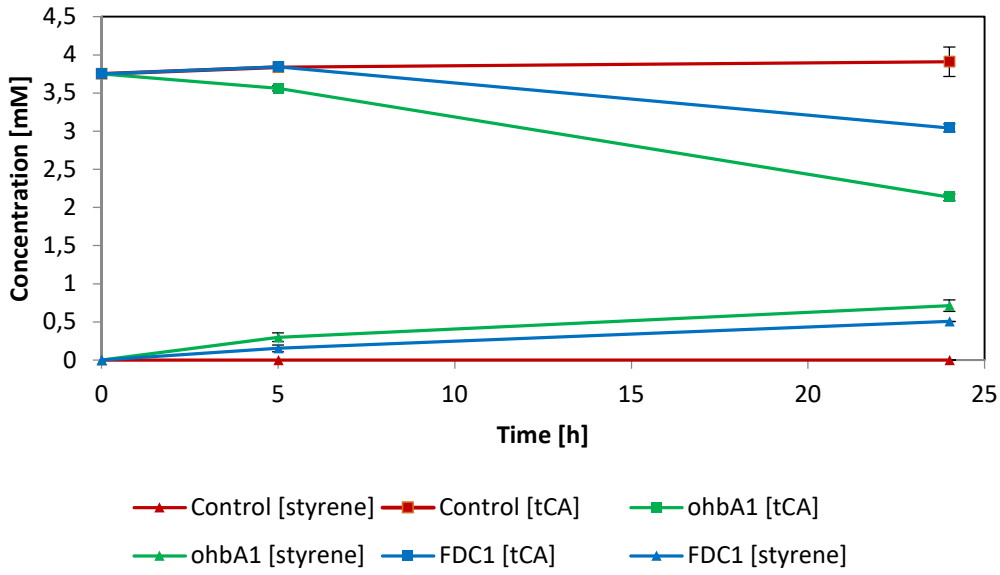


Figure 41: Comparison of activity of OHBA1 and FDC1 - Consumption of *trans*-cinnamic acid and formation of styrene performed in whole-cells expressing OHBA1 and FDC1.

Seed cultures of *E. coli* BL21 (DE3)pLysS strain transformed with the pETohbA1 and pETfdc1 were grown overnight in LB medium and used to inoculate fresh LB medium (400 mL). Cultures were incubated (37 °C, 200 rpm) until an OD_{600} of ~0.6 before protein expression was induced by the addition of IPTG (0.4 mM). Induced cultures were incubated (37 °C, 200 rpm) for further 20 h and the cells harvested by centrifugation (10,000 rpm, 20 min, 4 °C). Pellets were resuspended in fresh PBS solution and the cells were transferred to sealed glass vials (20 mL) with PTFE caps at a final concentration of OD_{600} ~1.0. *trans*-cinnamic acid was added (3.75 mM) and biotransformation performed for 24 h at 30 °C. Samples (2 mL) were removed at intervals and analysed by HPLC. Data and standard deviations are the mean of three replicates.

Therefore, based on the latter results and in efforts to search for an improved styrene biosynthetic pathway capable of synthesizing styrene from glucose, *A. thaliana* PAL2 was co-expressed with either *S. cerevisiae* FDC1 or *A. niger* OhbA1, in *E. coli* NST74. Strains transformed with either pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohbA1* were cultured in MM1 medium as described in the Materials and Methods section (4.4.4) and styrene production was measured 24 h after IPTG induction.

Styrene production by the strain expressing FDC1 was 0.69 mM; meanwhile the final styrene concentration reached by the strain expressing OhbA1 was 0.30 mM. Therefore, about 2.3-fold higher styrene production was achieved when

PAL2 is co-expressed with FDC1. However, previous results showed that when both enzymes were compared individually for the *trans*-cinnamic acid decarboxylation, *trans*-cinnamic acid consumption with OhbA1 was higher than with FDC1, suggesting a higher efficiency. Taken together, the latter results suggest that a low PAL activity might be limiting the production of styrene, which would be in accordance with previous observations made by McKenna and Nielsen (2011). It is presumed that the low productivity of PAL2 is due to both product inhibition and reversibility of the enzymatic reaction. It has been reported that compounds structurally related to L-phenylalanine and *trans*-cinnamic acid, such as L-tyrosine and both *m*- and *p*-coumaric acids, have inhibitory effects even at relatively low concentrations. Moreover, phenylalanine ammonia lyase enzyme was found to be inhibited by cinnamic acid, one of the products of the reaction (Koukol and Conn 1961). In addition, even though the equilibrium of the reaction favours the degradation of L-phenylalanine to *trans*-cinnamic acid and ammonia, at high pH and ammonia concentration the reaction can be reversed to produce L-phenylalanine (Takaç, Akay, and Özdamar 1995).

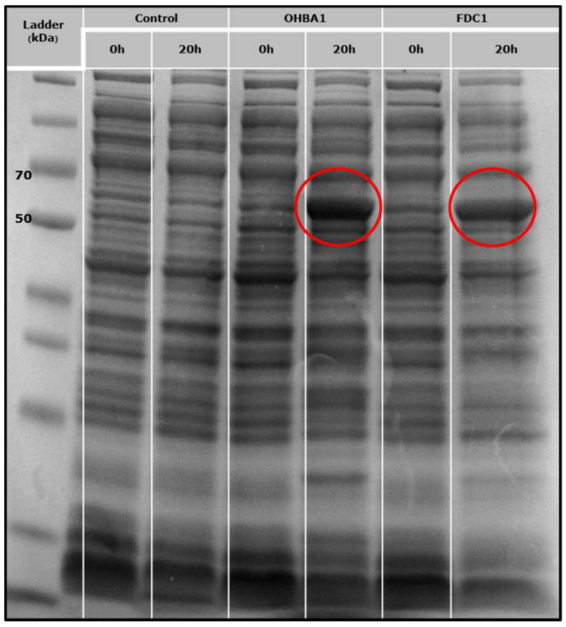


Figure 42: Protein expression in cultures used for of the biotransformation.
SDS-PAGE analysis of *A. niger* OhbA1 and *S. cerevisiae* FDC1 in crude extract (CE) Samples were taken at 0 and 20 h after induction with IPTG. The level of migration corresponding to OhbA1 and FDC1 are marked in red circles.

5.3.4 Bioproduction of styrene from L-phenylalanine by E. coli NST74 pSTV28_pal2 pTrc99A_fdc1 and E. coli NST74 pSTV28_pal2 pTrc99A_ohbA1

Since L-phenylalanine is the immediate precursor in our biosynthetic pathway, it is worth considering that its availability could also be constraining the styrene production in the present study. Therefore, the aim of this experiment was to measure the production of L-phenylalanine starting from glucose of the *E. coli* NST74 strain under the same conditions previously used to produce styrene.

Cultures were carried out as described in the Materials and Methods section (4.4.3). Briefly, seed cultures of *E. coli* NST74 were grown overnight in MM1 medium and used to inoculate fresh MM1 medium (50 mL) to a final OD_{600} of ~0.05. The cultures were incubated for 48 h (32 °C, 200 rpm).

Maximal titres of L-phenylalanine reached after 48 h of fermentation were 461.3 mg/L, which represents a yield of 0.031 $g/g_{glucose}$. McKenna and Nielsen (2011) achieved average yields of 0.057 g/g in the production of L-

phenylalanine when working with the *E. coli* NST74 strain under similar conditions. Even though McKenna & Nielsen achieved a higher production, both yields are quite far from the 0.151 g/g yield obtained by Choi (1981) when developed the *E. coli* NST74 strain. However, Choi added thiamine (0.1 g/L) to the medium to support the cells growth and worked in a 1 L batch fermentor, hence achieving much higher cell density. Nevertheless, all together the latter results suggest that the low L-phenylalanine production of the strain used in the present experiments could also, in conjunction with the low enzymatic activity of the enzymes involved in the pathway, be a constraint for the bioproduction of styrene in the current process.

In order to support our hypothesis that the low L-phenylalanine production by the NST74 strain hampered the net production of styrene in our pathway, the conversion of exogenous L-phenylalanine to styrene by the NST74 transformed strains with either pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohbA1* was investigated.

Cultures were performed as already described in the Material and methods section (4.4.6). Briefly, seed cultures *E. coli* NST74 harbouring either pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohbA1* plasmids were grown overnight in LB medium and used to inoculate fresh LB medium (100 mL). Cultures were grown (32 °C, 200 rpm) until an OD_{600} of ~0.6, when it was induced with IPTG and then incubated for additional 8 h. Cells were then centrifuged, and the pellet was re-suspended in PBS buffer (100 mL). Then cultures were diluted to a final OD_{600} of ~0.6 in fresh PBS buffer with a starting L-phenylalanine concentration of 900 mg/L. The biotransformation was performed for additional 24 h (32 °C, 200 rpm).

Final styrene titres reached by the strains expressing PAL2 with FDC1 or OhbA1 were 2.71 and 2.32 mM, correspondingly. The first outcome to be highlighted from the latter results is that a notable increase in the styrene production was achieved when exogenous L-phenylalanine was added. A final concentration of styrene almost four and seven-fold higher than previous experiments starting

from glucose were obtained when FDC1 and OhbA1 were expressed in MM1, respectively (*i.e.* 0.69 and 0.30 mM). Additionally, as expected from the previous results, higher concentrations of styrene were reached when PAL2 was co-expressed with FDC1 instead of OhbA1. These results confirm the suspicions regarding the low availability of L-phenylalanine being a constraint in the current bioproduction pathway, and also suggest that perhaps the production of L-phenylalanine by the NST74 strain has reached a physiological limit.

5.3.5 Biosynthesis of styrene E. coli NST74 pSTV28_pal2 pTrc99A_fdc1 and E. coli NST74 pSTV28_pal2 pTrc99A_ohbA1 in the presence of IL

One of the aims of the present project was to assess the possibility to solve the issues related with product toxicity during the biocatalytic production of styrene by using alternative solvents to conventional organic solvents for continuous product removal. Therefore, the aim of the following experiment was to assess the production of styrene starting from glucose in a biphasic system using the previously selected biocompatible ionic liquid $[P_{66614}][NTf_2]$ as a secondary extractant phase.

Biotransformations were carried out as described in the Materials and Methods section (4.4.4). Briefly, seed cultures of *E. coli* NST74 co-expressing either pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohbA1* were grown overnight in MM1 medium, then used to inoculate sealed glass vials containing fresh MM1 medium (10 mL) in the absence of ionic liquid as control and with $[P_{66614}][NTf_2]$ ionic liquid as the extracting phase (20% v/v); to a final OD_{600} of ~0.05. Cultures were grown until OD_{600} ~0.6 was reached (32 °C, 200 rpm), at which time cultures were induced with IPTG. Samples (1 mL) from both the aqueous and IL phases were taken 24 h after IPTG induction.

Samples of the aqueous phase were analysed by HPLC and GC-MS. Neither chromatograms showed distinguishable peaks corresponding to styrene,

suggesting that most of the styrene was extracted from the culture by the ionic liquid during the fermentation. When the ionic liquid phases were analysed by GC-FID, the styrene concentration produced by the strain expressing FDC1 was 1.96 mM, which represents a 2.8-fold increase compared with the same culture conditions but when no ionic liquid is present (*i.e.* 0.69 mM styrene production in MM1). Alternatively, the styrene production reached by the strain expressing OhbA1 was 0.77 mM, which corresponds to a 2.6-fold increase compared with the culture in absence of ionic liquid as an extracting phase (*i.e.* 0.30 mM styrene production in MM1). These results confirm the initial benefits of using ionic liquids as a secondary phase for the *in situ* extraction of toxic chemicals in fermentation broths.

Lastly, the conversion of exogenous L-phenylalanine to styrene only by the NST74 strain harbouring the plasmid pSTV28*_pal2* pTrc99A*_fdc1* was measured in the presence of $[P_{66614}][NTf_2]$ as a secondary phase. Cultures were performed as detailed in the Material and methods section (4.4.6). Briefly, seed cultures of *E. coli* NST74 pSTV28*_pal2* pTrc99A*_fdc1* were grown overnight in LB medium and used to inoculate fresh LB medium. Cultures were grown until an OD_{600} of ~0.6, at which point it was induced with IPTG and then incubated for additional 8 h. Cells were then centrifuged, and the pellet was re-suspended in PBS buffer. Cultures were then diluted to a final OD_{600} of ~0.6 in fresh PBS buffer with a starting L-phenylalanine concentration of 900 mg/L and ionic liquid $[P_{66614}][NTf_2]$ was added (20% v/v). The biotransformation was performed for an additional 24 h.

Similarly, to the previous biotransformation starting from glucose, when the aqueous phase was analysed by either HPLC or GC-MS, none of the chromatograms presented distinguishable peaks corresponding to styrene, which would confirm that styrene was mostly removed by the ionic liquid. Regarding the ionic liquid phase analysis, a styrene concentration of 3.53 mM was achieved. When compared to the concentration obtained in the biotransformation started from glucose performed in the presence of ionic liquid (*i.e.* 1.96 mM by *E. coli_pal2_fdc1*), this result represents a 1.8-fold

increment, which would support our hypothesis that the availability of L-phenylalanine is a constraint in the bioproduction of styrene. Furthermore, this final concentration is 1.3-fold higher than when styrene is produced from exogenous L-phenylalanine but in the absence of ionic liquid as a secondary phase (*i.e.* 2.71 mM by *E. coli_pal2_fdc1*). This result confirms the advantage of using an ionic liquid as an *in situ* extraction phase to increase the production levels of styrene and to overcome toxic levels of styrene whilst the biotransformation is being carrying out.

5.3.6 Conclusions

The L-phenylalanine overproducing *E. coli* NST74 strain was successfully transformed with either pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohbA1* plasmids, and tested for the biocatalytical production of styrene in two different minimal media, MSX and MM1, in absence and presence of ionic liquid $[P_{66614}][NTf_2]$ as a secondary extractant phase.

The styrene production when compared in the different experimental conditions tested (Table 16), it is observed that when cultures started at the same initial glucose concentrations (*i.e.* 15 g/L), a higher production of styrene was obtained in MM1 medium. It has been reported that in the presence of an excess of glucose *E. coli* switches the carbon flow toward acetic acid formation (Han, Lim, and Hong 1992), which inhibit cell growth and product synthesis. It is suggested that the higher buffer capacity of MM1 helps to alleviate the negative effects of acetic acid on the recombinant *E. coli* growth allowing higher styrene production. This is support by the higher final OD_{600} reached by the *E. coli_pal2_fdc1* strain for growth in MM1 medium instead of MSX, achieving a maximal OD of 2.5 and 1.9, respectively, when the stationary phase was reached (Figure 29). Additionally, when MSX medium was used with an initial glucose concentration of 5 g/L, a similar styrene concentration was achieved to that when 15 g/L of glucose were added to MSX (*i.e.* 0.35 and 0.38 mM,

respectively), suggesting that perhaps after reaching a specific styrene production limit the recombinant *E. coli* strain starts to diverge glucose to the synthesis of acetic acid and other metabolites that might inhibit the cell, which toxic effect, in the case of MM1 medium would be reduced by its stronger buffer capacity. Therefore, it would be appropriate to test this hypothesis in future work by investigating the styrene production in both MSX and MM1 media with an initial glucose concentration of 5 g/L and measuring periodically the production and accumulation of other metabolites, such as acetic acid.

Table 16: Comparison of styrene produced for the different fermentation media.

	Maximum styrene produced [mM]					
***E. coli* NST74 strain**	**MM1[(a)]**	**MSX[(a)]**	**MSX[(b)]**	**L-phe[(c)]**	**MM1[(a)]/IL**	**L-phe[(c)]/IL**
pal2_fdc1	0.69	0.35	0.38	2.71	1.96	3.53
pal2_ohbA1	0.30	--	--	2.32	0.77	--

[(a)] 15 g/L initial glucose concentration.
[(b)] 5 g/L initial glucose concentration.
[(c)] 900 mg/L L-phenylalanine initial concentration

It has been suggested that a low activity of the pathway enzymes constrain the productivity of the styrene-producing strain. In efforts to improve the current catalytic pathway, OHBA1, a putative 4-hydroxybenzoic acid decarboxylase required for the decarboxylation of sorbic acid to 1,3-pentadiene by *Aspergillus niger*, was tested for conversion of *trans*-cinnamic acid to styrene and compared to FDC1; and later was co-expressed with *A. thaliana* PAL2 and investigated for the production of styrene from glucose. When tested individually, *trans*-cinnamic acid consumption with OhbA1 was higher than with FDC1, suggesting a higher efficiency. However, when OhbA1 was co-expressed with PAL2, a lower concentration of styrene was achieved, suggesting that a low PAL activity might be limiting the production of styrene. Future work to improve the PAL activity could be the use of codon optimised variants, an

approach that has been shown to be successful in the development of pathways involving the expression of genes derived from plants in *E. coli* (Keasling 2008). The genetic code is composed of 64 codons with only 21 amino acids and "stop" assignments. Therefore, the code is redundant: some amino acids are represented by multiple transfer RNAs using different triplicate codes. Preferential usage of particular codons varies by organism which can create challenges for expressing recombinant proteins in heterologous expression systems, resulting in low and unreliable expression. Thus, in codon optimisation preferred codons are selected over rarely used codons in order to suit the host protein expression machinery aiming to increase the yield of heterologous expression (Plotkin and Kudla 2011). Additionally, it has been reported that FDC1 requires a cofactor for optimal performance, with that cofactor being synthesised by PAD1, an enzyme which had been defined as a phenylacrylic acid decarboxylase enzyme (Lin *et al*. 2015). Therefore, it would be appropriate to attempt the co-expression of PAD1 and FDC1 in the current strain and to investigate styrene production.

L-phenylalanine availability was confirmed to be a constraint for the bioproduction of styrene, and results demonstrate that if an increase amount of this precursor is supplied to the biocatalyst, a higher total production of styrene is achieved. However, the addition of exogenous L-phenylalanine is not an economical approach to improve styrene biosynthesis; thus, future work should focus on enhancing the endogenous production of L-phenylalanine in the host platform. For example, an interesting approach was developed by Báez-Viveros *et al.* (2004), reaching high yields of 0.33 g/g, by using the PB12 strain, a mutant derivative of *E. coli* JM101, which has an inactive phosphotransferase transport system (PTS) and overexpressed versions of feedback inhibition resistant chorismate mutase/prephenate dehydratase (CM-PDT).

Finally, the feasibility and capacity of using ILs as a secondary phase during the biocatalytic production of styrene was investigated. An increased production in both scenarios, starting from glucose and from exogenous L-phenylalanine was

obtained; even achieving styrene concentrations when starting from exogenous L-phenylalanine concentrations over the *E. coli* toxic threshold, thus supporting their potential use as alternatives to organic solvents in this biosynthesis of styrene to enhance its production and overcome issues related with the end-product toxicity. However, for the implementation of this process at large scale, due to the high cost of the starting materials for the synthesis of this particular ionic liquid (*i.e.* the salt [$LiNTf_2$] cost is around £3500/kg, Sigma Aldrich), evaluation of cheaper ionic liquids options would be necessary.

5.4 Fed-batch production of styrene in LUND media by *E. coli* NST74

5.4.1 Introduction

Biotransformations using engineered *E. coli* strains for the production of styrene was proved successfully in small scale. Therefore, to optimise styrene on a larger scale, fed-batch fermentations at 1.25 L scale were performed.

There are three classical operational modes that can be operated for cultures: continuous, batch, and semi-batch (or semi-continuous) (Lim and Shin 2013; Yamanè and Shimizu 1984; Minihane and Brown 1986). In continuous processes, an inflow of the necessary nutrients is constantly fed to the reactor, while an outflow mix of products, cells and residues is constantly removed such that the volume in the reactor is kept constant and all nutrients are present at constant concentrations, therefore establishing a steady state process. Even though continuous reactors are widely used in the chemical industry, continuous fermentations have not been commonly used yet, mainly because of the difficulty of keeping sterility during the process, and the occurrence of the genetic drift of the cell population, since there is a constant selection for faster-growing rather than more productive organisms (Gerson, Kole, and Ozum 1988).

In a batch fermentation process, all the required nutrients are present in the culture medium before inoculation, hence their concentrations are not controlled and vary as the cells consume them, whilst the products are collected at the end of the run (Yamanè and Shimizu 1984). Only the pH, temperature, dissolved oxygen and foam are corrected in order to hold them constant during the course of the batch culture. Therefore, only optimizations to the initial medium components can be made, whilst profile optimizations of pH and temperature would help to enhance productivity of the process.

In the case of a fed-batch (or semi-batch) process, one or more of the necessary nutrients are either constantly or intermittently supplied to the bioreactor, with no effluent during the course of the culture, and only collecting the products at the end of the run (Lim and Shin 2013). Hence, with this process the concentration of one or more of the nutrients can be manipulated by controlling the feed rates during the run with respect to the online measurement of control parameters such as pH and dissolved oxygen. Thus, the fed-batch culture is a dynamic process with the major advantage over the conventional batch operation that the concentration or yield of the target product can be maximised by altering the concentration of key components in the culture medium that control the main reaction rates. Under these controlled conditions, cells can grow to high cell density, achieving concentrations greater than 100 g (dry cell weight) per litre (Riesenberg *et al*. 1991), which is usually required to make many fermentation processes economically viable. Achieving high cell densities also increases the amount of catalyst per unit volume of culture, therefore increasing the volumetric yield and productivity (Makrides 1996). Increased volumetric productivity allows the use of smaller bioreactors, hence reducing the size of the plant required for production. Additionally, downstream process and operational costs are reduced since the purification equipment required can be of a smaller scale, and less total water would be required, resulting in less wastewater produced and lower cost in sterilisation of media (Lee 1996), with all the above allowing a reduction in the capital investment and improving the cost-effective

production of the desired products of the process. These bioreactors are usually used to process low-volume, high value products such as fine and specialty chemicals, amino acids and antibiotics, and recombinant DNA products. Due to the high value of this kind of product, the amount of profit can be greatly increased even with minimal improvements in the yield and productivity of the process (Lim and Shin 2013). Therefore, owing to the increasing demand for specialty chemicals, over the last three decades there has been enormous progress in the use and development of enhanced fed-batch bioreactors due to some inherent advantages that this kind of bioreactors present.

In general, a defined medium is used to obtain high cell-density cultures (HCDC), in which the nutrient concentrations are known and also contains all the inorganic salts and trace elements necessary to support cell growth. After inoculation, the microorganisms grow at the maximum specific growth rate until a medium component, usually the carbon source, is completely consumed and the cell growth stops. Then, a feed with concentrated nutrients is fed into the fermentor and growth is re-started and kept until high cell-density is achieved. In this regard, whilst certain nutrients, such as carbon or nitrogen sources, are fundamental for cell growth, they can become inhibitory when they are present above a certain concentration, explaining why increasing the concentration of nutrients in a batch culture media does not always generate HCDC (Lee 1996). Therefore, medium and feeding regimes need to be carefully designed in order to allow fast cell growth but prevent the production and accumulation of toxic compounds, such as acetate (Han *et al*. 1992).

Acetate formation is especially problematic in fed-batch fermentation. Its production is dependent on the medium used and the specific growth rate (Lee 1996). For instance, acetate forms when the specific growth of *E. coli* exceeds 0.2 or 0.35 h^{-1} in complex and defined media, respectively (Riesenberg *et al*. 1991). Additionally, a study using different recombinant microorganisms, including *E. coli*, *Saccharomyces cerevisiae* and *Bacillus*, grown in a defined medium showed that acetate formation varies depending on the microbe used

and, furthermore, acetate has a greater detrimental impact on recombinant cells than on non-recombinant cells, with the accumulation of acetate in the medium significantly reducing the expression of recombinant-protein (Bauer *et al*. 1990; Fieschko 1989). Hence, many different strategies have been developed to reduce acetate formation in fed-batch cultures, which, usually at concentrations higher than 10 g/L, becomes inhibitory (Pan, Rhee, and Lebeault 1987), such as controlling the specific growth rate by limiting essential nutrients such as carbon or nitrogen or reducing the nutrient uptake and growth, hence minimising the production of inhibitory side-products (Riesenberg *et al*. 1991); altering the media composition, using glycerol as carbon source for instance (Holms 1986); lowering the culture temperature in order to control the cell metabolism (MacDonald and Neway 1990), or even by means of metabolic engineering to remove the enzymes involved in the formation of acetate (Bauer *et al*. 1990).

A common strategy used to achieve HCDC and reduce the formation of inhibitory by-products, acetate in particular, is to develop a controlled feed method which can be started automatically or manually, once the key nutrients in the culture are depleted, with or without feed-back control (Minihane and Brown 1986). Simple feed methods with no feed-back control have been used to obtain high cell-density of *E. coli* in culture, where the feed rate is set up to a predetermined profile, such a constant-rate feeding where concentrated nutrients are fed into the fermentor at a constant level hence increasing the cell concentration in the fermentor. However, by maintaining a constant feeding rate the specific growth rate will continuously decrease over time, and the increase in cell density slows down (Jensen and Carlsen 1990). A stepwise increase in the feeding rate allows compensating for the decrease in specific growth rate during almost the entire fermentation period by gradually increasing the feed rate as biomass concentration increases (Konstantinov *et al*. 1991). An exponential feeding-rate allows cells to grow at a constant specific growth rate by increasing exponentially the feed rate as biomass concentration increases (Pan, Rhee, and Lebeault 1987).

Fully automated feeding rate controls have been developed by using feedback control in respond to different conditions in the fermentor. Indirect feedback loops couple the feeding with the measurement of various physical parameters related to cell growth, such as dissolved oxygen (DO) (Cutayar and Poillon 1989); pH (Lee and Chang 1993); and CO_2 evolution rate (CER) (Lee and Mohler 1988), which can change depending on the conditions in the fermentor. For instance, the DO-stat and pH-stat method are based, respectively, on the increase in the concentration of dissolved oxygen and pH, as a result of the depletion of the principal carbon source. The feed rate can then be increased to maintain both parameters at a set point. The CO_2 evolution rate method is based on the CER being roughly proportional to the consumption of the carbon source, hence the nutrient feeding is controlled by measuring on-line the concentration of CO_2 in the gas outlet. Direct feedback control operates with a direct signal of the concentration of the main carbon source in the fermentor by which the feed rate is controlled.

5.4.2 Fermentation design for the fed-batch production of styrene

The fermentation process was split in three characteristic phases that allowed biomass to be efficiently generated for the later production of styrene. Samples were taken regularly to measure OD_{600}, dry cell weight, glucose consumption and/or accumulation, and organic acid concentrations. It is worth noticing that for the calculation of total production or consumption, and/or conversion efficiencies of a particular compound, the change in the fermentor volume due to addition of feed, acid or base, and removal of culture during sampling was taken into account.

Phase one, the batch phase, comprises the inoculation of *E. coli* NST74 pSTV28_pal2 pTrc99A_fdc1 into the fermentation medium, containing glucose (11.9 g/L) and yeast extract (5 g/L) as the sources of carbon, with resultant growth at the maximum specific growth rate. Due to the uptake of oxygen by

the growing culture, the DO decreases until it reaches 30% saturation, when the feedback loop control for DO becomes operational and the stirrer speed and air flow cascade control were used to maintain the DO at 30%. The total consumption of the glucose and yeast extract initially added is denoted by a rapid increase in the DO, indicating that the cells had starved, which marks the end of the batch phase and phase two was started.

In phase two, the fed-batch phase, a stepwise glucose feeding regime was started and incrementally increased over a period of 3 h in order to provide additional nutrients and increase the cell density of the culture. As the feeding produces further growth, an immediate decrease in the DO to the control level is observed. The feed composition was a concentrated glucose solution containing $MgSO_4$, trace elements and yeast extracts. Due to the high concentration of mineral salts needed in the medium to support growth to a high cell density, higher concentrations of $MgSO_4$ and trace elements were added to the feed instead of to the initial culture medium, thus precipitation of phosphate salts during autoclaving of the batch medium was avoided nor toxic levels of these minerals were present in the fermentor at the beginning of the batch phase (Matsui *et al.* 1989; Riesenberg *et al.* 1990; Webb 2015). All four components of the feed were autoclaved separately and later mixed after cooling avoiding the denaturation of the medium. Yeast extract was added to reduce the likelihood of acetate being produced during the feeding stage, since it reduces the glucose uptake rate by providing most of the necessary nutrients for the synthesis of building blocks of cells, and thus glucose is mainly used as an energy source (Han *et al*., 1992; Panda, Khan, and Mishra, 2000), and also the presence of yeast extract in the medium helps to lower the inhibitory effect of acetic acid by promoting its utilisation during carbon limitation (Koh *et al*. 1992; Nancib, Mosrati, and Boudrant 1993). The stepwise feed regime helped to maintain the specific growth rate in the range of 0.2 – 0.3 h^{-1}, which is below the critical growth rate at which acetic acid is produced (Riesenberg 1991).

Phase three was started when the culture reached an OD_{600} ~50. Cells were induced by addition of 0.4 mM IPTG. The glucose feeding rate was maintained

throughout the rest of the fermentation at the same rate phase two was finished. Glucose presence was checked regularly, in which case the feed rate was slightly decreased each time that accumulation was detected.

5.4.3 Uninduced fed-batch fermentation of E. coli NST74 harbouring pSTV28_pal2 and pTrc99A_fdc1

In order to check if the conditions for the fermentation protocol designed previously were appropriate to obtain a high cell-density culture of recombinant cells, firstly an uninduced fermentation of the *E. coli* NST74 strain harbouring pSTV28_pal2 and pTrc99A_fdc1 was performed (Figure 43).

At the end of phase one, all the glucose initially added (11.9 g/L) had been consumed, the culture OD_{600} was 18.7, corresponding to 5.4 g/L DCW, and no acetate had accumulated; however low levels of lactate (0.2 g/L) had accumulated as the sole organic acid product detected by HPLC. Given that there was no induction with IPTG, phase two was continued for further 33 h, completing a total of 48 h of fermentation. Since the feed was started, it took a further 5 h to reach OD_{600} 46.7 (16.1 g/L DCW), and an additional 35.6 g of glucose were consumed. At this point, low levels of pyruvate (0.03 g/L), lactate (0.3 g/L), formate (0.1 g/L) and acetate (2.4 g/L) had accumulated. No glucose was accumulated during this period. Additionally, the presence of low levels of the aromatic amino acids tyrosine (0.03 g/L) and L-phenylalanine (0.54 g/L) were detected by HPLC. Unexpectedly, low levels of *trans*-cinnamic acid (0.3 g/L) and styrene (0.018 g/L) were also detected in the culture, meaning that low levels of recombinant proteins PAL2 and FDC1 were expressed even without induction. This expression in the absence of an inducer could be due to the presence of the *lac* and *trc* promoters in the plasmids pSTV28 and pTrc99A, respectively, which both are *lac*-derived promoters known for their intrinsic leaky expression in non-induced cells (Rosano and Ceccarelli 2014; Bernhard, Klammt, and Rüterjans 2007).

During the rest of the fermentation, a further 259.9 g of glucose were consumed to produce a final total biomass of 43.3 g/L, with a yield of 0.14 g/g. No glucose was accumulated during the entire fermentation. By the end of the fermentation low levels of pyruvate (0.05 g/L), lactate (0.7 g/L) and formate (1.66 g/L) were detected by HPLC. On the other hand, levels about the inhibition concentration of acetate (13.1 g/L) were accumulated, explaining the decrease in the OD_{600} during the last 6 h of fermentation. Additionally, the only amino acid detected was L-phenylalanine (1.32 g/L), since the tyrosine accumulated during the first 6 h of phase two was completely re-consumed by the end of the run. Finally, further accumulation of *trans*-cinnamic acid (1.03 g/L) and styrene (0.025 g/L) were detected. If taken into account the total accumulation of *trans*-cinnamic acid and styrene, and the final concentration of L-phenylalanine in the fermentor, a total of 2.50 g of L-phenylalanine was produced during the entire fermentation process, obtaining a conversion yield of 0.008 $g/g_{glucose}$. It is possible that the decrease in yield of the L-phenylalanine production when compared to the previous result obtained in shake flask might be due to the metabolic burden imposed on the microbe by the synthesis of the inserted plasmids.

This fermentation confirmed that the present used medium and the designed culture method are applicable for the high concentration cultivation of the selected recombinant *E. coli* strain.

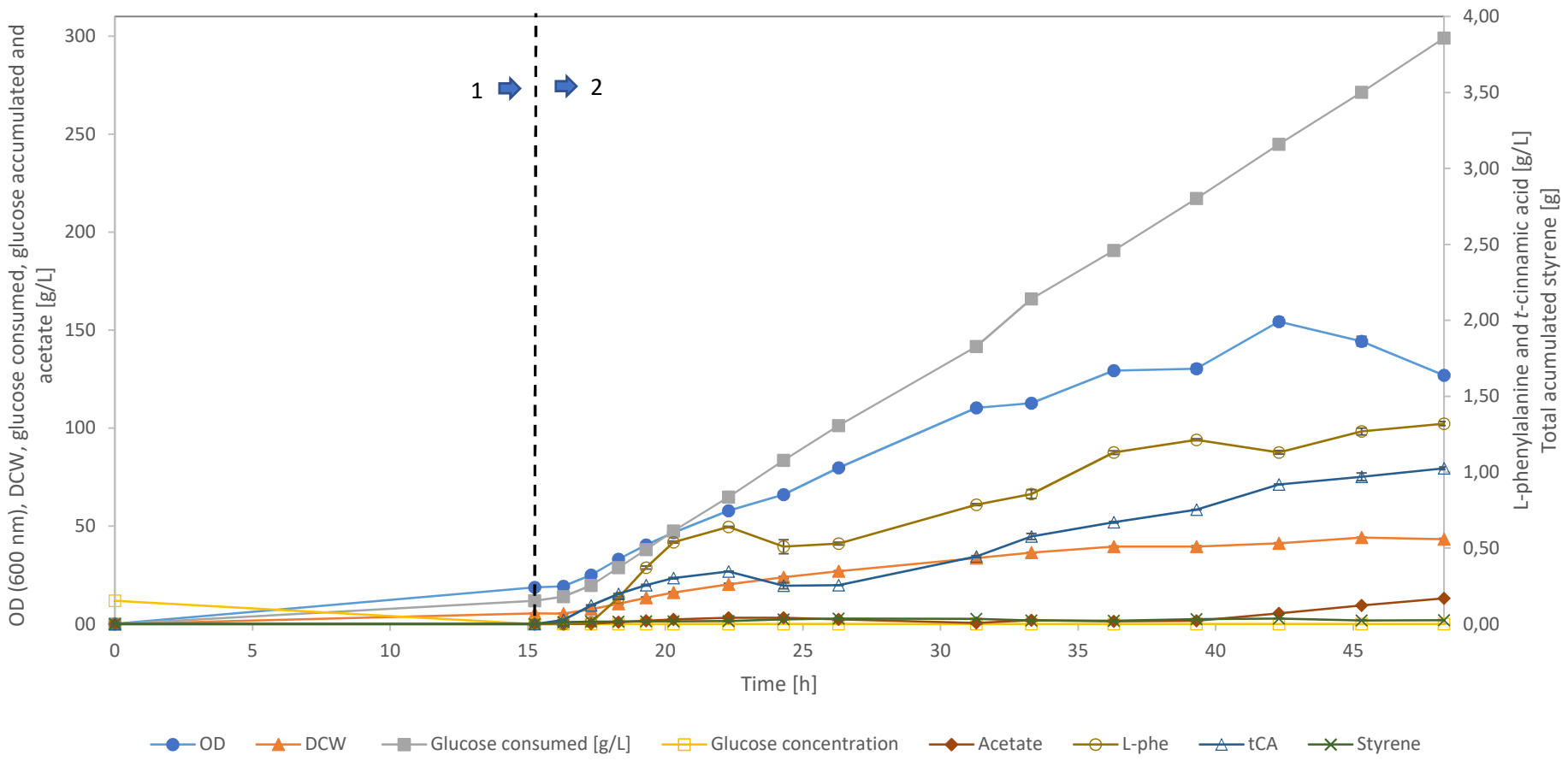


Figure 43: Control uninduced fed-batch fermentation.

E. coli NST74 harbouring pSTV28_pal2 and pTrc99A_fdc1 was grown to starvation on a single batch of glucose (1). Following this, a stepwise glucose feeding regime was used to grow the cells (2). During this control fermentation there it was no induction with IPTG, therefore phase two was continued for further 33 h. Samples were taken to measure OD_{600}, and concentrations of DCW, glucose consumed, actual glucose accumulated, acetate, L-phenylalanine, *trans*-cinnamic acid and styrene. Results and standard deviations are the average of triplicate experiments.

5.4.4 High cell-density fermentation of recombinant E. coli for the production of styrene

The fermentation method previously tested to produce a high cell-density culture of the recombinant *E. coli* NST74 strain harbouring pSTV28_pal2 and pTrc99A_fdc1 was repeated, this time with induction of the cells with IPTG for the production of styrene (Figure 44).

During phase one, 11.9 g of glucose were consumed and converted to 4.6 g of biomass (OD_{600} 18.1) and low levels of lactate (0.2 g/L). Phase two lasted 5 h, and an additional 35.5 g of glucose were converted to 10.4 g of biomass, with a conversion yield of 0.29 $g/g_{glucose}$. A practical yield value of 0.5 $g/g_{substrate}$, for both glucose and glycerol, have been previously established and reported (Herbert, Elsworth, and Telling 1956), showing that there is still scope to enhance this phase of the fermentation. Nevertheless, previous yields were determined working with non-recombinant cells, therefore the plasmid metabolic burden has to be considered in the microorganism used in this fermentation when trying to increase the dry cell weight yield. Low levels of lactate (0.3 g/L), formate (0.08 g/L) and acetate (2.7 g/L) had accumulated; and, in a similar fashion to the previous fermentation, tyrosine (0.04 g/L), L-phenylalanine (0.44 g/L), and *trans*-cinnamic acid (0.03 g/L) and styrene (0.08 g/L) had accumulated, confirming the leaky expression of the recombinant protein PAL2 and FDC1.

Phase three was initiated when the culture was at OD_{600} 44.6 (15.0 g/L DCW). At this point, the IPTG inducer was added to the medium, and phase three was allowed to continue for 28 h. A further 196.3 g of glucose were consumed and converted into 13.1 g of biomass, with a conversion yield of 0.067 $g/g_{glucose}$. However, in this case an accumulation of glucose (2.6 g) occurred by the end of the fermentation, perhaps due to a reduction in the metabolic activity at this stage, which coincides with the decrease in the DO during the same period of time. The decrease in the total biomass produced (*i.e.* 28.3 g/L) when compared

to the uninduced fermentation (*i.e.* 43.3 g/L) indicated the metabolic burden exhibited on cells due to gene expression and demonstrated that carbon was deviated away from biomass formation and instead styrene was produced in higher quantities. Consequently, the toxic effect of styrene also must be taken into consideration regarding a lower biomass production.

Low levels of pyruvate (0.15 g/L), lactate (1.56 g/L) and formate (0.43 g/L) were detected by HPLC. However, high levels of acetate (34.2 g/L) were accumulated probably due to the observed accumulation of glucose, explaining the decrease in the activity of the cells due to the acid inhibition. Additionally, as expected due to the induction of the cells, a low concentration of L-phenylalanine (0.01 g/L) had accumulated and no *trans*-cinnamic acid was detected by HPLC at the end of the fermentation run.

The highest concentration of styrene was accumulated during the first 4-8 h after induction, which also corresponded with the last period when the cells were actively growing, achieving a concentration of 0.14 g/L (*i.e.* 1.38 mM), a yield of 0.0017 $g/g_{glucose}$ and a production rate of 25.5 mg/L/h. Unexpectedly, very low levels of styrene (0.03 g/L) were accumulated in the medium after 48 h, achieving a very poor yield of 0.0001 $g/g_{glucose}$. It is hypothesised that the decrease in the styrene concentration could be due to two main factors; firstly, a decline in the metabolic activity of the cells as they reached the stationary phase, and, secondly, the loss of styrene from the medium due to the stripping by both the air flow injected to the reactor and the increased stirrer speed to maintain the DO at the set up level for the fermentation process. Therefore, a stripping test was carried out to verify if styrene was being lost by this mechanism.

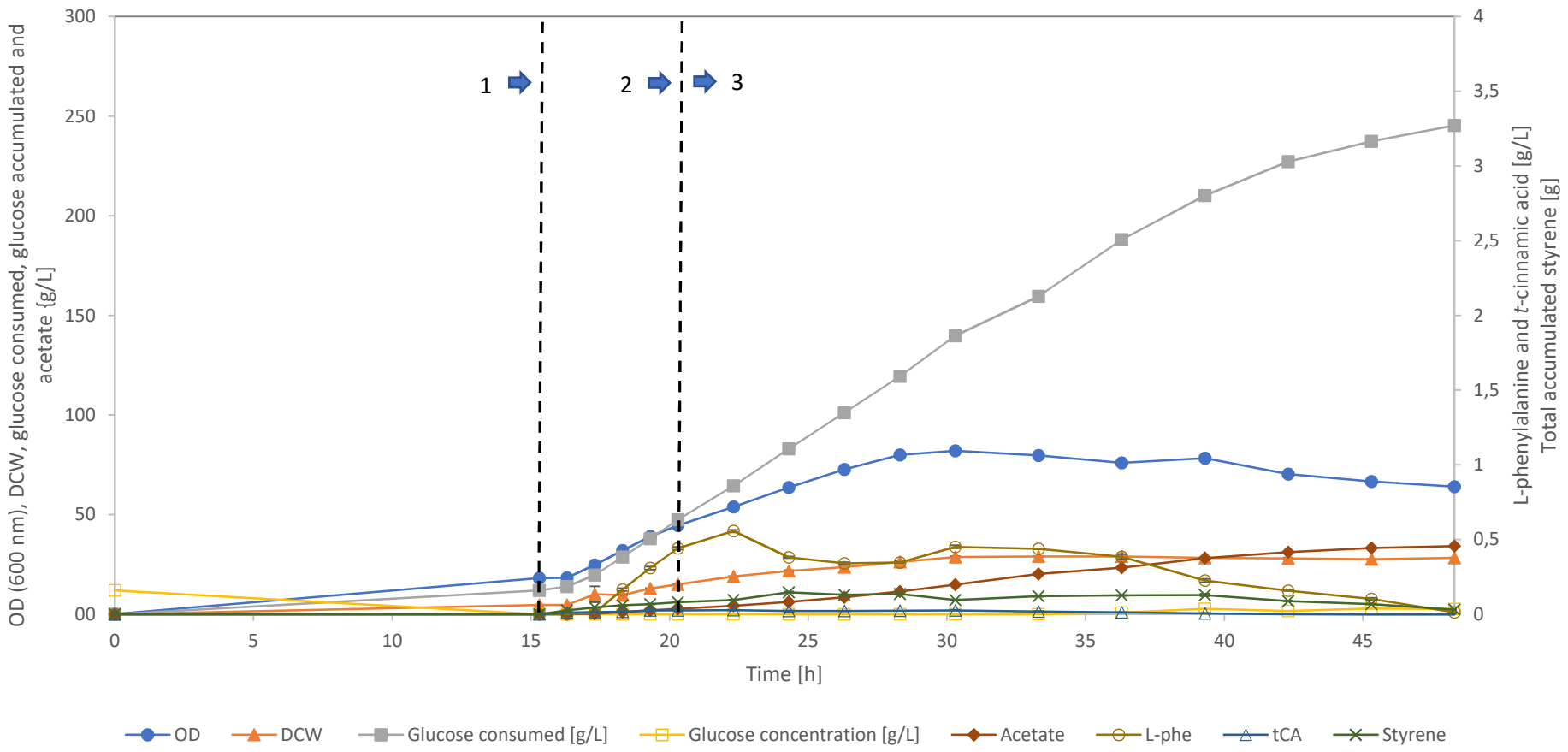


Figure 44: High cell-density fermentation for the production of styrene.

E. coli NST74 harbouring pSTV28_pal2 and pTrc99A_fdc1 was grown to starvation on a single batch of glucose (1). Following this, a stepwise glucose feeding regime was used to grow the cells to an OD_{600} of 50 (2), at which point cells were induced by the addition of IPTG (0.4 mM). The glucose feed was maintained for further 28 h (3). Samples were taken to measure OD_{600}, and concentrations of DCW, glucose consumed, actual glucose accumulated, acetate, L-phenylalanine, *trans*-cinnamic acid and styrene. Results and standard deviations are the average of triplicate experiments.

5.4.5 Styrene gas stripping tests from the bioreactor system

During the previous induced fermentation performed to produce styrene it was noticed that the highest concentrations were accumulated during the initial 4-8 hours after the induction and that most of the styrene was then lost from the medium by the end of the fermentation run. This phenomenon is suspected to be due, mostly, to the styrene being removed by both the stirrer mechanism of the reactor and the air flow injected to maintain the DO level for the fermentation. Therefore, to verify this hypothesis styrene stripping tests were carried out in the bioreactor uninoculated. The conditions of the test were the same as those used for the fermentation (*i.e.* 37 °C, 1 L/min air flow) and the lowest and highest agitation speeds, 400 and 1200 rpm, respectively, were tested. The bioreactor was initially charged with 1 L of medium and warmed up to 37 °C, whilst constant aeration and agitation were on. When the required temperature was achieved, aeration was turned off and 220 µL of styrene were added to reach an initial concentration of 200 mg/L, which is sufficiently lower than its solubility limit in water (*i.e.* 300 mg/L) to ensure it is fully dissolved in the aqueous phase. Complete mixing of the solution was achieved only by mild agitation for one minute, hence avoiding major gas stripping. Then aeration was restarted and samples (10 mL) were taken every 4 min and continuing up to 40 min (Figure 45).

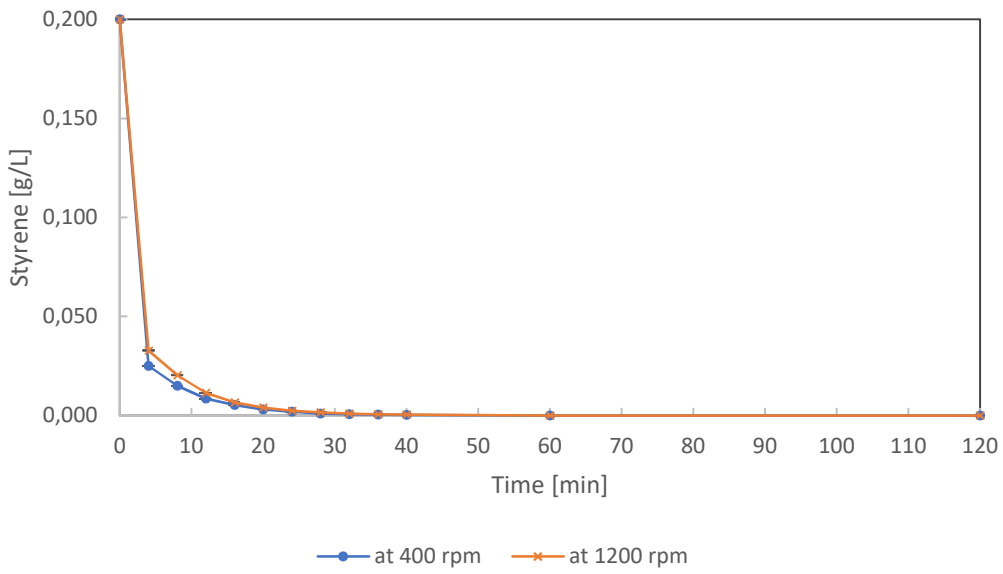


Figure 45: Styrene stripping test.
Bioreactor conditions were set up at 37 °C, 1 L/min air flow, and with a styrene concentration of 200 mg/L. Measurements were recorded at agitation speeds of 400 and 1200 rpm. Samples (10 mL) from the aqueous phase were taken every 4 min and analysed by HPLC. Results and standard deviations are the average of triplicate experiments.

Under either agitation conditions, more than 95% of the styrene initially present was stripped out within the first 16 minutes, with maximum stripping rates of 149 and 187 mg/L/h at 400 and 1200 rpm, respectively. These rates are, individually, almost 6-fold and 7.5-fold faster than the highest production rate achieved during the previous fermentation (*i.e.* 25.5 mg/L/h). Additionally, similar losses were generated at both stirrer speeds, suggesting that increasing the agitation rate above 400 rpm has no significant impact on the stripping rate of styrene in this specific system. These results are in agreement with previous report (McKenna *et al*. 2014), which suggests that above 400 rpm the system is already mixing at the turbulent flow regime, making any further speed increase to provide only minimal increment in the styrene removal. Consequently, these experiments have confirmed that styrene is being lost during the fermentation by gas stripping under the above conditions, and therefore, efforts to recover the stripped styrene were carried out.

5.4.6 Styrene gas recovery tests from the bioreactor system with an absorption column

In an attempt to recover the styrene stripped out from the fermentation, a gas absorption unit was developed and tested. Gas absorption is a unit operation in which a gas mixture is contacted with a liquid aiming to preferentially dissolve one or more components of the gas into the liquid (Perry, Green, and Maloney 2007). The following experiment focused on the development of an absorption system considering the simplest case whereby only one component of the gas mixture (*i.e.* styrene) is being absorbed by the liquid solvent; and all the other components of the gas stream are assumed to be inert, and that there is no mass transfer from the liquid to the gas phase. Additionally, it is assumed that no significant chemical reaction occurs between the solute and the absorbent, therefore only physical absorption occurs.

If the main purpose of the absorption operation is to produce a specific solution, then the solvent for absorption is selected with respect to the nature of the desired product. Alternatively, if the main purpose is to remove a specific component from the gas, as it is in this case, more options are frequently possible, but some properties, such as gas solubility and solvent volatility, are important considerations (Treybal 1980). First of all, the gas solubility should be high in order to increase the rate of absorption and minimise the amount of solvent required. Additionally, the solvent should be relatively non-volatile since the exit gas usually leaves the absorption operation saturated with solvent and therefore much may be lost. From an industrial point of view, the solvent should also be economical, non-corrosive, non-viscous, chemically stable, non-toxic and non-flammable.

For the purpose of these experiments, the two main characteristics taken into account were the high solubility of styrene in the solvent and a low volatile nature of the solvent. Hence, dodecane was selected since, firstly, it presents a similar Hildebrand solubility parameter to styrene, 7.9 and 9.3 $cal^{1/2}$ $cm^{-3/2}$, respectively, ensuring a total miscibility between these two materials (Barton

1975; Smallwood 1996). Secondly, it has a boiling point of 216.3 °C, ensuring that most of the dodecane will not be lost in the exit gas by volatilisation. Although cheaper organic solvents are available, such as the Isopar™ fluids, the complexity to analyse them due to their non-homogenous composition, including a mix of alkanes, isoalkanes and cycloalkanes, and the lack of data regarding their Hildebrand solubility parameter, make them an unsuitable option for this particular experiment. Therefore, an absorption column containing 120 mL of dodecane was attached to the exhaust gas of the bioreactor.

The bioreactor conditions were exactly the same as the ones used in the stripping tests (*i.e.* 37 °C, 1 L/min air flow), with an initial styrene concentration of 200 mg/L, and absorption measurements were recorded at agitation speeds of 400 and 1200 rpm. In a similar fashion, samples (250 µL) from the organic solvent were taken every 4 min and continuing up to 40 min and analysed by GC-FID (Figure 46).

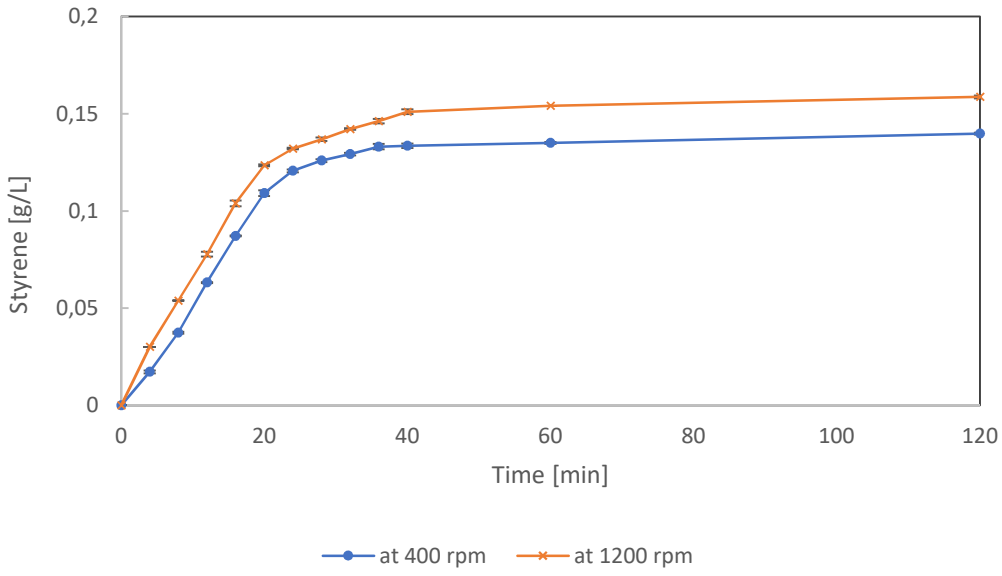


Figure 46: Styrene gas absorption with a dodecane absorption column.
Bioreactor conditions were set up at 37 °C, 1 L/min air flow, and with a styrene concentration of 200 mg/L. Measurements were recorded at agitation speeds of 400 and 1200 rpm. Samples (250 µL) from the organic solvent were taken every 4 min and analysed by GC-FID. Results and standard deviations are the average of triplicate experiments.

Analogous to the stripping experiments, the absorption mechanism behaves in a similar fashion for both agitation speeds, confirming that the stripping rate is independent of the agitation speed ratio when the system has already achieved a turbulent regime. Additionally, it is observed that not all the initial styrene added to the medium (*i.e.* 200 mg) was absorbed in the dodecane column, with a total of 140 mg and 159 mg recovered from the experiments carried out at 400 and 1200 rpm, respectively, after 2 h; representing a 70% and 79% recovery each. This could be due to the absorption capacity of the amount of dodecane used being insufficient to absorb all the styrene, or that the height of the column was not large enough to trap all the styrene contained in each air bubble ascending through the column. In order to check these hypotheses a higher initial concentration of styrene was tried to be absorbed by this same column.

Strain *E. coli* NST74_pSTV28pal2_pTRc99Afdc1 produced 1.32 g/L of L-phenylalanine when uninduced. This indicates that if all the L-phenylalanine were converted to styrene, in addition to the conversion of the accumulated *trans*-cinnamic acid (1.03 g/L), and the produced styrene (0.025 g/L) due to leaky expression, a total of 1.58 g should be produced. Therefore, an initial quantity slightly higher than this theoretical maximum of 2 g of styrene was added to the medium. The experimental conditions were kept identical to the previous stripping and absorption tests, and the agitation was set at 400 rpm, as it has been confirmed its small influence over the stripping rate above this speed ratio. Samples were taken from both the aqueous phase and organic solvent, and analysed by HPLC and GC-FID, respectively (Figure 47).

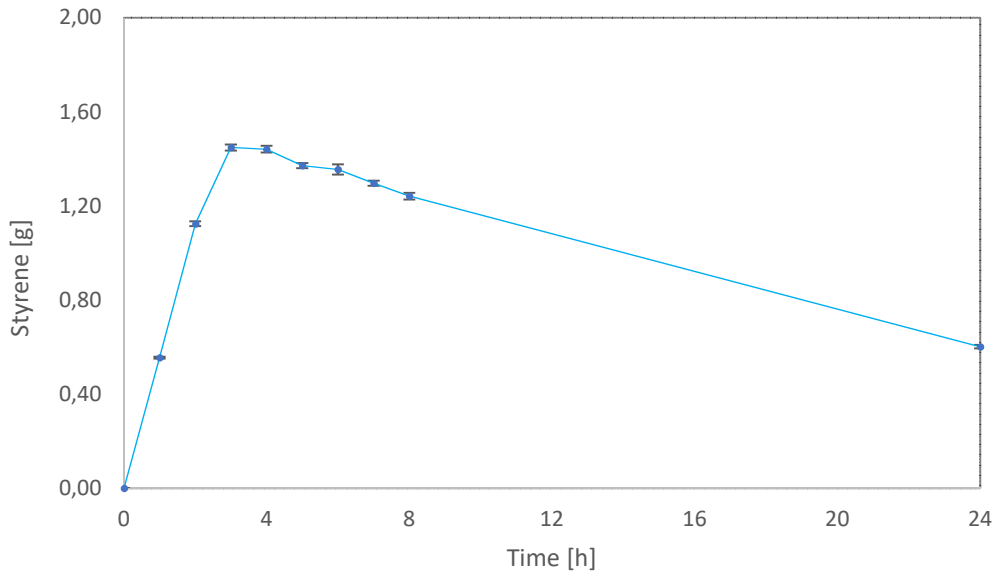


Figure 47: Styrene gas absorption with a dodecane absorption column. Bioreactor conditions were set up at 37 °C, 1 L/min air flow, and with an initial styrene concentration of 2 g/L. Absorption measurements were recorded at agitation speeds of 400 rpm. Samples from both the aqueous phase and the organic solvent were taken and analysed by HPLC and GC-FID, respectively. Results and standard deviations are the average of triplicate experiments.

During the first hour of the experiment, a total of 555 mg of styrene were absorbed in the dodecane column, hence refuting the assumption that the absorption capacity of the selected dodecane column (*i.e.* 120 mL) was not sufficient to absorb the initial 200 mg of styrene added in the previous experiments. A maximal quantity of 1.45 g of styrene was accumulated in the organic phase after 3 h, corresponding to a 72% recovery, which also coincided with no styrene being detected in the aqueous phase by HPLC. After that point, the styrene was slowly stripped out from the dodecane over the next 21 h period at an average rate of 40 mg/L/h. These results taken together with the outcomes from the prior stripping and absorption experiments, suggest that in fact the height of the column is not sufficiently tall to absorb the styrene from the air bubbles with their current ascending velocity and/or bubble diameter. Nevertheless, on average in all these absorption experiments, almost 75% of the styrene stripped out from the bioreactor was recovered by the designed absorption column. Further experiments could be carried out to determine

different kinetic parameters for this particular absorption system, such as the styrene gas transfer coefficient and the air bubble ascending velocity, to then by means of a chemical process simulation software, such as HYSYS or CHEMCAD, estimate more accurately the height of the column needed to absorb all the styrene produced. However, these sets of experiments are beyond the scope of this project.

5.4.7 Capture of styrene from the fed-batch fermentation system by liquid absorption

The recovery of styrene was proven successfully by means of an absorption column, using dodecane as solvent, attached to the end of the exit gas of the fermentation equipment. To examine the implementation of this recovery system to capture the styrene stripped out during a fermentation process, the same high cell-density fermentation protocol was repeated, this time with the absorption column connected to the exhaust gas of the bioreactor (Figure 48).

During phase one and two, prior to induction of PAL2 and FDC1, a total of 42.9 g of glucose were consumed by the cells and converted to 16.4 g of biomass representing a yield of 0.38 $g/g_{glucose}$. Additionally, low levels of the organic acids lactate (0.17 g/L), formate (0.15 g/L) and acetate (0.53 g/L) were detected by HPLC; likewise, low levels of tyrosine (0.03 g/L), L-phenylalanine (0.49 g/L), *trans*-cinnamic acid (0.27 g/L) and styrene (0.01 g/L) were accumulated in the medium. Remarkably, by this time a total of 0.36 g styrene was accumulated in the dodecane solvent.

At this point the culture reached an OD_{600} of 55.9 (DCW 16.4 g/L), hence the cells were induced with IPTG and phase three was started. Phase three was continued for 28 h, during which further 168.2 g of glucose were consumed and converted to a surprising amount of 67.1 g of biomass, for a final concentration

of 83.3 g/L of DCW. There was no glucose accumulation by the end of the fermentation. This atypical high quantity of biomass could be due to the observed increase in the emulsification of the culture and accumulation of cell debris in samples, affecting both the OD and DCW measurements. Moreover, when compared with the DCW/OD_{600} ratio of all previous fermentations both uninduced and induced, this coefficient was kept consistently within a range of 0.3-0.4 throughout all the duration of the fermentation run. However, during this fermentation, this coefficient was kept within this range only until 30 h of fermentation, when it increased up to 0.5 and later even reached a maximal value of 0.7 by the end of the 48 h. The increment of this coefficient coincides with an unknown and unusual viscous material found over the supernatant and fixed to the walls of the Eppendorf vials after centrifugation of the samples at ages over 30 h, which could explain the rise of the DCW.

As expected from previous fermentations, low levels of pyruvate (1.73 g/L), lactate (1.55 g/L) and formate (3.30 g/L) were detected by HPLC; on the other hand, high inhibitory levels of acetate (31.2 g/L) were accumulated. Even though all the fed glucose was consumed by the end of the fermentation, glucose was accumulated over a 9 h period between 33 and 42 h, which coincides with the increase of the acetate concentration to toxic levels in the culture. This outcome reinforces the suspicions that the high level of emulsion and accumulated cell debris could have affected the measurements of OD and total biomass.

In a similar fashion, low levels of tyrosine (0.28 g/L), L-phenylalanine (0.04 g/L) and styrene (0.004 g/L) were accumulated in the aqueous phase, and no *trans*-cinnamic acid was detected by HPLC in the medium. However, a remarkable amount of 2.35 g of styrene were accumulated in the dodecane column by the end of the fermentation, achieving a yield of 0.011 $g/g_{glucose}$. This result illustrates first that, even though the height of the column was not sufficient to capture all the styrene stripped under the current fermentation conditions, the capacity of the column was proven to be larger than previously estimated. Secondly, the total styrene production was shown to be greater than the

theoretical estimations based on the L-phenylalanine produced during the uninduced fermentation. Additionally, in a similar way to previous fermentations and stripping/absorption experiments, concentrations of styrene in both the aqueous phase and the absorption column were higher before the end of the fermentation, confirming that styrene is also stripped out from the dodecane once the production rate of styrene starts to decline. Moreover, a maximal amount of styrene combined between the two phases of 3.35 g was obtained at the 39 h of fermentation, with a yield of 0.017 $g/g_{glucose}$, which represents a 100-fold increase in the yield with respect the previous fermentation with no absorption system. This result confirms the possibility to absorb high concentrations of styrene with the use of the column.

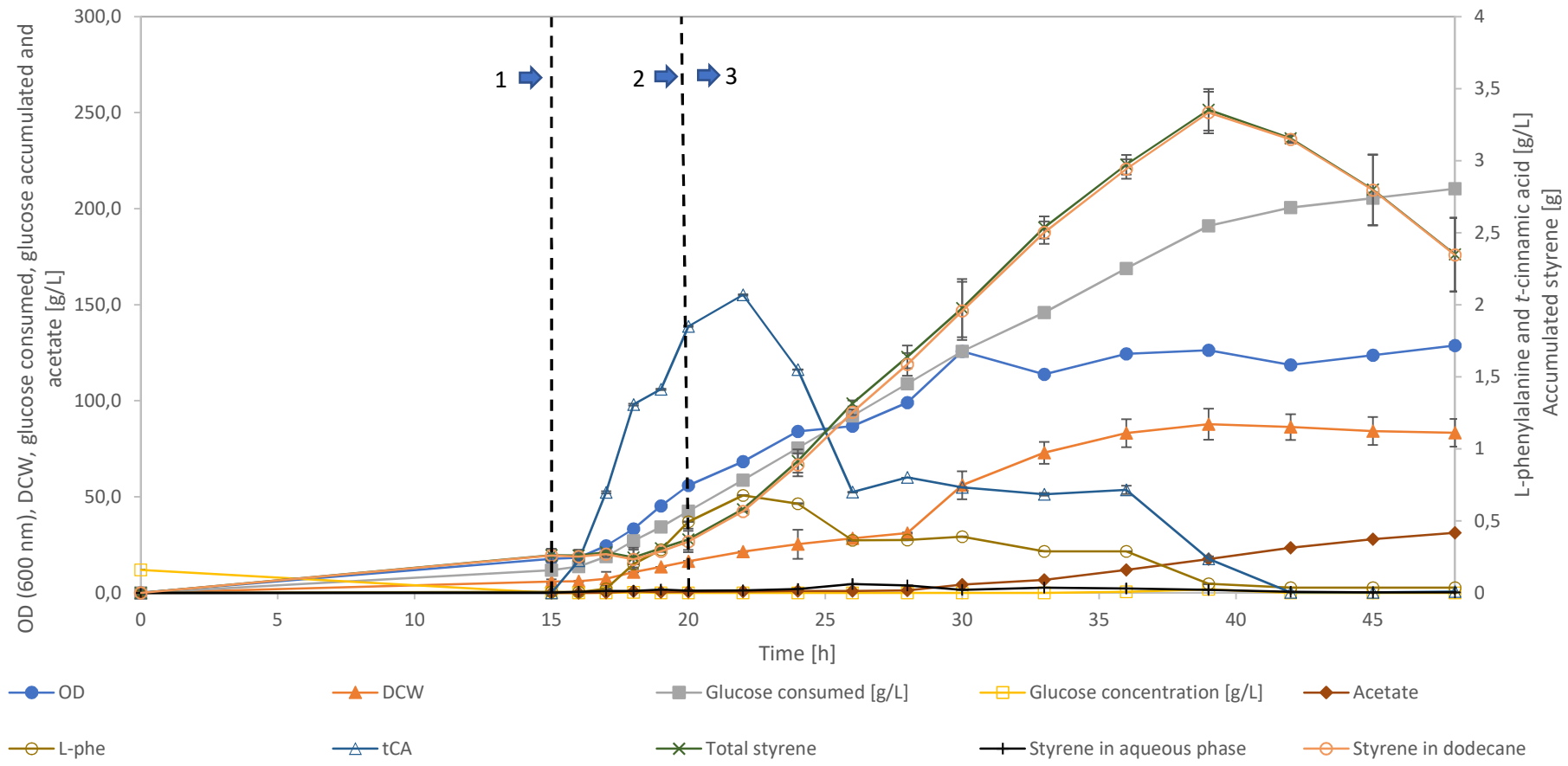


Figure 48: Capture of styrene from the fed-batch fermentation system by liquid absorption.

E. coli NST74 harbouring pSTV28_pal2 and pTrc99A_fdc1 was grown to starvation on a single batch of glucose (1). Following this, a stepwise glucose feeding regime was used to grow the cells to an OD_{600} of 50 (2), at which point cells were induced by the addition of IPTG (0.4 mM). The glucose feed was maintained for further 28 h (3). Samples were taken to measure OD_{600}, and concentrations of DCW, glucose consumed, actual glucose accumulated, acetate, L-phenylalanine, *trans*-cinnamic acid and total styrene (i.e. the quantity of styrene accumulated in both the aqueous phase and the dodecane column). Results and standard deviations are the average of duplicate experiments.

5.4.8 Fed-batch production of styrene using ionic liquids as solvents for in situ product removal

The capturing system developed using a dodecane column has been proven to be highly effective to accumulate the styrene lost from the bioreactor by stripping whilst the fermentation is being carried out. Finally, in order to evaluate if the use of a biocompatible ionic liquid as second extractant phase would improve the production of styrene, the ionic liquid $[P_{66614}][NTf_2]$ was used for the *in situ* extraction of styrene (Figure 49).

Phase one was carried out as normal and 11.9 g of glucose were converted to 6.1 g of biomass. No accumulation in the aqueous phase of any of the analysed metabolites was detected by HPLC. However, 0.27 g of styrene had already accumulated in the dodecane column. The glucose feed was then started and phase two was initiated. Culture was grown until an OD_{600} of ~30, at which point $[P_{66614}][NTf_2]$ (250 mL, 20% v/v) was pumped into the bioreactor, so that the final total culture volume was 1.25 L. The OD was continuously monitored to confirm that the cells were growing as expected. Phase two lasted 5 h, and additional 31 g of glucose were converted to 11.0 g of biomass to reach an OD_{600} of 50. It is worth noticing that, regardless of the addition of the ionic liquid to the culture, it took the same time as previous fermentations with no ionic liquid to reach similar levels of bacterial concentration, highlighting the biocompatibility of the selected IL. At this point, low levels of lactate (0.16 g/L), formate (0.13 g/L) and acetate (0.79 g/L) had accumulated; and no tyrosine, L-phenylalanine, and *trans*-cinnamic acid nor styrene had accumulated in the aqueous phase. In a remarkable outcome, 0.48 g of styrene had already accumulated in the absorption column and 0.68 g in the IL phase, for a total combined of 1.36 g.

IPTG was then added to the culture and phase three was started, which continued for further 28 h. During this period additional 115.6 g of glucose were consumed, however no increase in the biomass concentration was observed,

indicating that the culture did not use the glucose for further growth. An accumulation of 3.3 g of glucose was detected by the end of the fermentation. Additionally, lactate (10.2 g/L), formate (0.6 g/L) and acetate (24.5 g/L) were detected by HPLC. The high concentration of acetate was probably result of the glucose accumulation previously indicated, which occurred over two periods of time, first between 30 and 36 h, and later between 42 and 48 h. When compared to the previous fermentations, neither L-phenylalanine nor *trans*-cinnamic acid were accumulated during the whole fermentation process, suggesting that the continuous extraction of styrene by the ionic liquid was acting as the pathway driving force by shifting the pathway equilibrium toward styrene production. Additionally, no styrene was detected by HPLC in the aqueous phase by the end of the fermentation; with all the styrene accumulated in the dodecane column (1.41 g) and the ionic liquid phase (1.26 g), supporting the latter assumptions. Furthermore, during the whole fermentation process, the highest quantity accumulated of styrene combined between both the dodecane and ionic liquid was 3.85 g at 30 h, with a yield 0.031 $g/g_{glucose}$, which represents a yield increase of 200-fold when compared to the fermentation in the absence of ionic liquid and no dodecane column, and an almost 2-fold increase when the absorption system is attached to the bioreactor, but no ionic liquid is present.

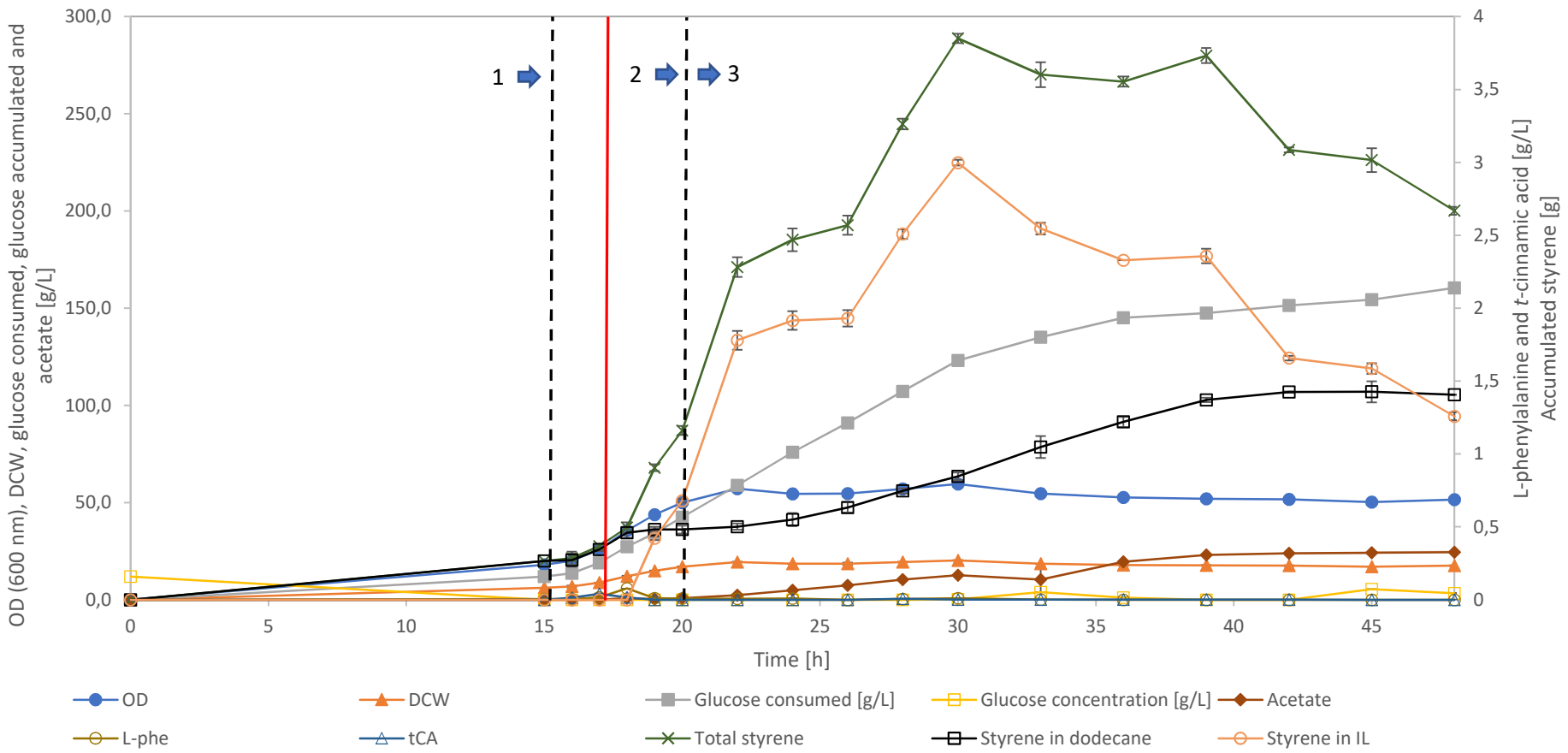


Figure 49: Fed-batch production of styrene using ionic liquids as solvents for *in situ* product removal.

E. coli NST74 harbouring pSTV28_pal2 and pTrc99A_fdc1 was grown to starvation on a single batch of glucose (1). Following this, a stepwise glucose feeding regime was started. Culture was grown until an OD_{600} of ~30, at which point $[P_{66614}][NTf_2]$ (250 mL, 20% v/v) was pumped into the bioreactor (red line). Cell were then grown to an OD_{600} of 50 (2), at which point cells were induced by the addition of IPTG (0.4 mM). The glucose feed was maintained for further 28 h (3). Samples were taken to measure OD_{600}, and concentrations of DCW, glucose consumed, actual glucose accumulated, acetate, L-phenylalanine, *trans*-cinnamic acid and total styrene (i.e. the quantity of styrene accumulated in the aqueous phase, the dodecane column and the ionic liquid phase). Results and standard deviations are the average of duplicate experiments.

5.4.9 Discussions and conclusions for fed-batch production of styrene

Comparison of the styrene produced after the batch phase of each fermentation (Table 17), showed that the fermentation in the presence of ionic liquid as a secondary extractant phase and an absorption column to capture the styrene stripped out during the fermentation process resulted in the highest overall concentration of styrene.

Table 17: Comparison of glucose consumed and styrene produced for all fermentations.

Fermentation	Glucose consumed [g] [(a)]	Glucose consumption rate [g/L/h] [(a)]	Maximum styrene produced [g] [(b)]	Maximum production rate [g/L/h] [(b)]	Maximum conversion efficiency [$g/g_{glucose}$] [(b)]
Induced control	231.8	7.1	0.146	0.0255	0.00173
Induced test with absorption column	199.2	6.0	3.35	0.125	0.0173
Induced test with IL phase and absorption column	146.6	4.5	3.85	0.232	0.031

[(a)] Total glucose consumed after the batch phase (*i.e.* phase one).
[(b)] Production rates and conversion efficiencies were calculated with respect to the period of the maximal styrene concentration during the fermentation.

However, similar maximum quantities of styrene were achieved in the fermentation with no presence of ionic liquid but with an absorption column attached to the exhaust gas of the bioreactor. In this case, it can also be observed that styrene is being produced at a slower rate than the calculated stripping rates (*i.e.* 0.149 and 0.187 g/L/h at 400 and 1200 rpm, respectively),

then accumulation of styrene to inhibitory levels should not occur. Taking together, these outcomes would suggest that there it would be no need for the use of an extractant phase; however, the strain production rate still decreased throughout the fermentation, suggesting that there is still some styrene in the medium in contact with the cells, inhibiting the biocatalyst over time. Therefore, it is believed that the *in situ* extraction with ionic liquid is more efficient in keeping styrene out of contact with the cells compared to gas stripping, thus reducing its inhibitory effects. This assumption is supported by the findings that, when ionic liquid is present, no styrene was accumulated in the aqueous phase during the fermentation, and that the maximum production rate is almost 2-fold higher.

In fermentations using ionic liquids there was no accumulation of either L-phenylalanine or *trans*-cinnamic acid during the all fermentation process. It is believed that the presence of ionic liquid in the culture acts as a driving force in the styrene metabolic pathway, pulling both precursors towards styrene synthesis. Additionally, it is observed that a 25% and 35% less total glucose was consumed when compared to the fermentations with and without an absorption column, respectively; also achieving even higher productions rates and yields of styrene, and, moreover, 30% less acetate was produced. On the other hand, it was observed that growth was halted after cell induction only in the presence of ionic liquid. All the above suggests that less carbon was diverted towards cells growth and mainly used for styrene production; however, regarding cell growth, it also must be taken into account that the presence of ionic liquid might inhibit the cells to continue growing.

Overall, fermentation in the presence of ionic liquids as an extractant phase has been shown to be more efficient with respect to feedstock consumption. However, a recovery system was also shown to be of great importance to increase the production yields when producing volatile compounds. Future work will focus on comparing the use of an organic solvent as a secondary phase in order to have a more comprehensive assessment regarding the benefits of using ionic liquids in this fermentation process.

6. Conclusions, Discussion and Future work

The possibility of reducing the environmental impact of the well-established chemical processes by means of biocatalytic processes at industrial scale is of great interest at the current time. However, in order for biocatalysis to become a feasible alternative option to the current chemical processes there are various constraints that need to be overcome first; with the susceptibility of the selected biocatalysts to either the substrate or end product being one of the most critical needing to be addressed.

Therefore, the work in this thesis aimed to enhance the biocatalytic production of styrene from renewable resources by overcoming the end-product toxicity constrain using ionic liquids to *in situ* remove the styrene from the culture media. For this, first, a wide range of ionic liquids were selected and tested for their toxicity towards *E. coli* using a high throughput screening method. The biocompatible ionic liquids were chosen and their capacity for styrene extraction was investigated. Later, styrene production using a metabolically modified *E. coli* strain was successfully performed in the presence of a previously selected ionic liquid in both flask and fed-batch fermentations.

Firstly, the styrene toxicity towards the *E. coli* MG1655 was determined in minimal media, showing that at a 2.5 mM styrene concentration cell growth was completely inhibited. The obtained toxic threshold was found to be different than the levels established in previous works towards *E. coli* (McKenna and Nielsen 2011); however, its toxic level was found to be dependent on both the kind of strain selected and media used, highlighting the different behaviours shown by even similar microorganisms against the same compound, thus exposing the necessity to experimentally evaluate the toxic effects of the end-product towards the selected biocatalyst.

Investigation of the use of ionic liquids as alternative biocompatible solvents for the *in situ* extraction of styrene was proven to be successful, by removing almost completely the styrene from the aqueous phase, and even increasing

the toxic threshold of styrene over a 100-fold when compared to the toxicity limit established in the absence of ionic liquids. With regards to the investigation of the biocompatibility of ionic liquids, this study suggests that its toxicity is highly influenced by the ionic liquids chemical structure, *i.e.* the alkyl chain length, cation family and anionic moiety, therefore there is a huge potential for the design of a wide range of non-toxic ionic liquids based on the current results and understanding of their structure-toxicity relationship presented in this thesis. Furthermore, there is also the opportunity to investigate the mechanism of toxicity of ionic liquids, since results in chapter 5.1 suggested the interaction between the studied ionic liquids and components of the culture media, affecting the overall pH of the culture, resulting in inhibitory effects towards the cells; and its influence seems to be related to the ionic liquid structure. The fluctuations in the pH of the culture medium could be due to either the extraction of essential components out of the growth medium by the ionic liquids, or a biochemical reaction between specific components of the media and the ionic liquids. The ability of ILs to extract metal salts from liquid media has been reported in the literature (Han and Row 2010; Matsumoto *et al*. 2004; Simoni *et al.* 2010), therefore, future work should contemplate the analysis of both the IL and the aqueous phases of the system to help elucidate if some components were transferred from the aqueous phase into the IL layer or if any reaction took place.

The interaction between the selected ionic liquids and styrene was suggested to be mainly due to strong van der Waals and π—π stacking interactions between the aromatic rings of styrene and the different anions moieties of the selected ILs; however, the mechanism of interaction between styrene and ILs requires further investigation to understand the interactions responsible for the high extraction efficiencies in to order to produce better task-specific ILs (TSILs) for this extraction process. Additionally, this would help to select the most suitable method for the recovery of styrene from ILs.

The distillation of solutes from ILs is in general considered as a simple process (Poole and Poole 2010; Oliveira *et al*. 2012); since ILs present a negligible

vapour pressure, they cannot be distilled under normal pressure conditions, hence product recovery from them is generally easy. In particular, the volatile nature of styrene would ease the recovery process by distillation from the IL; however, distillation at industrial scale is an expensive and energy intensive process, therefore alternative recovery methods should be considered. Results in the chapter 5.4 showed that styrene was also stripped from the IL phase during the fed-batch fermentation process, decreasing its concentration by the end of the run. This outcome suggests that, if aeration of the culture was maintained for longer, eventually all of the styrene would have been stripped from the IL. Thus, an alternative approach to recovery of the styrene from the IL could be to remove it by aeration, and then recover the stripped styrene by cryogenic condensation using liquid nitrogen, which is a well-established technology for controlling emissions of organic solvent vapours classified as hazardous air pollutants (HAPs) and volatile organic compounds (VOCs) (BNP Media 2014). Its low power consumption and the opportunity to recycle the nitrogen would offer an economical alternative to the distillation process. However, safety operational precautions must be taken when using cryogenic liquids, since the low temperature of liquid nitrogen may cause gaseous oxygen to condense from the atmosphere and accumulate as liquid oxygen, thus creating an oxygen-enriched environment. The presence of condense oxygen significantly increases the risk of fire or explosion since it may combine with organic compounds which could produce an explosive mixture. This method could also be used for the recovery and/or purification of the IL in order for it to be recycled in the process. This point raises questions about the recyclability of the ILs, since their currently high cost makes their use at industrial scale only viable if the IL can be reused without loss of productivity during the process. Therefore, it would be important to study the repeated use of the ILs as a secondary phase for the extraction of styrene, before the efficiency of the process decreases.

The *in situ* extraction of styrene *via* liquid-liquid extraction using the selected biocompatible ionic liquid was one of the most critical steps in the development

of this integrated process, and it was successfully performed, achieving to increase the production of styrene of the fermentation process compared to the production in absence of a secondary extractant phase. According to the experimental results of the fermentation without *in situ* extraction, even though the styrene concentration did not reach the toxic threshold towards the cell, it was observed that their productivity decreased over time when compared to the productivities of the fermentation in the presence of ionic liquids as a secondary extractant phase. Moreover, the ionic liquid acted as a driving force of the biosynthetic pathway, pulling the majority of the precursors towards the production of styrene, hence validating the use of ionic liquid in this particular bioprocess. Nevertheless, the use of an organic solvent as a potential extractant cannot be ruled out without further experiments. Thus, future work should consider the use of a suitable organic solvent as a secondary phase, assessing its toxicity towards the biocatalyst and styrene extraction capability. In this work dodecane was shown to have a great affinity towards styrene; additionally, it is immiscible with water, and, most important, it has been reported to be biocompatible with *E. coli* in both complex and minimal media (Disley 2016), hence, it seems like a suitable solvent to be tested for this fermentation process. Fed-batch production of styrene should be carried out repeating the same operation conditions that were used in the presence of ionic liquid as an extractant in order to make a comprehensive analysis and comparison of all the key variables involved in this process. Due to the high affinity previously showed by dodecane it is expected that a high extraction of styrene would be achieved; hence the importance that other variables such as glucose consumption and acetic acid formation will have for a thorough comparison of the benefits and disadvantages of each solvent as a secondary phase for the *in situ* extraction of styrene.

It was shown that the highest total production of styrene was achieved when the stripped styrene was captured by means of liquid absorption using a dodecane column attached to the exhaust gas of the bioreactor; therefore, a production process combining both the *in situ* extraction of styrene and its

capture with a liquid column appears to be the best approach to obtain higher bioproduction yields of styrene. In this respect, during the fermentation and stripping/absorption experiments it was revealed that the height of the used column was not sufficient to absorb the total amount of styrene stripped from the bioreactor at the current air flow rate and air bubbles diameter. As stated earlier, further experiments need to be performed in order to be able to establish key absorption kinetic parameters of this system, to then by using computational modelling establish the precise column's height needed. Another approach for this absorption system could be the use of various smaller absorption columns operating either in a parallel or in series configuration. Additionally, changes to the column design (*i.e.* height and diameter) could also be tested, together with different aeration rates. In this regard, the possibility of using pure oxygen aeration could be an alternative to reduce the stripping of styrene since lower aeration rates would be needed for the bacterial growth; thus, possibly, reducing both the amount of absorbent solvent needed and the dimensions of the absorption column since more styrene could be extracted *in situ*. However, as stated earlier, careful must be taken when working with pure oxygen since an oxygen enriched environment, combined with a volatile and flammable compound such as styrene increases the risk of fire. The coefficient of performance of each configuration and its sensitivity to different operation conditions should be then measured and compared in order to determine the advantages and disadvantages of each different configuration absorption systems. At an industrial scale, gas absorption is most commonly practiced in a packed tower (Cussler 1985), which is basically a column filled with inert material. A gas mixture enters the bottom of the tower and flows out to the top. The target compound is absorbed by the solvent flowing countercurrently through the packing, achieving in this way an intimate contact between gas and liquid, thus enhancing the absorption process. Therefore, a more exhaustive cost-benefit analysis of the different analysed systems should be carried out in order to select the most effective absorption strategy.

Other alternatives to increase the bioproduction of styrene could be achieved by further engineering the current used biocatalyst and the styrene metabolic pathway. The low activity of the pathway enzymes was suggested to be hampering the production of styrene. For instances, PAL expression could be enhanced by use of a codon optimisation approach, which has been shown to be successful to develop pathways in *E. coli* involving the expression of genes derived from plants (Keasling 2008). Moreover, FDC1 has been reported to require a cofactor, synthesised by PAD1, an enzyme which had been defined as a phenylacrylic acid decarboxylase enzyme, for optimal performance (Lin *et al*. 2015); therefore, co-expression of PAD1 and FDC1 in the current strain should be carried out. Additionally, the production of L-phenylalanine was confirmed to be limiting the styrene production, therefore additional modifications in the genome of the current host platform that have previously been shown to increase the L-phenylalanine yield could be incorporated (de Boer and Dijkhuizen 1990; Liu *et al*. 2014; Báez-Viveros *et al*. 2004).

Finally, the bioproduction of styrene needs to improve on the poor yields obtained so far to achieve an economically viable process and be able to compete against the petrochemical process currently used. Nevertheless, the work presented in this thesis gives a proof of concept for the feasibility to use ionic liquids to overcome the toxicity issues of styrene towards the biocatalyst, which could be ultimately used for the production of styrene from renewable feedstocks. The work also demonstrated, that, due to the highly volatile nature of styrene, a combined process using a capture system for the styrene lost by the exhaust gas and *in situ* extraction seems to be the best option to increase productivity of the current biosynthetic process.

7. References

Åkesson, M., E. Karlsson, P. Hagander, J. Axelsson, and A. Tocaj. 1999. "On-Line Detection of Acetate Formation in *Escherichia Coli* Cultures Using Dissolved Oxygen Responses to Feed Transients." *Biotechnology and Bioengineering* 64 (5): 590–98.

Aki, S., J. Brennecke, and A. Samanta. 2001. "How Polar Are Room-Temperature Ionic Liquids?" *Chemical Communications*, no. 5: 413–14.

Anastas, P., and N. Eghbali. 2010. "Green Chemistry: Principles and Practice." *Chemical Society Reviews* 39 (1): 301–12.

Aono, R. 1998. "Improvement of Organic Solvent Tolerance Level of *Escherichia Coli* by Overexpression of Stress-Responsive Genes." *Extremophiles* 2 (3): 239–48.

Armstrong, D., L. He, and Y. Liu. 1999. "Examination of Ionic Liquids and Their Interaction with Molecules, When Used as Stationary Phases in Gas Chromatography." *Analytical Chemistry* 71 (17): 3873–76.

Asako, H., H. Nakajima, K. Kobayashi, M. Kobayashi, and R. Aono. 1997. "Organic Solvent Tolerance and Antibiotic Resistance Increased by Overexpression of marA in *Escherichia Coli*." *Applied and Environmental Microbiology* 63 (4): 1428–33.

Atwood, J. 1984. "Liquid Clathrates." In *Inclusion Compounds, Vol. 1*, edited by J. Atwood, J. Davies, and D. MacNicol. London: Academic Press.

Ba, H., Y. Liu, X. Mu, W. Doh, J. Nhut, P. Granger, and C. Pham-Huu. 2015. "Macroscopic Nanodiamonds/β-SiC Composite as Metal-Free Catalysts for Steam-Free Dehydrogenation of Ethylbenzene to Styrene." *Applied Catalysis A: General* 499: 217–26.

Ba, H., S Podila, Y. Liu, X. Mu, J. Nhut, V. Papaefthimiou, S. Zafeiratos, P. Granger, and C. Pham-Huu. 2015. "Nanodiamond Decorated Few-Layer

Graphene Composite as an Efficient Metal-Free Dehydrogenation Catalyst for Styrene Production." *Catalysis Today* 249: 167–75.

Báez-Viveros, J., J. Osuna, G. Hernández-Chávez, X. Soberón, F. Bolívar, and G. Gosset. 2004. "Metabolic Engineering and Protein Directed Evolution Increase the Yield of L-Phenylalanine from Glucose in *E. Coli*." *Biotechnology and Bioengineering* 87 (4): 516–24.

Baker, S., G. Baker, M. Kane, and F. Bright. 2001. "The Cybotactic Region Surrounding Fluorescent Probes Dissolved in 1-Butyl-3-Methylimidazolium Hexafluorophosphate. Effects of Temperature and Added Carbon Dioxide." *Journal of Physical Chemistry B* 105 (39): 9663–68.

Balderas-Hernández, V., A. Sabido-Ramos, P. Silva, N. Cabrera-Valladares, G. Hernández-Chávez, J. Báez-Viveros, A. Martínez, F. Bolívar, and G. Gosset. 2009. "Metabolic Engineering for Improving Anthranilate Synthesis from Glucose in *Escherichia Coli*." *Microbial Cell Factories* 12: 1–12.

Bar, R. 1988. "Effect of Interphase Mixing on a Water-Organic Solvent Two-Liquid Phase Microbial System - Ethanol Fermentation." *J. Chem. Tech. Biotechnol.* 43: 49–62.

Barker, J., and J. Frost. 2001. "Microbial Synthesis of *p*-Hydroxybenzoic Acid from Glucose." *Biotechnology and Bioengineering* 76 (4): 376–90.

Barton, A. 1975. "Solubility Parameters." *Chemical Reviews* 75 (6): 731–53.

Bassetti, L., and J. Tramper. 1994. "Organic Solvent Toxicity in Morinda Citrifolia Cell Suspensions." *Enzyme and Microbial Technology* 16 (8): 642–48.

Bauer, K., A. Ben-Bassat, M. Dawson, V. de la Puente, and J. Neway. 1990. "Improved Expression of Human Interleukin-2 in High-Cell-Density Fermentor Cultures of *Escherichia Coli* K-12 by a Phosphotransacetylase Mutant." *Applied and Environmental Microbiology* 56 (5): 1296–1302.

Bechtold, M., and S. Panke. 2009. “In Situ Product Recovery Integrated with Biotransformations.” *CHIMIA International Journal for Chemistry* 63 (6): 345–48.

Becker, J., and C. Wittmann. 2015. “Advanced Biotechnology. Metabolically Engineered Cells for the Bio-Based Production of Chemicals and Fuels, and Health-Care Products.” *Angewandte Chemie International Edition*, no. 150: 3328–50.

Ben-Bassat, A., W. Qi, F. Sariaslani, X. Tang, and T. Vannelli. 2003. Microbial conversion of glucose to *para*-hydroxystyrene. PCT/US03/17926, issued 2003.

Berg, C. van den. 2010. “In-Situ Product Recovery from Fermentation Broths. The Application of Solvent-Impregnated Resins as a Product Recovery Tool.” Technical University of Delft.

Berndes, Goran, Monique Hoogwijk, and Richard Van Den Broek. 2003. “The Contribution of Biomass in the Future Global Energy Supply - a Review of 17 Studies.” *Biomass and Bioenergy* 25: 1–28.

Bernhard, F., C. Klammt, and H. Rüterjans. 2007. “Recombinant Deoxyribonucleic Acid and Protein Expression.” In *Comprehensive Medicinal Chemistry II*, edited by John B. Taylor and David J. Triggle, 107–28. Elsevier.

Biczak, R., B. Pawłowska, P. Bałczewski, and P. Rychter. 2014. “The Role of the Anion in the Toxicity of Imidazolium Ionic Liquids.” *Journal of Hazardous Materials* 274: 181–90.

Blundell, R., and P. Licence. 2014. “Quaternary Ammonium and Phosphonium Based Ionic Liquids. A Comparison of Common Anions.” *Physical Chemistry Chemical Physics : PCCP* 16 (29): 15278–88.

BNP Media. 2014. “Using Cryogenic Condensation to Control Organic Solvent Vapor Emissions.” *Processing Cooling*.

Boer, L. de, and L. Dijkhuizen. 1990. “Microbial and Enzymatic Processes for L-Phenylalanine Production.” In *Microbial Bioproducts. Advances in Biochemical Engineering/Biotechnology*, edited by A. Fiechter, 1st ed., 41:1–27. New York: Springer, Berlin, Heidelberg.

Branco, L., J. Crespo, and C. Afonso. 2002. “Studies on the Selective Transport of Organic Compounds by Using Ionic Liquids as Novel Supported Liquid Membranes.” *Chemistry. A European Journal.* 8 (17): 3865–71.

British Petrolium. 2017. “BP Statistical Review of World Energy 2017.” London.

Bruce, L., and A. Daugulis. 1991. “Solvent Selection Strategies for Extractive Biocatalysis.” *Biotechnology Progress* 7: 116–24.

Bruschi, F., M. Dundar, P. B. Gahan, K. Gartland, M. Szente, M. P. Viola-Magni, and Y. Akbarova. 2011. “Biotechnology Worldwide and the ‘European Biotechnology Thematic Network’ Association (EBTNA).” *Current Opinion in Biotechnology* 22 (SUPPL. 1): 4–11. doi:10.1016/j.copbio.2011.05.506.

Campo, P., G. Loquet, V. Blachère, and M. Roure. 1999. “Toluene and Styrene Intoxication Route in the Rat Cochlea.” *Neurotoxicology and Teratology* 21 (4): 427–34.

Cantone, S., U. Hanefeld, and A. Basso. 2007. “Biocatalysis in Non-Conventional Media-Ionic Liquids, Supercritical Fluids and the Gas Phase.” *Green Chemistry* 9 (9): 954–71.

Cao, L., J. Lee, W. Chen, and T. Wood. 2006. “Enantioconvergent Production of (R)-1-Phenyl-1,2-Ethanediol From Styrene Oxide by Combining the Solanum Tuberosum and an Evolved Agrobacterium Radiobacter AD1 Epoxide Hydrolases.” *Biotechnology and Bioengineering* 94 (3): 522–29.

Carter, E., S. Culver, P. Fox, R. Goode, I. Ntai, M. Tickell, R. Traylor, N. Hoffman, and J. Davis. 2004. “Sweet Success. Ionic Liquids Derived from Non-Nutritive Sweeteners.” *Chemical Communications (Cambridge, England)* 6: 630–31.

Carvalho, P., S. Ventura, M. Batista, B. Schröder, F. Gonçalves, J. Esperança, F. Mutelet, and J. Coutinho. 2014. “Understanding the Impact of the Central Atom on the Ionic Liquid Behavior. Phosphonium vs Ammonium Cations.” *Journal of Chemical Physics* 140 (6): 064505 (1-12).

Cascon, H., S. Choudhari, G. Nisola, E. Vivas, D. Lee, and W. Chung. 2011. “Partitioning of Butanol and Other Fermentation Broth Components in Phosphonium and Ammonium-Based Ionic Liquids and Their Toxicity to Solventogenic Clostridia.” *Separation and Purification Technology* 78 (2): 164–74.

Chen, D., X. OuYang, Y. Wang, L. Yang, and C. He. 2013. “Liquid–liquid Extraction of Caprolactam from Water Using Room Temperature Ionic Liquids.” *Separation and Purification Technology* 104: 263–67.

Cherubini, Francesco. 2010. “The Biorefinery Concept - Using Biomass instead of Oil for Producing Energy and Chemicals.” *Energy Conversion and Management* 51: 1412–21.

Chiappe, C., and D. Pieraccini. 2005. “Ionic Liquids. Solvent Properties and Organic Reactivity.” *Journal of Physical Organic Chemistry* 18 (4): 275–97.

Choi, Y. 1981. “Phenylalanine Production by *Escherichia Coli*. A Feasibility Study.” University of South Wales.

Cieniecka-Rosłonkiewicz, A., J. Pernak, J. Kubis-Feder, A. Ramani, A. Robertson, and K. Seddon. 2005. “Synthesis, Anti-Microbial Activities and Anti-Electrostatic Properties of Phosphonium-Based ILs.” *Green Chemistry* 7 (12): 855–62.

Clark, D., and J. Beard. 1979. “Altered Phospholipid Composition in Mutants of *Escherichia Coli* Sensitive or Resistant to Organic Solvents.” *Journal of General Microbiology* 113: 267–74.

Collins, A., J. Woodley, and J. Liddell. 1995. “Determination of Reactor Operation for the Microbial Hydroxylation of Toluene in a Two-Liquid

Phase Process." *Journal of Industrial Microbiology* 14 (5): 382–88.

Cornmell, R., C. Winder, S. Schuler, R. Goodacre, and G. Stephens. 2008. "Using a Biphasic IL-Water Reaction System to Improve Oxygenase-Catalysed Biotransformation with Whole Cells." *Green Chemistry* 10 (6): 685–91.

Crabbe, P., C. Tse, and P. Munro. 1986. "Effect of Microorganisms on Rate of Liquid Extraction of Ethanol from Fermentation Broths." *Biotechnology and Bioengineering* 28 (7): 939–43.

Crabtree, G., and N. Lewis. 2007. "Solar Energy Conversion." *Physics Today* 60 (3): 37–42.

Craig, T., and A. Daugulis. 2013. "Polymer Characterization and Optimization of Conditions for the Enhanced Bioproduction of Benzaldehyde by Pichia Pastoris in a Two-Phase Partitioning Bioreactor." *Biotechnology and Bioengineering* 110 (4): 1098–1105.

Crutzen, P .J., A. R. Mosier, K. A. Smith, and W. Winiwarter. 2008. "N2O Release from Agro-Biofuel Production Negates Global Warming Reduction by Replacing Fossil Fuels." *Atmospheric Chemistry and Physics* 8: 389–95.

Cull, S., J. Holbrey, V. Vargas-Mora, K. Seddon, and G. Lye. 2000. "Room-Temperature Ionic Liquids as Replacements for Organic Solvents in Multiphase Bioprocess Operations." *Biotechnology and Bioengineering* 69 (2): 227–33.

Cussler, E. 1985. *Diffusion: Mass Transfer in Fluid Systems*. 2nd ed. Cambridge: Cambridge University Press.

Cutayar, J., and D. Poillon. 1989. "High Cell Density Culture of *E. Coli* in a Fed-Batch System with Dissolved Oxygen as Substrate Feed Indicator." *Biotechnology Letters* 11 (3): 155–60.

Dafoe, J., and A. Daugulis. 2014. "In Situ Product Removal in Fermentation Systems. Improved Process Performance and Rational Extractant

Selection.” *Biotechnology Letters* 36: 443–60.

Darwin, A. 2005. “The Phage-Shock-Protein Response.” *Molecular Microbiology* 57 (3): 621–28.

Daugulis, A. 2001. “Two-Phase Partitioning Bioreactors. A New Technology Platform for Destroying Xenobiotics.” *Trends in Biotechnology* 19 (11): 457–62.

Daugulis, A., M. Tomei, and B. Guieysse. 2011. “Overcoming Substrate Inhibition during Biological Treatment of Monoaromatics. Recent Advances in Bioprocess Design.” *Applied Microbiology and Biotechnology* 90 (5): 1589–1608.

Davies, W., and B. Davidson. 1982. “The Nucleotide Sequence of aroG, the Gene for 3-Deoxy-D-Arabinoheptulosonate-7-Phosphate Synthetase (Phe) in *Escherichia Coli* K12.” *Nucleic Acids Research* 10 (13): 4045–58.

Davis, W., and T. Stout. 1971. “Disc Plate Method of Microbiological Antibiotic Assay. I. Factors Influencing Variablity and Error.” *Journal of Microbiology* 22 (4): 659–65.

Deive, F., M. Rivas, and A. Rodríguez. 2013. “Study of Thermodynamic and Transport Properties of Phosphonium-Based Ionic Liquids.” *The Journal of Chemical Thermodynamics* 62: 98–103.

Demberelnyamba, D., K. Kim, S Choi, S. Park, H. Lee, C. Kim, and I. Yoo. 2004. “Synthesis and Antimicrobial Properties of Imidazolium and Pyrrolidinonium Salts.” *Bioorganic and Medicinal Chemistry* 12 (5): 853–57.

Deng, Y., I. Beadham, M. Ghavre, M. Costa, N. Gathergood, P. Husson, B. Légeret, B. Quilty, M. Sancelme, and P. Besse-Hoggan. 2015. “When Can Ionic Liquids Be Considered Readily Biodegradable. Biodegradation Pathways of Pyridinium, Pyrrolidinium and Ammonium-Based Ionic Liquids.” *Green Chemistry* 17 (3): 1479–91.

Dennewald, D., W. Pitner, and D. Weuster-Botz. 2011. “Recycling of the Ionic Liquid Phase in Process Integrated Biphasic Whole-Cell Biocatalysis.” *Process Biochemistry* 46 (5). Elsevier Ltd: 1132–37.

Deyko, A., S. Hessey, P. Licence, E. Chernikova, V. Krasovskiy, L. Kustov, and R. Jones. 2012. “The Enthalpies of Vaporisation of Ionic Liquids: New Measurements and Predictions.” *Physical Chemistry Chemical Physics* 14 (9): 3181–93.

Ding, J., X. Huang, and L. Zhang. 2009. “Tolerance and Stress Response to Ethanol in the Yeast *Saccharomyces Cerevisiae*.” *Applied Microbiology and Biotechnology* 85: 253–63.

Disley, Z. 2016. “Solving the Problem of Product Toxicity during the Bioproduction of Methacrylic Acid.” The Univervisity of Nottingham.

Docherty, K., and C. Kulpa, Jr. 2005. “Toxicity and Antimicrobial Activity of Imidazolium and Pyridinium Ionic Liquids.” *Green Chemistry* 7 (4): 185–89.

Doelle, H., K. Ewings, and N. Hollywood. 1982. “Regulation of Glucose Metabolism in Bacterial Systems.” In *Microbial Reactions*, 1–35. Springer, Berlin, Heidelberg.

Draths, K., and J. Frost. 1995. “Environmentally Compatible Synthesis of Catechol from D-Glucose.” *Journal of the American Chemical Society* 117 (8): 2395–2400.

Dupont, J., C. Consorti, and J. Spencer. 2000. “Room Temperature Molten Salts. Neoteric ‘Green’ Solvents for Chemical Reactions and Processes.” *Journal of the Brazilian Chemical Society* 11 (4): 337–44.

Dzyuba, S., and R. Bartsch. 2003. “Recent Advances in Applications of Room-Temperature Ionic Liquid-Supercritical CO2 Systems.” *Angewandte Chemie (International Ed. in English)* 42 (2): 148–50.

Earle, M., and K. Seddon. 2000. “Ionic Liquids. Green Solvents for the Future.”

Pure and Applied Chemistry 72 (7): 1391–98.

Ernst, M., B. Kaup, M. Müller, S. Bringer-Meyer, and H. Sahm. 2005. "Enantioselective Reduction of Carbonyl Compounds by Whole-Cell Biotransformation, Combining a Formate Dehydrogenase and a (R)-Specific Alcohol Dehydrogenase." *Applied Microbiology and Biotechnology* 66 (6): 629–34.

Eyles, S., and L. Gierasch. 2010. "Nature's Molecular Sponges. Small Heat Shock Proteins Grow into Their Chaperone Roles." *Proceedings of the National Academy of Sciences* 107 (7): 2727–28.

Ezeji, T. C., N. Qureshi, and H. Blaschek. 2004. "Acetone Butanol Ethanol (ABE) Production from Concentrated Substrate. Reduction in Substrate Inhibition by Fed-Batch Technique and Product Inhibition by Gas Stripping." *Applied Microbiology and Biotechnology* 63 (6): 653–58.

Ezeji, T., N. Qureshi, and H. Blaschek. 2003. "Production of Acetone, Butanol and Ethanol by Clostridium Beijerinckii BA101 and in Situ Recovery by Gas Stripping." *World Journal of Microbiology and Biotechnology* 19 (6): 595–603.

Fang, Z., R. Smith, and X. Qi. 2014. *Production of Biofuels and Chemicals with Ionic Liquids*. Springer.

Feng, X., and R. Huang. 1997. "Liquid Separation by Membrane Pervaporation. A Review." *Industrial & Engineering Chemistry Research* 36 (4): 1048–66.

Fernandez, X., L. Lizzani-Cuvelier, A. Loiseau, C. Perichet, C. Delbecque, and J. Arnaudo. 2005. "Chemical Composition of the Essential Oils from Turkish and Honduras Styrax." *Flavour and Fragrance Journal* 20 (1): 70–73.

Fieschko, J. 1989. "Fermentation Technology Using Recombinant Microrganisms." *Biotechnology* 76. VCH Verlagsgesellschaft: 114–40.

Fraser, K., and D. MacFarlane. 2009. "Phosphonium-Based Ionic Liquids. An

Overview.” *Australian Journal of Chemistry* 62 (4): 309.

Freeman, A., J. Woodley, and M. Lilly. 1993. “In Situ Product Removal as a Tool for Bioprocessing.” *Nature Biotechnology* 11 (9): 1007–12.

Freemantle, M. 1998. “Designer Solvents - Ionic Liquids May Boost Clean Technology Development.” *Chemical & Engineering News* 76 (13): 32–37.

Freire, M., L. Santos, A. Fernandes, J. Coutinho, and I. Marrucho. 2007. “An Overview of the Mutual Solubilities of Water-Imidazolium-Based Ionic Liquids Systems.” *Fluid Phase Equilibria* 261 (1–2): 449–54.

Fukaya, Y., Y. Iizuka, K. Sekikawa, and H. Ohno. 2007. “Bio Ionic Liquids. Room Temperature Ionic Liquids Composed Wholly of Biomaterials.” *Green Chemistry* 9 (11): 1155–57.

Fukumoto, K., M. Yoshizawa, and H. Ohno. 2005. “Room Temperature Ionic Liquids from 20 Natural Amino Acids.” *Journal of the American Chemical Society* 127 (8): 2398–99.

Gamenara, D., and P. Domínguez de María. 2009. “Candida Spp. Redox Machineries. An Ample Biocatalytic Platform for Practical Applications and Academic Insights.” *Biotechnology Advances* 27 (3). Elsevier Inc.: 278–85.

Gao, F., and A. Daugulis. 2009. “Bioproduction of the Aroma Compound 2-Phenylethanol in a Solid-Liquid Two-Phase Partitioning Bioreactor System by Kluyveromyces Marxianus.” *Biotechnology and Bioengineering* 104 (2): 332–39.

Garcia, M., N. Gathergood, and P. Scammells. 2005. “Biodegradable Ionic Liquids - Part II. Effect of the Anion and Toxicology.” *Green Chemistry* 7 (1): 9–14.

Garcia, S., N. Lourenço, D. Lousa, A. Sequeira, P. Mimoso, J. Cabral, C. Afonso, and S. Barreiros. 2004. “A Comparative Study of Biocatalysis in Non-Conventional Solvents. Ionic Liquids , Supercritical Fluids and Organic

Media.” *Green Chemistry* 6: 466–70.

Gathergood, N., P. Scammells, and M. Garcia. 2006. “Biodegradable Ionic Liquids - Part III. The First Readily Biodegradable Ionic Liquids.” *Green Chemistry* 8 (2): 156–60.

Gavin, J. 1957. “Analytical Microbiology. II. The Diffusion Methods.” *Applied Microbiology* 5 (1): 25–33.

Gerson, D., M. Kole, and B. Ozum. 1988. “Substrate Concentration Control in Bioreactors.” *Biotechnology and Genetic Engineering Reviews* 6 (1): 67–150.

Gosset, G. 2009. “Production of Aromatic Compounds in Bacteria.” *Current Opinion in Biotechnology* 20 (6): 651–58.

Gouveia, W., T. Jorge, S. Martins, M. Meireles, M. Carolino, C. Cruz, T. Almeida, and M. Araújo. 2014. “Toxicity of Ionic Liquids Prepared from Biomaterials.” *Chemosphere* 104. Elsevier Ltd: 51–56.

Gowrishankar, J., and J. Pittard. 1982. “Regulation of Phenylalanine Biosynthesis in *E. Coli* K-12. Control of Transcription of the pheA Operon.” *Journal of Bacteriology* 150 (3): 1130–37.

Gyamerah, M., and J. Glover. 1996. “Production of Ethanol by Continuous Fermentation and Liquid-Liquid Extraction.” *Journal of Chemical Technology & Biotechnology* 66: 145–52.

Han, D., and K. Row. 2010. “Recent Applications of Ionic Liquids in Separation Technology.” *Molecules (Basel, Switzerland)* 15 (4): 2405–26.

Han, K., H. Lim, and J. Hong. 1992. “Acetic Acid Formation in *Escherichia Coli* Fermentation.” *Biotechnology and Bioengineering* 39 (6). John Wiley & Sons, Inc.: 663–71.

Handy, S., M. Okello, and G. Dickenson. 2003. “Solvents from Biorenewable Sources: Ionic Liquids Based on Fructose.” *Organic Letters* 5 (14): 2513–

15.

Hansch, C., and A. Leo. 1979. *Substituent Constants for Correlation Analysis in Chemistry and Biology*. New York: Wiley.

Harjani, J., J. Farrell, M. Garcia, R. Singer, and P. Scammells. 2009. “Further Investigation of the Biodegradability of Imidazolium Ionic Liquids.” *Green Chemistry* 11 (6): 821.

Harrop, A., M. Hocknull, and M. Lilly. 1989. “Biotransformations in Organic Solvents. A Difference between Gram-Positive and Gram-Negative Bacteria.” *Biotechnology Letters* 11 (11). Kluwer Academic Publishers: 807–10.

Hayashi, S., R. Aono, T. Hanai, H. Mori, T. Kobayashi, and H. Honda. 2003. “Analysis of Organic Solvent Tolerance in *Escherichia Coli* Using Gene Expression Profiles from DNA Microarrays.” *Journal of Bioscience and Bioengineering* 95 (4): 379–83.

Hecke, W. Van, G. Kaur, and H. De Wever. 2014. “Advances in in-Situ Product Recovery (ISPR) in Whole Cell Biotechnology during the Last Decade.” *Biotechnology Advances* 32 (7). Elsevier Inc.: 1245–55.

Heipieper, H., R. Diefenbach, and H. Keweloh. 1992. “Conversion of Cis Unsaturated Fatty Acids to Trans, a Possible Mechanism for the Protection of Phenol-Degrading *Pseudomonas Putida* P8 from Substrate Toxicity.” *Applied and Environmental Microbiology* 58 (6): 1847–52.

Heipieper, H., G. Neumann, S. Cornelissen, and F. Meinhardt. 2007. “Solvent-Tolerant Bacteria for Biotransformations in Two-Phase Fermentation Systems.” *Applied Microbiology and Biotechnology* 74 (5): 961–73.

Heipieper, H., F. Weber, J. Sikkema, H. Keweloh, and J. de Bont. 1994. “Mechanisms of Resistance of Whole Cells to Toxic Organic Solvents.” *Trends in Biotechnology* 12 (10): 409–15.

Held, M., A. Schmid, J. van Beilen, and B. Witholt. 2000. “Biocatalysis. Biological Systems for the Production of Chemicals.” *Pure and Applied Chemistry* 72 (7): 1337–43.

Herbert, D., R. Elsworth, and R. Telling. 1956. “The Continuous Culture of Bacteria. A Theoretical and Experimental Study.” *J. Gen. Microbiol* 14 (3): 601–22.

Higgins, C. 2007. “Multiple Molecular Mechanisms for Multidrug Resistance Transporters.” *Nature* 446: 749–57.

Hobbs, H., and N. Thomas. 2007. “Biocatalysis in Supercritical Fluids, in Fluorous Solvents, and under Solvent-Free Conditions.” *Chemical Reviews* 44 (107): 2786–2820.

Holbrey, J., W. Reichert, M. Nieuwenhuyzen, O. Sheppard, C. Hardacre, and R. Rogers. 2003. “Liquid Clathrate Formation in Ionic Liquid-Aromatic Mixtures.” *Chem. Commun.*, no. 4. The Royal Society of Chemistry: 476–77.

Holbrey, J., and R. Rogers. 2002. “Green Chemistry and Ionic Liquids : Synergies and Ironies Green Chemistry Approach.” *ACS Symposium Series*, 2–14.

Holms, W. 1986. “The Central Metabolic Pathways of *Escherichia Coli*: Relationship between Flux and Control at a Branch Point, Efficiency of Conversion to Biomass, and Excretion of Acetate.” In *Current Topics in Cellular Regulation*, edited by Bernard L. Horecker and Earl R. B. T. Stadtman, 28:69–105. Academic Press.

Hu, Q., Y. Xu, and Y. Nie. 2010. “Enhancement of Candida Parapsilosis Catalyzing Deracemization of (R,S)-1-Phenyl-1,2-Ethanediol to Its (S)-Enantiomer by a Highly Productive ‘two-in-One’ resin-Based in Situ Product Removal Strategy.” *Bioresource Technology* 101 (21). Elsevier Ltd: 8461–63.

Hua, D., S. Lin, Y. Li, H. Chen, Z. Zhang, Y. Du, X. Zhang, and P. Xu. 2010. “Enhanced 2-Phenylethanol Production from L-Phenylalanine via in Situ

Product Adsorption." *Biocatalysis and Biotransformation* 28 (4): 259–66.

Huddleston, J., H. Willauer, R. Swatloski, A. Visser, and R. Rogers. 1998. "Room Temperature Ionic Liquids as Novel Media for 'clean' Liquid–liquid Extraction." *Chem. Commun.*, no. 16. The Royal Society of Chemistry: 1765–1766.

Ikegami, T., H. Yanagishita, D. Kitamoto, K. Haraya, T. Nakane, H. Matsuda, N. Koura, and T. Sano. 1997. "Production of Highly Concentrated Ethanol in a Coupled Fermentation-Pervaporation Process Using Silicalite Membranes." *Biotechnology Techniques* 11 (12): 921–24.

Ingram, L. 1977. "Changes in Lipid Composition of *Escherichia Coli* Resulting from Growth with Organic Solvents and with Food Additives." *Applied and Environmental Microbiology* 33 (5): 1233–36.

Inokuma, K., J. Liao, M. Okamoto, and T. Hanai. 2010. "Improvement of Isopropanol Production by Metabolically Engineered *Escherichia Coli* Using Gas Stripping." *Journal of Bioscience and Bioengineering* 110 (6). The Society for Biotechnology, Japan: 696–701.

Inoue, A., and K. Horikoshi. 1991. "Estimation of Solvent-Tolerance of Bacteria by the Solvent Parameter Log P." *Journal of Fermentation and Bioengineering* 71 (3): 194–96.

International Energy Agency. 2008. "World Energy Outlook 2008." Paris.

International Energy Agency. 2016. "Key World Energy Statistics." Paris.

Isken, S., and J. de Bont. 1998. "Bacteria Tolerant to Organic Solvents." *Extremophiles* 2 (3). Springer-Verlag: 229–38.

Jain, A., T. Khan, and A. Daugulis. 2010. "Bioproduction of Benzaldehyde in a Solid-Liquid Two-Phase Partitioning Bioreactor Using Pichia Pastoris." *Biotechnology Letters* 32 (11): 1649–54.

James, D., and W. Castor. 1994. "Styrene - Ullmann's Encyclopedia of Industrial

Chemistry." In *Ullmann's Encyclopedia of Industrial Chemistry*, edited by B. Elvers, S. Hawkins, and W. Russey, 5th rev. E, 329–344. New York: VCH Publishers.

Jang, Y., B. Kim, J. Shin, Y. Choi, S. Choi, C. Song, J. Lee, H. Park, and S. Lee. 2012. "Bio-Based Production of C2 – C6 Platform Chemicals." *Biotechnology and Bioengineering* 109 (10): 2437–59.

Jarsch, I., F. Daste, and J. Gallop. 2016. "Membrane Curvature in Cell Biology. An Integration of Molecular Mechanisms." *Journal of Cell Biology* 214 (4): 375–87.

Jensen, E., and S. Carlsen. 1990. "Production of Recombinant Human Growth Hormone in *Escherichia Coli*. Expression of Different Precursors and Physiological Effects of Glucose, Acetate, and Salts." *Biotechnology and Bioengineering* 36 (1): 1–11.

Jeong, S., S. Ha, S. Han, M. Lim, S. Kim, Y. Kim, Y. Koo, J. So, and T. Jeon. 2012. "Elucidation of Molecular Interactions between Lipid Membranes and Ionic Liquids Using Model Cell Membranes." *Soft Matter* 8 (20): 5501.

Kabelitz, N., P. Santos, and H. Heipieper. 2003. "Effect of Aliphatic Alcohols on Growth and Degree of Saturation of Membrane Lipids in Acinetobacter Calcoaceticus." *FEMS Microbiology Letters* 220: 223–27.

Keasling, J. 2008. "Synthetic Biology for Synthetic Chemistry." *ACS Chemical Biology* 3 (1): 64–76.

Keasling, Jay D. 2008. "Synthetic Biology for Synthetic Chemistry." *ACS Chemical Biology* 3 (1): 64–76. doi:10.1021/cb7002434.

Kempf, B., and E. Bremer. 1998. "Uptake and Synthesis of Compatible Solutes as Microbial Stress Responses to High-Osmolality Environments." *Arch Microbiology* 170: 319–30.

Kerr, R. 2007. "The Looming Oil Crisis Could Arrive Uncomfortably Soon."

Science 316 (5823): 351.

Khan, T., and A. Daugulis. 2010. “Application of Solid–liquid TPPBs to the Production of L-Phenylacetylcarbinol from Benzaldehyde Using Candida Utilis.” *Biotechnology and Bioengineering* 107 (4). Wiley Subscription Services, Inc., A Wiley Company: 633–41.

Kieboom, J., J. Dennis, J. de Bont, and G. Zylstra. 1998. “Identification and Molecular Characterization of an Efflux Pump Involved in *Pseudomonas Putida* S12 Solvent Tolerance.” *The Journal of Biological Chemistry* 273 (1): 85–91.

Klass, D. 1998. *Biomass for Renewable Energy, Fuels, and Chemicals*. Edited by Donald L. Klass. San Diego: Academic Press.

Kobayashi, H., M. Yamamoto, and R. Aono. 1998. “Appearance of a Stress-Response Protein, Phage-Shock Protein A, in *Escherichia Coli* Exposed to Hydrophobic Organic Solvents.” *Microbiology* 144 (2): 353–59.

Koh, B., U. Nakashimada, M. Pfeiffer, and M. Yap. 1992. “Comparison of Acetate Inhibition on Growth of Host and Recombinant *E. Coli* K12 Strains.” *Biotechnology Letters* 14 (12). Springer: 1115–18.

Konstantinov, K., N. Nishio, T. Seki, and T. Yoshida. 1991. “Physiologically Motivated Strategies for Control of the Fed-Batch Cultivation of Recombinant *Escherichia Coli* for Phenylalanine Production.” *Journal of Fermentation and Bioengineering* 71 (5): 350–55.

Koonin, Steven E. 2006. “Getting Serious About Biofuels.” *Science* 311.

Koukol, J., and E. Conn. 1961. “The Metabolism of Aromatic Compounds in Higher Plants.” *The Journal of Biological Chemistry* 236 (10): 2692–98.

Kralisch, D., A. Stark, S. Körsten, G. Kreisel, and B. Ondruschka. 2005. “Energetic, Environmental and Economic Balances: Spice up Your Ionic Liquid Research Efficiency.” *Green Chemistry* 7 (5). The Royal Society of

Chemistry: 301–9.

Kumar, P., R. Upadhyay, and R. Pandey. 2004. “Asymmetric Dihydroxylation Route to (R)-Isoprenaline, (R)-Norfluoxetine and (R)-Fluoxetine.” *Tetrahedron: Asymmetry* 15 (24): 3955–59.

Kurnia, K., T. Sintra, C. Neves, K. Shimizu, J. Canongia, F. Gonçalves, S. Ventura, M. Freire, L. Santos, and J. Coutinho. 2014. “The Effect of the Cation Alkyl Chain Branching on Mutual Solubilities with Water and Toxicities.” *Physical Chemistry Chemical Physics : PCCP* 16 (37): 19952–63.

Laane, C., S. Boeren, K. Vos, and C. Veeger. 1987. “Rules for Optimization of Biocatalysis in Organic Solvents.” *Biotechnology and Bioengineering* 30: 81–87.

Lee, S. 1996. “High Cell-Density Culture of *Escherichia Coli*.” *Trends in Biotechnology* 14 (3): 98–105.

Lee, S., and H. Chang. 1993. “High Cell Density Cultivation of *Escherichia Coli* W Using Sucrose as a Carbon Source.” *Biotechnology Letters* 15 (9): 971–74.

Lee, S., and R. Mohler. 1988. “Controlled Growth Rate Fermentation.” *EP 0* 315 (944): A2.

León, R., P. Fernandes, H. Pinheiro, and J. Cabral. 1998. “Whole-Cell Biocatalysis in Organic Media.” *Enzyme and Microbial Technology* 23: 483–500.

Li, W., D. Xie, and J. Frost. 2005. “Benzene-Free Synthesis of Catechol. Interfacing Microbial and Chemical Catalysis.” *Journal of the American Chemical Society* 278 (5): 2874–82.

Lian, J., R. McKenna, M. Rover, D. Nielsen, Z. Wen, and L. Jarboe. 2016. “Production of Biorenewable Styrene - Utilization of Biomass-Derived Sugars and Insights into Toxicity.” *Journal of Industrial Microbiology & Biotechnology* 43 (5). Springer, Berlin, Heidelberg: 595–604.

Lide, D., and G. Milne. 1993. *CRC Handbook of Data on Organic Compounds*. 3rd

ed. Boca Raton: CRC Press.

Liese, A., K. Seelbach, and C. Wandrey. 2006. *Industrial Biotransformations*. 2nd ed. John Wiley & Sons.

Lilly, M. 1982. “Two-Liquid-Phase Biocatalytic Reactions.” *Journal of Chemical Technology and Biotechnology* 32 (1): 162–69.

Lim, H., and H. Shin. 2013. *Fed-Batch Cultures - Principles and Applications of Semi-Batch Bioreactors*. *Cambridge University Press*. New York: Cambridge University Press.

Lin, F., K. Ferguson, D. Boyer, X. Lin, and E. Marsh. 2015. “Isofunctional Enzymes PAD1 and UbiX Catalyze Formation of a Novel Cofactor Required by Ferulic Acid Decarboxylase and 4-Hydroxy-3-Polyprenylbenzoic Acid Decarboxylase.” *ACS Chemical Biology* 10 (4): 1137–44.

Liu, S., R. Liu, M. Xiao, L. Zhang, Z. Ding, Z. Gu, and G. Shi. 2014. “A Systems Level Engineered *E. Coli* Capable of Efficiently Producing L-Phenylalanine.” *Process Biochemistry* 49 (5). Elsevier Ltd: 751–57.

Liu, S., Q. Wu, J. Zhang, and S. Mo. 2011. “Production of E-Poly-L-Lysine by Streptomyces Sp. Using Resin-Based, in Situ Product Removal.” *Biotechnology Letters* 33 (8): 1581–85.

Lomovskaya, O., M. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, et al. 2001. “Identification and Characterization of Inhibitors of Multidrug Resistance Efflux Pumps in Pseudomonas Aeruginosa. Novel Agents for Combination Therapy.” *Antimicrobial Agents and Chemotherapy* 45 (1): 105–16.

Los, D., and N. Murata. 2004. “Membrane Fluidity and Its Roles in the Perception of Environmental Signals.” *Biochimica et Biophysica Acta - Biomembranes* 1666 (1–2): 142–57.

Löser, C., A. Schröder, S. Deponte, and T. Bley. 2005. “Balancing the Ethanol

Formation in Continuous Bioreactors with Ethanol Stripping." *Engineering in Life Sciences* 5 (4): 325–32.

Lou, W., M. Zong, and T. Smith. 2006. "Use of Ionic Liquids to Improve Whole-Cell Biocatalytic Asymmetric Reduction of Acetyltrimethylsilane for Efficient Synthesis of Enantiopure (S)-1-Trimethylsilylethanol." *Green Chemistry* 8 (2): 147–55.

Luli, G., and W. Strohl. 1990. "Comparison of Growth, Acetate Production, and Acetate Inhibition of *Escherichia Coli* Strains in Batch and Fed-Batch Fermentations." *Appl. Environ. Microbiol.* 56 (4): 1004–11.

Lye, G., and J. Woodley. 1999. "Application of in Situ Product-Removal Techniques to Biocatalytic Processes." *Trends in Biotechnology* 17: 395–402.

MacDonald, H., and J. Neway. 1990. "Effects of Medium Quality on the Expression of Human Interleukin-2 at High Cell Density in Fermentor Cultures of *Escherichia Coli* K-12." *Applied and Environmental Microbiology* 56 (3): 640–45.

Makrides, S. 1996. "Strategies for Achieving High-Level Expression of Genes in *Escherichia Coli*." *Microbiol. Rev.* 60 (3): 512–38.

Malinowski, J. 2001. "Two-Phase Partitioning Bioreactors in Fermentation Technology." *Biotechnology Advances* 19: 525–38.

Matsuda, T., Y. Yamagishi, S. Koguchi, N. Iwai, and T. Kitazume. 2006. "An Effective Method to Use Ionic Liquids as Reaction Media for Asymmetric Reduction by Geotrichum Candidum." *Tetrahedron Letters* 47 (27): 4619–22.

Matsui, T., H. Yokota, S. Sato, S. Mukataka, and J. Takahashi. 1989. "Pressurized Culture of *Escherichia Coli* for a High Concentration." *Agricultural and Biological Chemistry* 53 (8): 2115–20.

Matsumoto, M., K. Mochiduki, K. Fukunishi, and K. Kondo. 2004. “Extraction of Organic Acids Using Imidazolium-Based Ionic Liquids and Their Toxicity to Lactobacillus Rhamnosus.” *Separation and Purification Technology* 40 (1). ELSEVIER SCIENCE BV: 97–101.

Matsumoto, M., K. Mochiduki, and K. Kondo. 2004. “Toxicity of Ionic Liquids and Organic Solvents to Lactic Acid-Producing Bacteria.” *Journal of Bioscience and Bioengineering* 98 (5): 344–47.

McKenna, R., L. Moya, M. McDaniel, and D. Nielsen. 2014. “Comparing in Situ Removal Strategies for Improving Styrene Bioproduction.” *Bioprocess and Biosystems Engineering* 38 (1): 165–74.

McKenna, R., and D. Nielsen. 2011. “Styrene Biosynthesis from Glucose by Engineered *E. Coli*.” *Metabolic Engineering* 13 (5). Elsevier: 544–54.

Meindersma, G., L. Galán, A. Hansmeier, and A. de Haan. 2007. “Application of Task-Specific Ionic Liquids for Intensified Separations.” *Monatshefte Für Chemie - Chemical Monthly* 138: 1125–36.

Merchant Research & Consulting. 2013. “Styrene: 2013 World Market Outlook and Forecast up to 2017.” Birmingham, United Kingdom.

Miller, J. 1972. “Experiments in Molecular Genetics.” In . New York: Cold Spring Harbour Laboratory Press.

Mimura, N., I. Takahara, M. Saito, T. Hattori, K. Ohkuma, and M. Ando. 1998. “Dehydrogenation of Ethylbenzene over Iron Oxide-Based Catalyst in the Presence of Carbon Dioxide.” *Catalysis Today* 45 (1–4): 61–64.

Mingardon, F., C. Clement, K. Hirano, M. Nhan, E. Luning, A. Chanal, and A. Mukhopadhyay. 2015. “Improving Olefin Tolerance and Production in *E. Coli* Using Native and Evolved AcrB.” *Biotechnology and Bioengineering* 112 (5): 879–88.

Minihane, B., and D. Brown. 1986. “Fed-Batch Culture Technology.”

Biotechnology Advances 4 (2): 207–18.

Mirata, M., D. Heerd, and J. Schrader. 2009. “Integrated Bioprocess for the Oxidation of Limonene to Perillic Acid with *Pseudomonas Putida* DSM 12264.” *Process Biochemistry* 44 (7): 764–71.

Mutelet, F., J. Jaubert, M. Rogalski, M. Boukherissa, and A. Dicko. 2006. “Thermodynamic Properties of Mixtures Containing Ionic Liquids - Activity Coefficients at Infinite Dilution of Organic Compounds in 1-Propyl Boronic Acid-3-Alkylimidazolium Bromide and 1-Propenyl-3-Alkylimidazolium Bromide Using Inverse Gas Chromatography.” *Journal of Chemical & Engineering Data* 51: 1274–79.

Nancib, N., R. Mosrati, and J. Boudrant. 1993. “Modelling of Batch Fermentation of a Recombinant *Escherichia Coli* Producing Glyceraldehyde-3-Phosphate Dehydrogenase on a Complex Selective Medium.” *The Chemical Engineering Journal* 52 (2). Elsevier: B35–48.

National Institute of AIST. 1999. “Spectral Database for Organic Compounds.” https://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi.

Nestl, B., S. Hammer, B. Nebel, and B. Hauer. 2014. “New Generation of Biocatalysts for Organic Synthesis.” *Angewandte Chemie - International Edition* 53: 3070–95.

Nicolaou, Sergios A., Stefan M. Gaida, and Eleftherios T. Papoutsakis. 2010. “A Comparative View of Metabolite and Substrate Stress and Tolerance in Microbial Bioprocessing. From Biofuels and Chemicals, to Biocatalysis and Bioremediation.” *Metabolic Engineering* 12 (4). Elsevier: 307–31. doi:10.1016/j.ymben.2010.03.004.

Nielsen, D., A. Daugulis, and P. McLellan. 2007a. “Dynamic Simulation of Benzene Vapor Treatment by a Two-Phase Partitioning Bioscrubber. Part I - Model Development, Parameter Estimation, and Parametric Sensitivity.” *Biochemical Engineering Journal* 36 (3): 239–49.

Nielsen, D., A. Daugulis, and P. McLellan. 2007b. “Dynamic Simulation of Benzene Vapor Treatment by a Two-Phase Partitioning Bioscrubber. Part II - Model Calibration, Validation, and Predictions.” *Biochemical Engineering Journal* 36 (3): 250–61.

O’Brien, D., and J. Craig. 1996. “Ethanol Production in a Continuous Fermentation-Membrane Pervaporation System.” *Applied Microbiology and Biotechnology* 44 (6): 699–704.

Oethinger, M., I. Podglajen, W. Kern, and S. Levy. 1998. “Overexpression of the marA or soxS Regulatory Gene in Clinical Topoisomerase Mutants of *Escherichia Coli*.” *Antimicrobial Agents and Chemotherapy* 42 (8): 2089–94.

Oliveira, F., J. Araújo, R. Ferreira, L. Rebelo, and I. Marrucho. 2012. “Extraction of L-Lactic, L-Malic, and Succinic Acids Using Phosphonium-Based Ionic Liquids.” *Separation and Purification Technology* 85. Elsevier B.V.: 137–46.

Olivier-Bourbigou, H., L. Magna, and D. Morvan. 2010. “Ionic Liquids and Catalysis. Recent Progress from Knowledge to Applications.” *Applied Catalysis A: General* 373 (1–2): 1–56.

Organisation for Economic Co-operation and Development. 2003. “OECD Guidelines for the Testing of Chemicals. Proposal for a New Guideline 310.” Paris.

Osborne, S., J. Leaver, M. Turner, and P. Dunnill. 1990. “Correlation of Biocatalytic Activity in an Organic-Aqueous Two-Liquid Phase System with Solvent Concentration in the Cell Membrane.” *Enzyme and Microbial Technology* 12 (4): 281–91.

Pacheco, A., C. Pereira, M. Almeida, and M. Sousa. 2009. “Small Heat-Shock Protein Hsp12 Contributes to Yeast Tolerance to Freezing Stress.” *Microbiology* 155: 2021–28.

Pagot, Y., J. Belin, F. Husson, and H. Spinnler. 2007. “Metabolism of

Phenylalanine and Biosynthesis of Styrene in Penicillium Camemberti." *The Journal of Dairy Research* 74 (2): 180–85.

Pan, J., J. Rhee, and J. Lebeault. 1987. "Physiological Constraints in Increasing Biomass Concentration of *Escherichia Coli* B in Fed-Batch Culture." *Biotechnology Letters* 9 (2). Springer: 89–94.

Panda, A., R. Khan, and S. Mishra. 2000. "Influences of Yeast Extract on Specific Cellular Yield of Ovine Growth Hormone during Fed-Batch Fermentation of *E. Coli*." *Bioprocess Engineering* 22: 379–83.

Panke, S., M. Wubbolts, A. Schmid, and B. Witholt. 2000. "Production of Enantiopure Styrene Oxide by Recombinant *Escherichia Coli* Synthesizing a Two-Component Styrene Monooxygenase." *Biotechnology and Bioengineering* 69 (1): 91–100.

Parent, J., M. Capela, J. Dafoe, and A. Daugulis. 2012. "A First Principles Approach to Identifying Polymers for Use in Two-Phase Partitioning Bioreactors." *Journal of Chemical Technology & Biotechnology* 87 (8): 1059–65.

Park, J., B. Buhler, T. Habicher, B. Hauer, S. Panke, B. Witholt, and A. Schmid. 2006. "The Efficiency of Recombinant *Escherichia Coli* as Biocatalyst for Stereospecific Epoxidation." *Biotechnology and Bioengineering* 95 (3): 501–12.

Paulechka, Y., G. Kabo, A. Blokhin, O. Vydrov, J. Magee, and M. Frenkel. 2003. "Thermodynamic Properties of 1-Butyl-3-Methylimidazolium Hexafluorophosphate in the Ideal Gas State." *Journal of Chemical & Engineering Data* 48: 457–62.

Pernak, J., I. Goc, and I. Mirska. 2004. "Anti-Microbial Activities of Protic Ionic Liquids with Lactate Anion." *Green Chemistry* 6 (7): 323.

Pernak, J., K. Sobaszkiewicz, and I. Mirska. 2002. "Anti-Microbial Activities of Ionic Liquids." *Green Chemistry* 5 (1): 52–56.

Perry, R., D. Green, and J. Maloney. 2007. *Perry's Handbook of Chemical Engineering*. 8th ed. McGraw-Hill.

Petkovic, Marija, Kenneth R. Seddon, Luís Paulo N. Rebelo, and Cristina Silva Pereira. 2011. "Ionic Liquids: A Pathway to Environmental Acceptability." *Chemical Society Reviews* 40: 1383–1403.

Pfruender, H., M. Amidjojo, U. Kragl, and D. Weuster-Botz. 2004. "Efficient Whole-Cell Biotransformation in a Biphasic Ionic Liquid/water System." *Angewandte Chemie - International Edition* 43 (34): 4529–31.

Pfruender, H., R. Jones, and D. Weuster-Botz. 2006. "Water Immiscible Ionic Liquids as Solvents for Whole Cell Biocatalysis." *Journal of Biotechnology* 124 (1): 182–90.

Pinkart, H., and D. White. 1997. "Phospholipid Biosynthesis and Solvent Tolerance in *Pseudomonas Putida* Strains." *Journal of Bacteriology* 179 (13): 4219–26.

Pittard, J., and J. Yang. 2008. "Biosynthesis of the Aromatic Amino Acids." *EcoSal Plus* 3 (1): 1–39.

Plotkin, J., and G. Kudla. 2011. "Synonymous but Not the Same: The Causes and Consequences of Codon Bias." *Nature Reviews Genetics* 12 (1): 32–42.

Plumridge, A., P. Melin, M. Stratford, M. Novodvorska, L. Shunburne, P. Dyer, J. Roubos, et al. 2010. "The Decarboxylation of the Weak-Acid Preservative, Sorbic Acid, Is Encoded by Linked Genes in Aspergillus Spp." *Fungal Genetics and Biology : FG & B* 47 (8). Elsevier Inc.: 683–92.

Poole, C., and S. Poole. 2010. "Extraction of Organic Compounds with Room Temperature Ionic Liquids." *Journal of Chromatography A* 1217 (16). Elsevier B.V.: 2268–86.

Prpich, G., and A. Daugulis. 2007. "Solvent Selection for Enhanced Bioproduction of 3-Methylcatechol in a Two-Phase Partitioning

Bioreactor." *Biotechnology and Bioengineering* 97 (3): 536–43.

Qi, W., T. Vannelli, S. Breinig, A. Ben-Bassat, A. Gatenby, S. Haynie, and F. Sariaslani. 2007. "Functional Expression of Prokaryotic and Eukaryotic Genes in *E. Coli* for Conversion of Glucose to *p*-Hydroxystyrene." *Metabolic Engineering* 9 (3): 268–76.

Quan, X., R. Rassadi, B. Rabie, N. Matusiewicz, and U. Stochaj. 2004. "Regulated Nuclear Accumulation of the Yeast hsp70 Ssa4p in Ethanol-Stressed Cells Is Mediated by the N-Terminal Domain, Requires the Nuclear Carrier Nmd5p and Protein Kinase C." *The FASEB Journal* 18 (7): 899–901.

Ramos, J., E. Duque, M. Gallegos, P. Godoy, M. Ramos-González, A. Rojas, W. Terán, and A. Segura. 2002. "Mechanisms of Solvent Tolerance in Gram-Negative Bacteria." *Annual Review of Microbiology* 56: 743–68.

Rantwijk, F. van, R. Madeira, and R. Sheldon. 2003. "Biocatalytic Transformations in Ionic Liquids." *Trends in Biotechnology* 21 (3): 131–38.

Rebros, M., H. Gunaratne, J. Ferguson, K. Seddon, and G. Stephens. 2009. "A High Throughput Screen to Test the Biocompatibility of Water-Miscible Ionic Liquids." *Green Chemistry* 11 (3): 402.

Reiling, H., H. Laurila, and A. Fiechter. 1985. "Mass Culture of *Escherichia Coli*: Medium Development for Low and High Density Cultivation of *Escherichia Coli* B/r in Minimal and Complex Media." *Journal of Biotechnology* 2 (3–4): 191–206.

Research and Markets. 2015. "Global Styrene Market 2015-2019." Dublin, Ireland.

Riesenberg, D. 1991. "High-Cell-Density Cultivation of *Escherichia Coli*." *Current Opinion in Biotechnology* 2 (3): 380–84.

Riesenberg, D., K. Menzel, V. Schultz, K. Schumann, G. Veith, G. Zuber, and W. Knorre. 1990. "High Cell Density Fermentation of Recombinant *Escherichia*

Coli Expressing Human Interferon Alpha 1." *Applied Microbiology and Biotechnology* 34: 77–82.

Riesenberg, D., V. Schulz, W. Knorre, H. Pohl, D. Korz, E. Sanders, A. Roβ, and W. Deckwer. 1991. "High Cell Density Cultivation of *Escherichia Coli* at Controlled Specific Growth Rate." *Journal of Biotechnology* 20: 17–28.

Rodriguez, M., N. Kelessidou, Z. Law, J. Gardiner, and G. Stephens. 2008. "Effect of Solvents on Obligately Anaerobic Bacteria." *Anaerobe* 14 (1): 55–60.

Rosano, G., and E. Ceccarelli. 2014. "Recombinant Protein Expression in *Escherichia Coli*. Advances and Challenges." *Frontiers in Microbiology* 5 (APR): 1–17.

Salminen, J., N. Papaiconomou, R. Kumar, J. Lee, J. Kerr, J. Newman, and J. Prausnitz. 2007. "Physicochemical Properties and Toxicities of Hydrophobic Piperidinium and Pyrrolidinium Ionic Liquids." *Fluid Phase Equilibria* 261 (1–2): 421–26.

Sardessai, Y., and S. Bhosle. 2002. "Tolerance of Bacteria to Organic Solvents." *Research in Microbiology* 153 (5): 263–68.

Sariaslani, F. 2007. "Development of a Combined Biological and Chemical Process for Production of Industrial Aromatics from Renewable Resources." *Annual Review of Microbiology* 61: 51–69.

Schmid, A., A. Kollmer, R. Mathys, and B. Witholt. 1998. "Developments toward Large-Scale Bacterial Bioprocesses in the Presence of Bulk Amounts of Organic Solvents." *Extremophiles* 2: 249–56.

Seymour, I., and P. Piper. 1999. "Stress Induction of HSP30, the Plasma Membrane Heat Shock Protein Gene of *Saccharomyces Cerevisiae*, Appears Not to Use Known Stress-Regulated Transcription Factors." *Microbiology*, no. 145: 231–39.

Shao, P., and R. Huang. 2007. "Polymeric Membrane Pervaporation." *Journal of*

Membrane Science 287 (2): 162–79.

Sikkema, J., J. de Bont, and B. Poolman. 1995. “Mechanisms of Membrane Toxicity of Hydrocarbons.” *Microbiol. Rev.* 59 (2): 201–22.

Sikkema, J., J. de Bontt, and B. Poolman. 1994. “Interactions of Cyclic Hydrocarbons with Biological Membranes.” *The Journal of Biological Chemistry* 269 (11): 8022–28.

Simoni, L., A. Chapeaux, J. Brennecke, and M. Stadtherr. 2010. “Extraction of Biofuels and Biofeedstocks from Aqueous Solutions Using Ionic Liquids.” *Computers and Chemical Engineering* 34 (9). Elsevier Ltd: 1406–12.

Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. Slonczewski. 1994. “Acid and Base Resistance in *Escherichia Coli* and *Shigella Flexneri*. Role of rpoS and Growth pH.” *Journal of Bacteriology* 176 (6): 1729–37.

Smallwood, I. 1996. *Handbook of Organic Solvent Properties*. London: Arnold.

Smith, L., J. Ravel, S. Lax, and W. Shive. 1962. “The Control of 3-Deoxy-D-Arabino-Heptulosonic Acid 7-Phosphate by Phenylalanine and Tyrosine.” *The Journal of Biological Chemistry* 237 (11): 3566–70.

Srirangan, K., L. Akawi, M. Moo-Young, and C. Chou. 2012. “Towards Sustainable Production of Clean Energy Carriers from Biomass Resources.” *Applied Energy* 100. Elsevier Ltd.: 172–86.

Stark, A., and K. Seddon. 2007. “Ionic Liquids.” In *Kirk-Othmer Encyclopaedia of Chemical Technology*, 26:836–920. Wiley Online Library.

Stolte, S., M. Matzke, J. Arning, A. Böschen, W. Pitner, U. Welz-Biermann, B. Jastorff, and J. Ranke. 2007a. “Effects of Different Head Groups and Functionalised Side Chains on the Aquatic Toxicity of Ionic Liquids.” *Green Chemistry* 9 (11): 1170–79.

Stolte, S., M. Matzke, J. Arning, A. Böschen, W. Pitner, U. Welz-Biermann, B. Jastorff, and J. Ranke. 2007b. “Effects of Different Head Groups and

Functionalised Side Chains on the Cytotocity of Ionic Liquids." *Green Chemistry* 9 (11): 760–67.

Straathof, A. 2003. "Auxiliary Phase Guidelines for Microbial Biotransformations of Toxic Substrate into Toxic Product." *Biotechnology Progress* 19: 755–62.

Straathof, A. 2014. "Transformation of Biomass into Commodity Chemicals Using Enzymes or Cells." *Chemical Reviews* 114 (3): 1871–1908.

Stratford, M., A. Plumridge, M. Pleasants, M. Novodvorska, C. Baker-Glenn, G. Pattenden, and D. Archer. 2012. "Mapping the Structural Requirements of Inducers and Substrates for Decarboxylation of Weak Acid Preservatives by the Food Spoilage Mould *Aspergillus Niger*." *International Journal of Food Microbiology* 157. Elsevier B.V.: 375–83.

Strom, A., and I. Kaasen. 1993. "Trehalose Metabolism in *Escherichia Coli*. Stress Protection and Stress Regulation of Gene Expression." *Molecular Microbiology* 8 (2): 205–10.

Takaç, S., B. Akay, and T. Özdamar. 1995. "Bioconversion of *trans*-Cinnamic Acid to L-Phenylalanine by L-Phenylalanine Ammonia-Lyase of Rhodotorula Glutinis. Parameters and Kinetics." *Enzyme and Microbial Technology* 17 (5): 445–52.

Tomas, C., N. Welker, and E. Papoutsakis. 2003. "Overexpression of groESL in Clostridium Acetobutylicum Results in Increased Solvent Production and Tolerance, Prolonged Metabolism, and Changes in the Cell's Transcriptional Program." *Applied and Environmental Microbiology* 69 (8): 4951–65.

Tossavainen, A. 1978. "Styrene Use and Occupational Exposure in the Plastics Industry." *Scandinavian Journal of Work, Environment and Health* 4 (2): 7–13.

Treybal, R. 1980. *Mass Transfer Operations*. 3rd ed. Singapore: McGraw-Hill.

Tribe, D. 1987. Novel microorganism and method. 459,302, issued 1987.

Tsunashima, K., and M. Sugiya. 2007. “Physical and Electrochemical Properties of Low-Viscosity Phosphonium Ionic Liquids as Potential Electrolytes.” *Electrochemistry Communications* 9 (9): 2353–58.

U.S. Department of Energy. 2012. “New Process for Producing Styrene Cuts Costs, Saves Energy, and Reduces Greenhouse Gas Emissions.” Washington D.C.

U.S. Environmental Protection Agency. 1994. “Styrene Fact Sheet.”

U.S. Environmental Protection Agency. U.S. Environmental Protection Agency. 2000. “Styrene Oxide.” In *Health Effects Notebook for Hazardous Air Pollutants*, 4.

United Nations Development Programme. 2000. “World Energy Assessment - Energy and the Challenge of Sustainability.” New York.

Urata, K., and N. Takaishi. 2001. “A Perspective on the Contribution of Surfactants and Lipids toward ‘Green Chemistry’. Present States and Future Potential.” *Journal of Surfactants and Detergents* 4 (2): 191–200.

Van Sonsbeek, H., H. Beeftink, and J. Tramper. 1993. “Two-Liquid-Phase Bioreactors.” *Enzyme and Microbial Technology* 15 (9): 722–29.

Vane, L. 2005. “A Review of Pervaporation for Product Recovery from Biomass Fermentation Processes.” *Journal of Chemical Technology and Biotechnology* 80 (6): 603–29.

Vedel, G., M. Peyret, J. Gayral, and P. Millot. 1996. “Evaluation of an Expert System Linked to a Rapid Antibiotic Susceptibility Testing System for the Detection of β-Lactam Resistance Phenotypes.” *Research in Microbiology* 147 (4): 297–309.

Ventura, S., A. Gonçalves, T. Sintra, J. Pereira, F. Gonçalves, and J. Coutinho. 2013. “Designing Ionic Liquids - The Chemical Structure Role in the

Toxicity." *Ecotoxicology* 22 (1): 1–12.

Ventura, S., C. Marques, A. Rosatella, C. Afonso, F. Gonçalves, and J. Coutinho. 2012. "Toxicity Assessment of Various Ionic Liquid Families towards Vibrio Fischeri Marine Bacteria." *Ecotoxicology and Environmental Safety* 76 (1): 162–68.

Verhoef, S., H. Ruijssenaars, J. de Bont, and J. Wery. 2007. "Bioproduction of *p*-Hydroxybenzoate from Renewable Feedstock by Solvent-Tolerant *Pseudomonas Putida* S12." *Journal of Biotechnology* 132 (1): 49–56.

Verhoef, S., N. Wierckx, R. Westerhof, J. de Winde, and H. Ruijssenaars. 2009. "Bioproduction of *p*-Hydroxystyrene from Glucose by the Solvent-Tolerant Bacterium *Pseudomonas Putida* S12 in a Two-Phase Water-Decanol Fermentation." *Applied and Environmental Microbiology* 75 (4): 931–36.

Vermuë, M., J. Sikkema, A. Verheul, R. Bakker, and J. Tramper. 1993. "Toxicity of Homologous Series of Organic Solvents for the Gram-Positive Bacteria Arthrobacter and Nocardia Sp. and the Gram-Negative Bacteria Acinetobacter and Pseudomonas Sp." *Biotechnology and Bioengineering* 42: 747–58.

Viboud, S., N. Papaiconomou, A. Cortesi, G. Chatel, M. Draye, and D. Fontvieille. 2012. "Correlating the Structure and Composition of Ionic Liquids with Their Toxicity on Vibrio Fischeri. A Systematic Study." *Journal of Hazardous Materials* 215–216. Elsevier B.V.: 40–48.

Vishniac, W., and M. Santer. 1957. "The Thiobacilli." *Bacteriological Reviews* 21 (3): 195–213.

Wahbi, L., D. Gokhale, S. Minter, and G. Stephens. 1996. "Construction and Use of Recombinant *Escherichia Coli* Strains for the Synthesis of Toluene Cis-Glycol." *Enzyme Microbial Technology* 19 (4): 297–306.

Wallace, B., and J. Pittard. 1969. "Regulator Gene Controlling Enzymes Concerned in Tyrosine Biosynthesis in *Escherichia Coli*." *Journal of*

Bacteriology 97 (3): 1234–41.

Wasserscheid, P., and T. Welton. 2008. *Ionic Liquids in Synthesis*. Edited by Peter Wasserscheid and Tom Welton. 2nd ed. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Webb, J. 2015. "Bio-Production of C5 Dicarboxylic Acids for Chemical Conversion to Methyl Methacrylate." The Univervisity of Nottingham.

Webber, M., and L. Piddock. 2003. "The Importance of Efflux Pumps in Bacterial Antibiotic Resistance." *Journal of Antimicrobial Chemotherapy* 51: 9–11.

Weber, F., and J. de Bont. 1996. "Adaptation Mechanisms of Microorganisms to the Toxic Effects of Organic Solvents on Membranes." *Biochimica et Biophysica Acta - Reviews on Biomembranes* 1286 (3): 225–45.

Weber, F., S. Isken, and J. de Bont. 1994. "Cis-Trans Isomerization of Fatty Adds as a Defence Mechanism of *Pseudomonas Putida* Strains to Toxic Concentrations of Toluene." *Microbiology* 140 (8): 2013–17.

Weber, F., L. Ooijkaas, R. Schemen, S. Hartmans, and J. de Bont. 1993. "Adaptation of *Pseudomonas Putida* S12 to High Concentrations of Styrene and Other Organic Solvents." *Applied and Environmental Microbiology* 59 (10): 3502–4.

Weber, H., T. Polen, J. Heuveling, V. Wendisch, and R. Hengge. 2005. "Genome-Wide Analysis of the General Stress Response Network in *Escherichia Coli* - Sigma S-Dependent Genes, Promoters, and Sigma Factor Selectivity." *Journal of Bacteriology* 187 (5): 1591–1603.

Weilnhammer, C., and E. Blass. 1994. "Continuous Fermentation with Product Recovery by in-Situ Extraction." *Chemical Engineering & Technology* 17 (6): 365–73.

Weuster-Botz, D. 2007. "Process Intensification of Whole-Cell Biocatalysis with Ionic Liquids." *The Chemical Record* 7 (6): 334–40.

White, D., J. Goldman, B. Demple, and S. Levy. 1997. “Role of the acrAB Locus in Organic Solvent Tolerance Mediated by Expression of marA, soxS, or robA in *Escherichia Coli*.” *Journal of Bacteriology* 179 (19): 6122–26.

Wierckx, N., H. Ballerstedt, J. de Bont, and J. Wery. 2005. “Engineering of Solvent-Tolerant *Pseudomonas Putida* S12 for Bioproduction of Phenol from Glucose.” *Applied and Environmental Microbiology* 71 (12): 8221–27.

Wilkes, J., J. Levisky, R. Wilson, and C. Hussey. 1982. “Dialkylimidazolium Chloroaluminate Melts. A New Class of Room-Temperature Ionic Liquids for Electrochemistry, Spectroscopy, and Synthesis.” *Inorganic Chemistry* 21: 1263–64.

Wilkes, J., and M. Zarawotko. 1992. “Air and Water Stable 1-Ethyl-3-Methylimidazolium Based Ionic Liquids.” *J. Chem. Soc. Chem. Commun.*, no. 13: 965–67.

Wilkinson, D., J. Woodley, and J. Ward. 1994. “Process Implications of Solvent Interaction with Microbial Catalysts.” In *IChemE Research Event*, 162–64.

Wood, N. 2011. “Toxicity of Ionic Liquids and Organic Solvents towards *Escherichia Coli* and *Pseudomonas Putida*.” The University of Manchester.

Wood, N., J. Ferguson, H. Gunaratne, K. Seddon, R. Goodacre, and G. Stephens. 2011. “Screening Ionic Liquids for Use in Biotransformations with Whole Microbial Cells.” *Green Chemistry* 13: 1843–51.

Xue, C., G. Du, J. Sun, L. Chen, S. Gao, M. Yu, S. Yang, and F. Bai. 2014. “Characterization of Gas Stripping and Its Integration with Acetone-Butanol-Ethanol Fermentation for High-Efficient Butanol Production and Recovery.” *Biochemical Engineering Journal* 83. Elsevier B.V.: 55–61.

Yamanè, T., and S. Shimizu. 1984. “Fed-Batch Techniques in Microbial Processes.” In *Bioprocess Parameter Control*, 30:147–94. Berlin, Heidelberg: Springer, Berlin, Heidelberg.

Yaws, C., H. Yang, and X. Pan. 1991. “Henry’s Law of Constants for 362 Organic Compounds in Water.” *Chemical Engineering* 98 (11). Access Intelligence LLC: 179.

Yeom, S., and A. Daugulis. 1999. “A New Method for the Determination of Microbial Activity and Critical logP in the Presence of Organic Solvents.” *Biotechnology Techniques* 13: 549–53.

Yu, X. 2012. Enzymatic system for monomer synthesis. PCT/US2012/043898, issued 2012.

Zhao, Z., Y. Dai, G. Ge, X. Guo, and G. Wang. 2015. “Facile Simultaneous Defect Production and O,N-Doping of Carbon Nanotubes with Unexpected Catalytic Performance for Clean and Energy-Saving Production of Styrene.” *Green Chemistry* 17 (7). Royal Society of Chemistry: 3723–27.

Zou, S., E. Du, Z. Hu, and Y. Zheng. 2013. “Enhanced Biotransformation of 1,3-Dichloro-2-Propanol to Epichlorohydrin via Resin-Based in Situ Product Removal Process.” *Biotechnology Letters* 35 (6): 937–42.

Zurawski, G., R. Gunsalus, K. Brown, and C. Yanofsky. 1981. “Structure and Regulation of aroH, the Structural Gene for the Tryptophan-Repressible 3-Deoxy-D-Arabino-Heptulosonic Acid-7-Phosphate Synthetase of *Escherichia Coli*.” *Journal of Molecular Biology* 145 (1): 47–73.

8. Appendices

8.1 Biocompatibility of $[P_{66614}][NTf_2]$ with *E. coli* MG1655

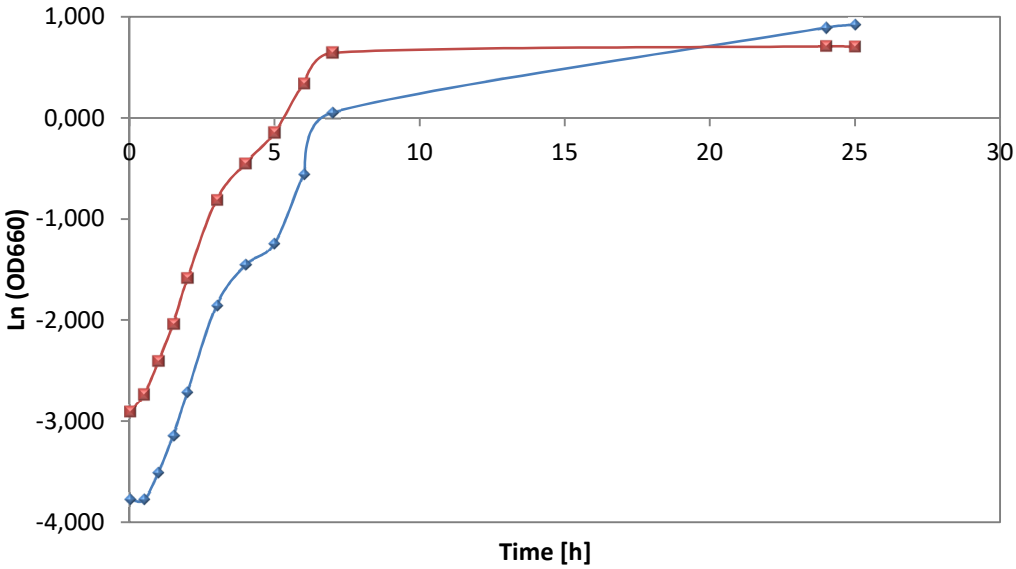


Figure 50: Growth curve of *E. coli* MG1655 in MSX in presence of $[P_{66614}][NTf_2]$ (blue diamond) and in absence of ionic liquid (red square).

8.2 Nuclear magnetic resonance spectroscopy (NMR)

Figures 51-53 show the NMR spectra of the ILs solutions with 1, 10, and 100 mM initial concentrations of styrene, respectively. Figures 54-55 show the NMR spectrum of the ionic liquid phase from the biotransformation cultures of glucose to styrene by *E. coli* NST74 harbouring pSTV28_*pal2* pTrc99A_*fdc1* or pSTV28_*pal2* pTrc99A_*ohba1,* respectively.

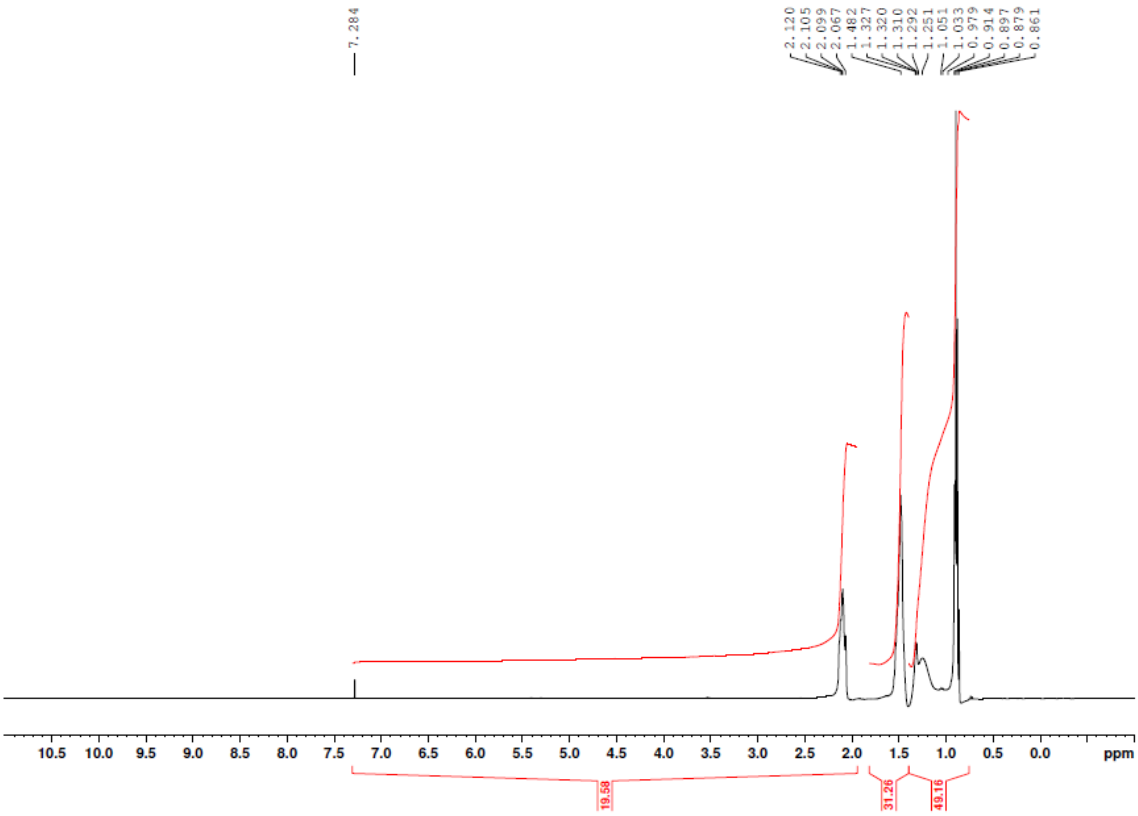


Figure 51: NMR spectrum of the 1 mM styrene solution in $[P_{66614}][NTf_2]$.

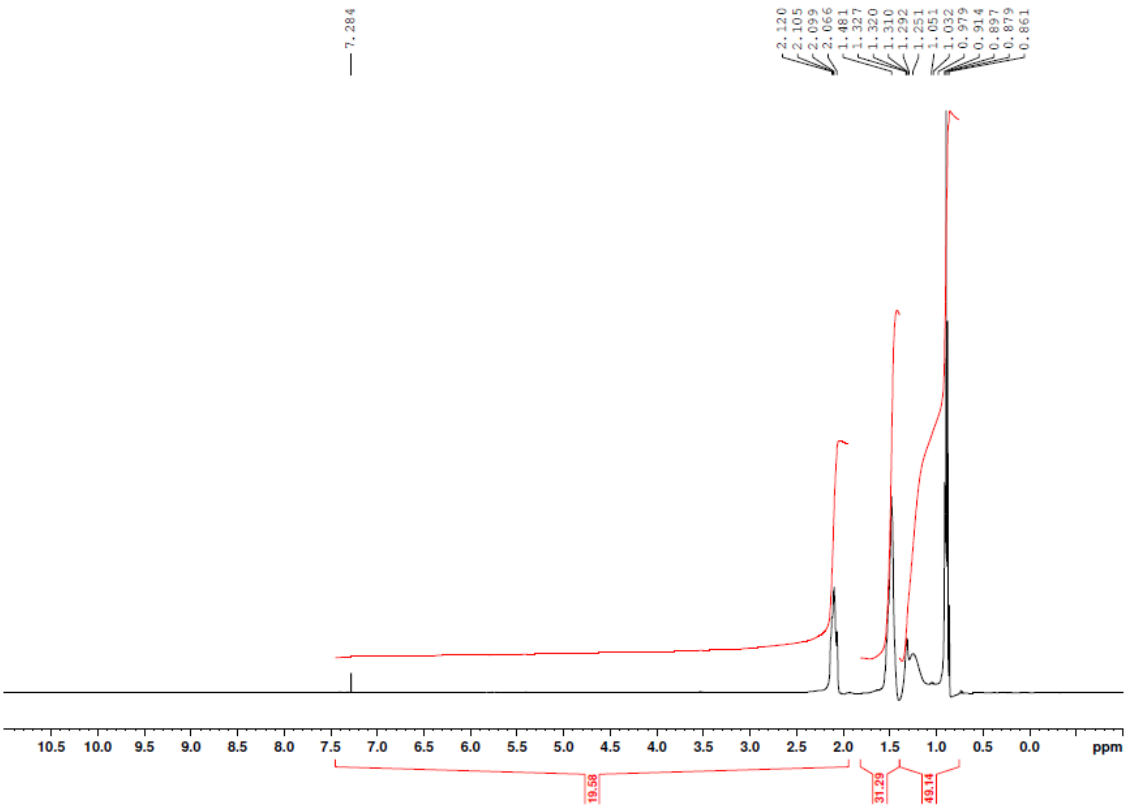


Figure 52: NMR spectrum of the 10 mM styrene solution in $[P_{66614}][NTf_2]$.

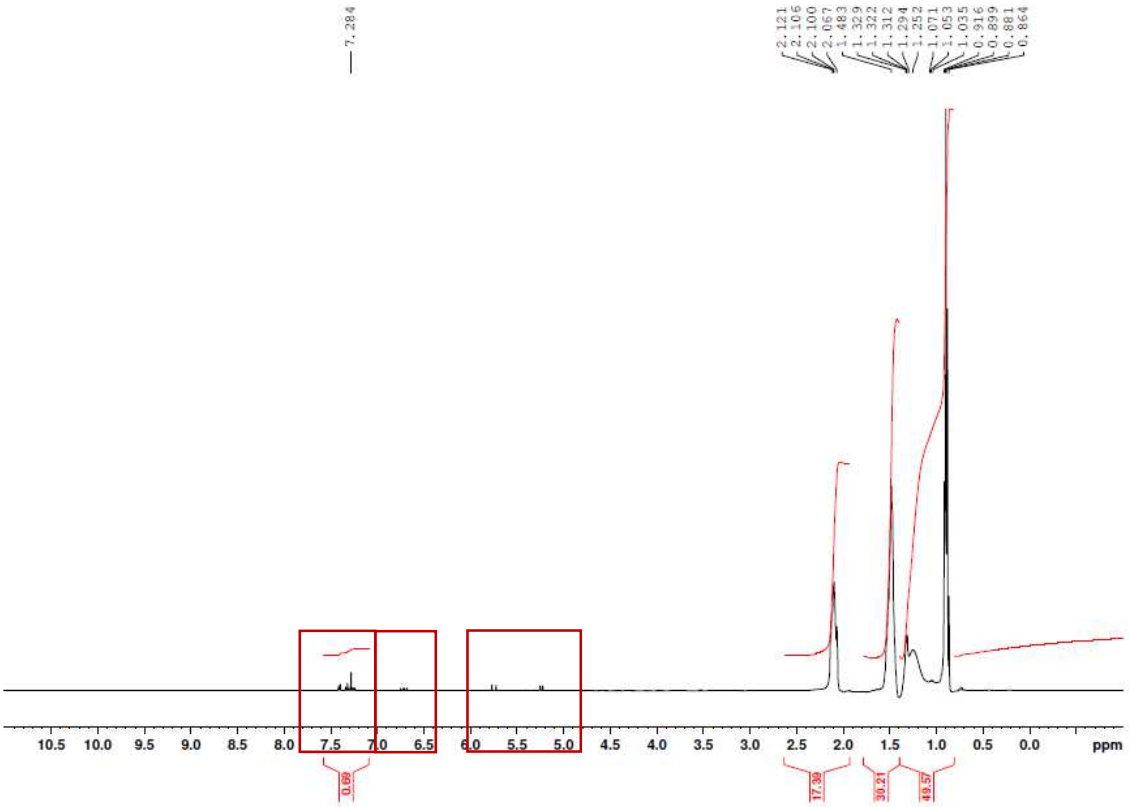


Figure 53: NMR spectrum of the 100 mM styrene solution in [P_{66614}][NTf_2].

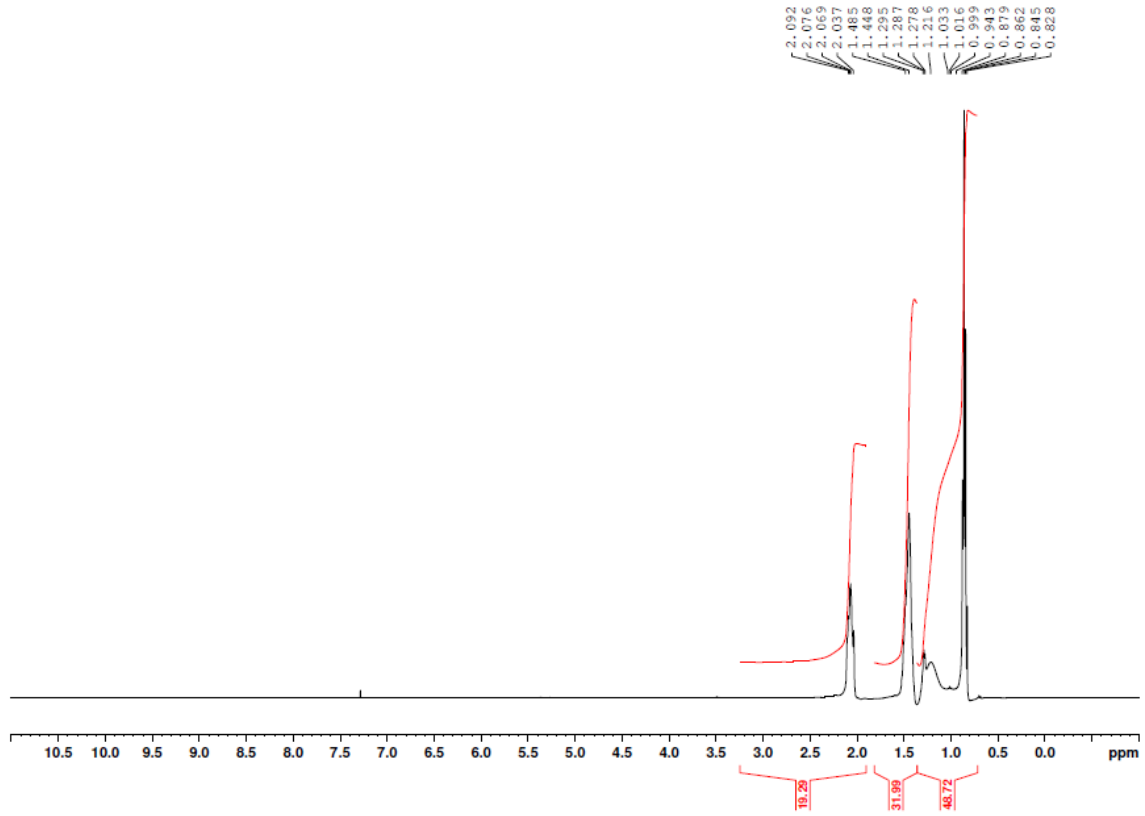


Figure 54: NMR spectrum of samples from the ionic liquid phase from the biotransformation of glucose to styrene by *E. coli* NST74 pSTV28_*pal2* pTrc99A_*fdc1*.

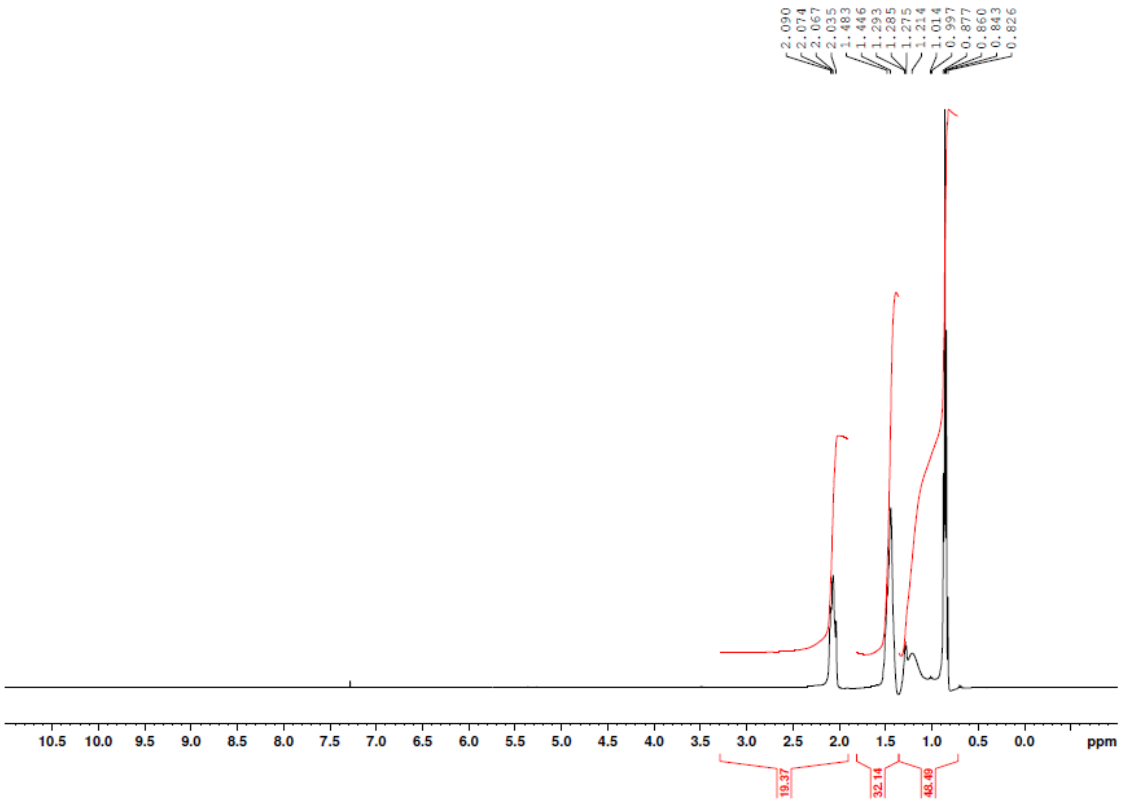
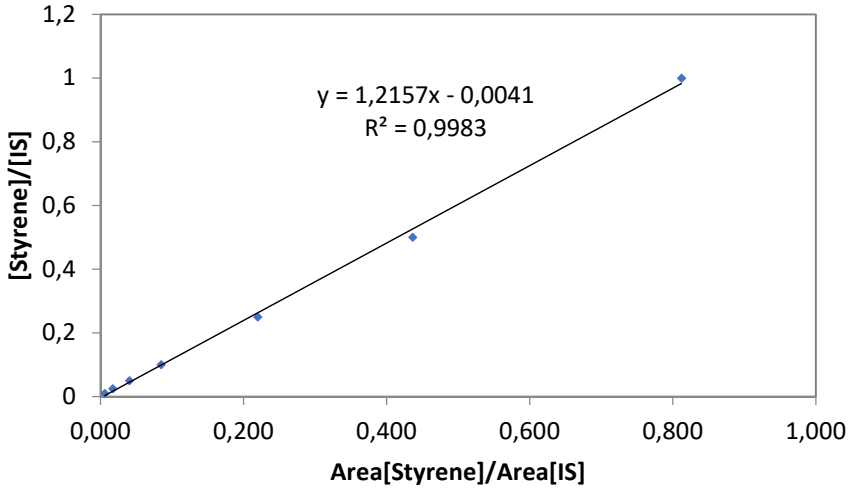


Figure 55: NMR spectrum of samples from the ionic liquid phase from the biotransformation of glucose to styrene by *E. coli* NST74 pSTV28_*pal2* pTrc99A_*ohba1*.

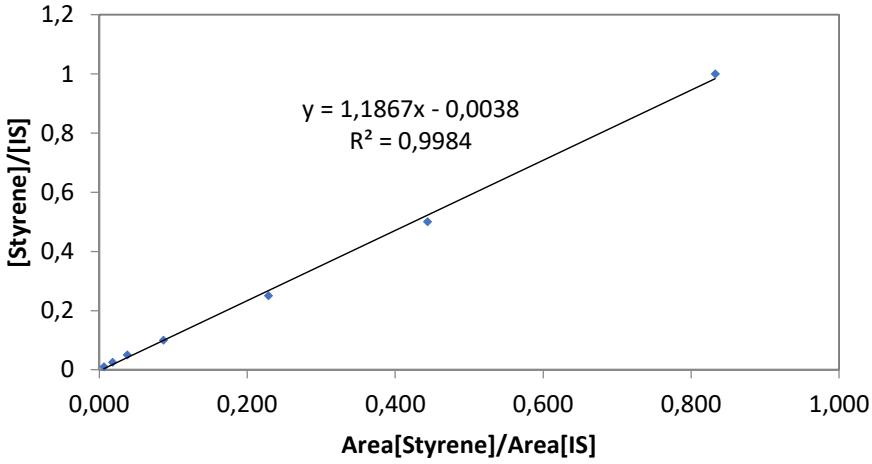
8.3 GC-MS calibration curves

Calibration curves were prepared in organic solvent using authentic styrene (Figure 56). Detection limit of styrene with the used column was 0.1 mM, therefore styrene solutions were prepared in concentration range of 0.1 to 10 mM.

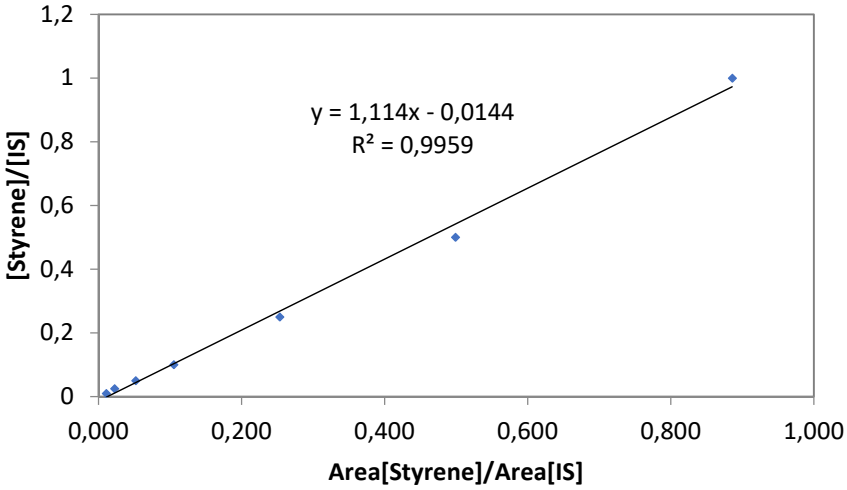
Hexane



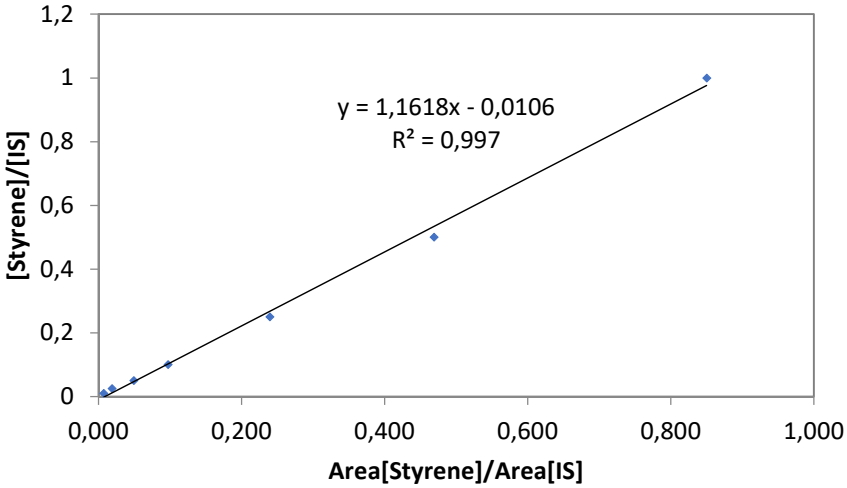
Ethyl Acetate



Dichloromethane



Toluene



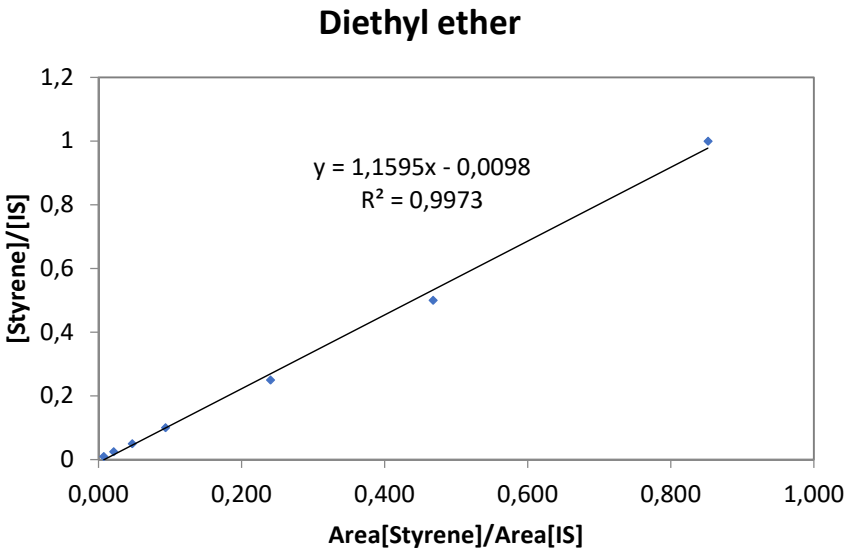


Figure 56: Calibration curves of styrene for the 5 organic solvents used in the extraction from aqueous phase.

8.4 Silica gel column

A silica gel column (Figure 57) was used to try to separate the ionic liquid from the homogenous solution generated by the mixing with each organic solvent and the styrene.



Figure 57: Silica gel column.

Solvent/IL–styrene mixtures in a 1:1 ratio were passed through the silica column. Increasing concentrations of styrene in IL were used.

8.5 GC-FID calibration curves

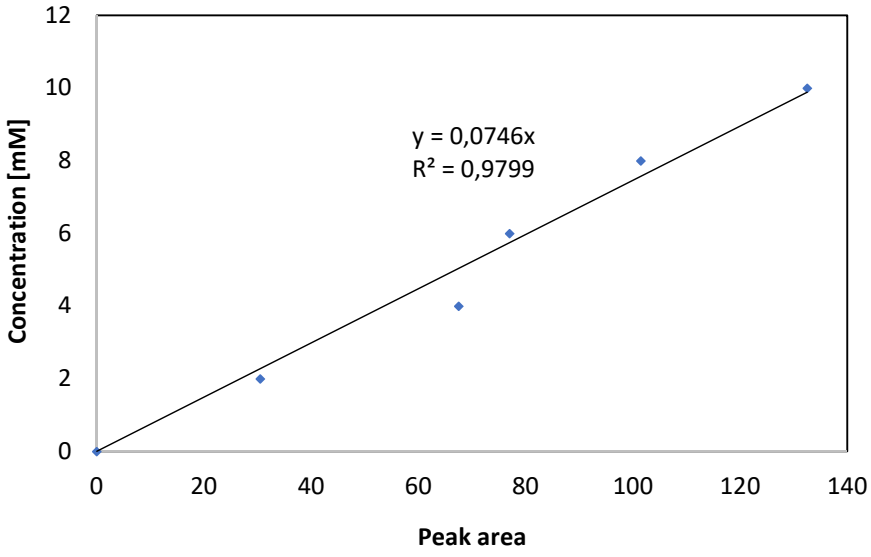


Figure 58: Styrene calibration curve in $[P_{66614}][NTf_2]$.

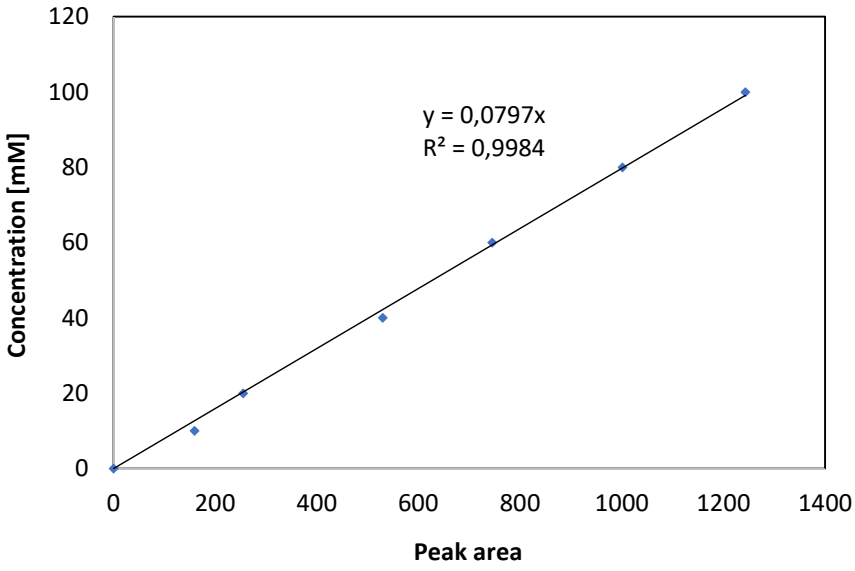


Figure 59: Styrene calibration curve in $[P_{66614}][NTf_2]$

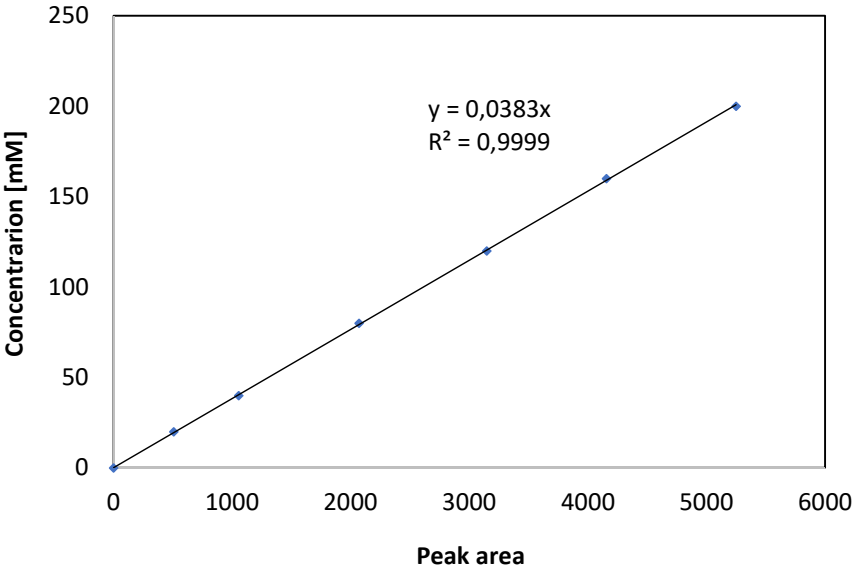


Figure 60: Styrene calibration curve in dodecane

8.6 HPLC calibration curves

To calculate the concentration of compounds consumed and produced during the different fermentation experiments, HPLC standard curves were generated using known concentrations of authentic compounds purchased

from Sigma-Aldrich (Figures 61-70). This was achieved by weighing out a known amount of the compound and diluting it in a known amount of water. Various solutions of known concentrations were produced for each compound, and serial dilutions were done and injected onto the HPLC column.

The peak area for each injection volume was measured and used to plot standard curves of the compound concentration against the peak area. The amount of compound used to generate the standard curve was varied depending on the expected titre of substrate and products, so that the amount produced would fall within the range of the calibration curve.

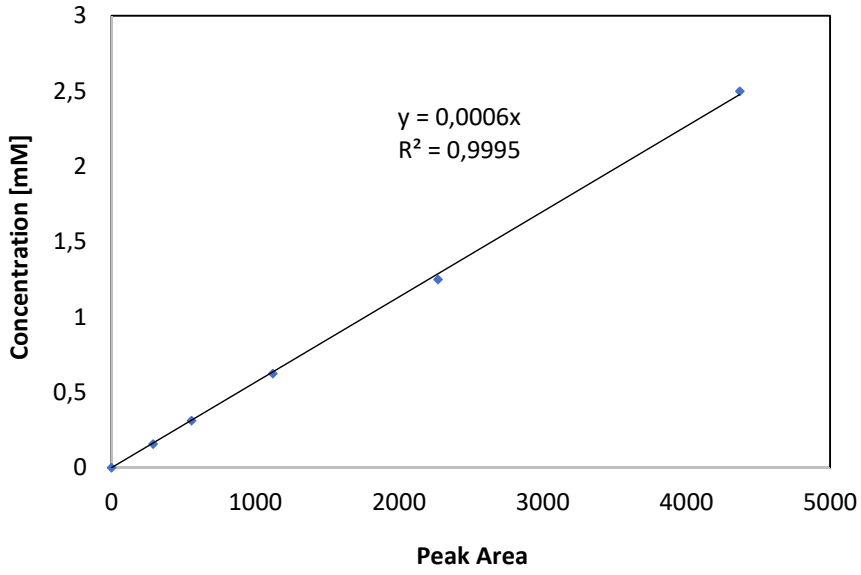


Figure 61: Tyrosine calibration curve.

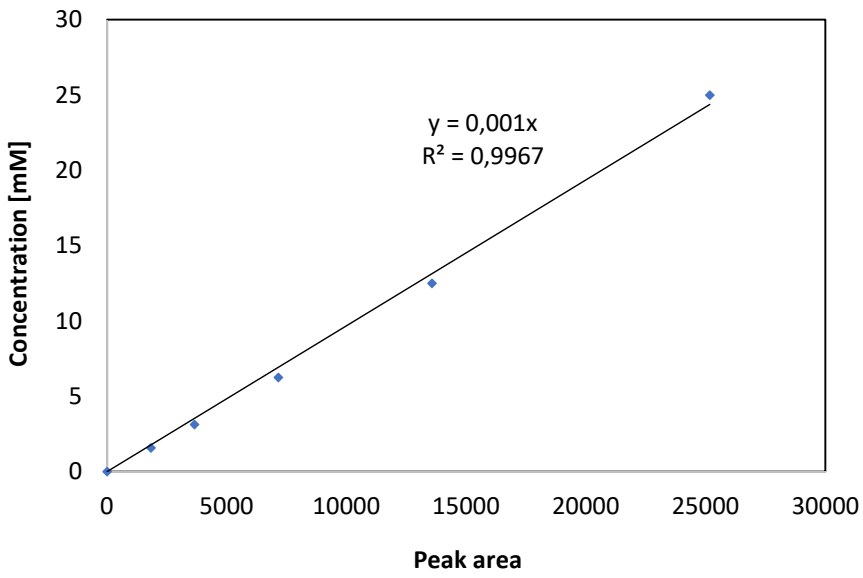


Figure 62: L-phenylalanine calibration curve.

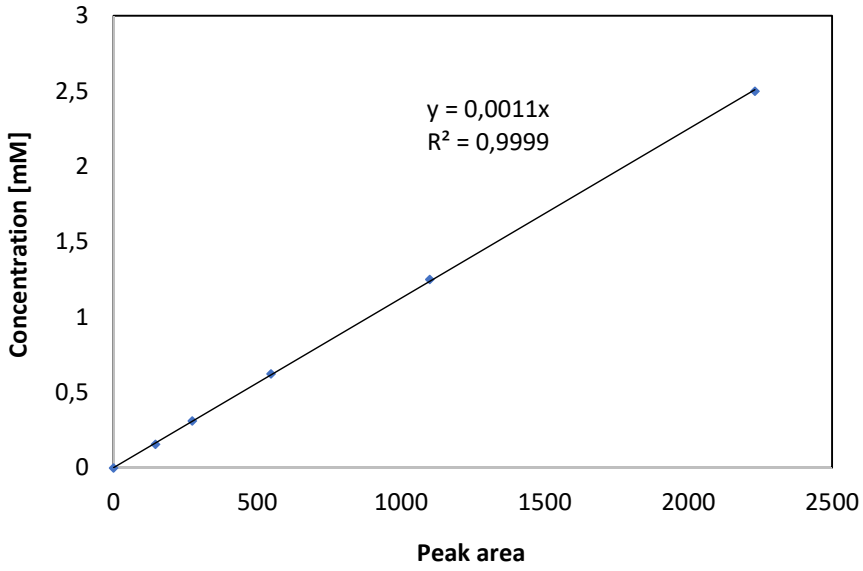


Figure 63: *p*-coumaric calibration curve.

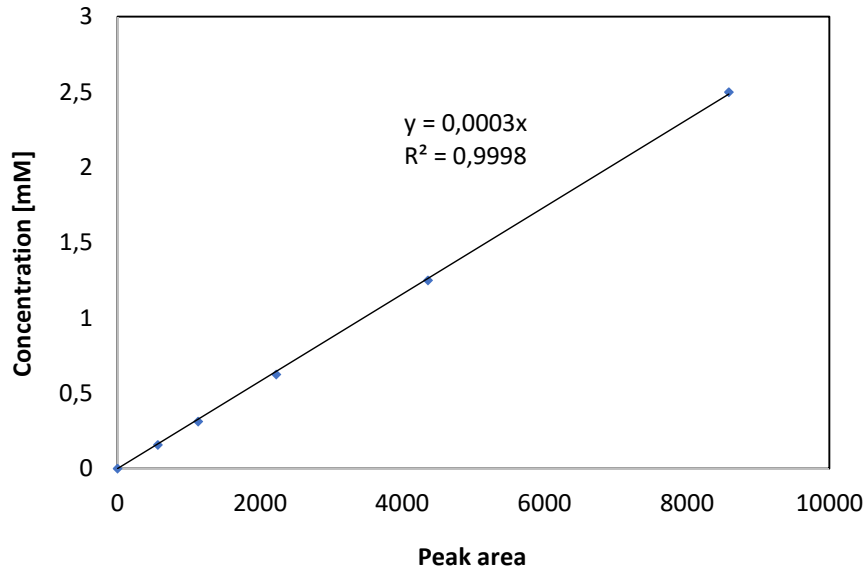


Figure 64: *trans*-cinnamic acid calibration curve.

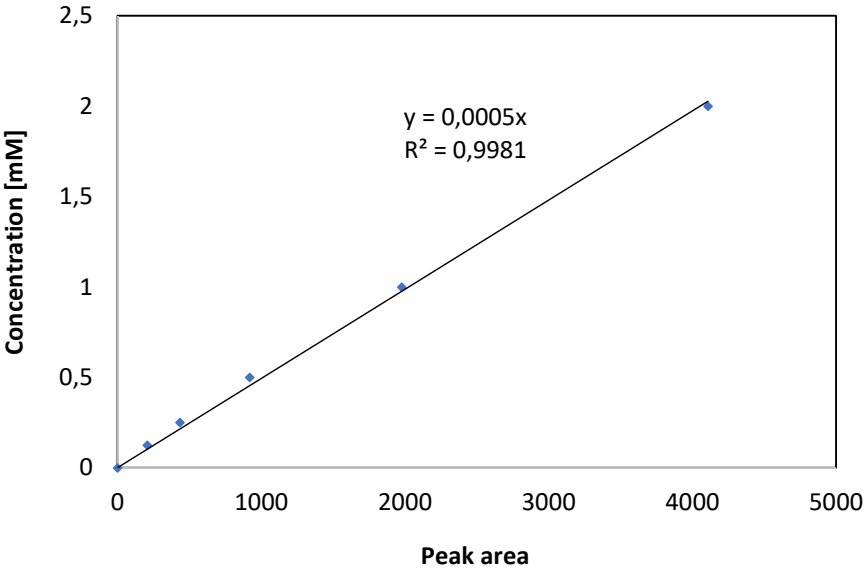


Figure 65: Styrene calibration curve.

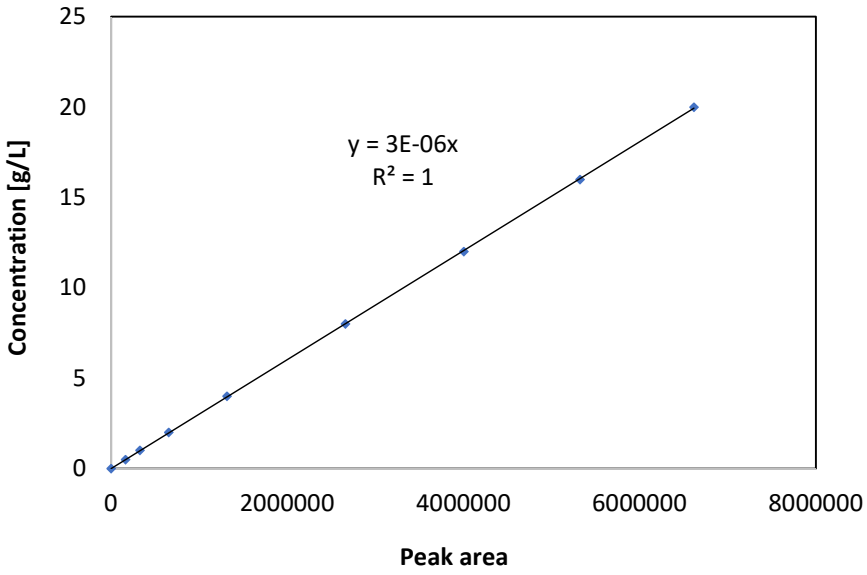


Figure 66: Glucose calibration curve.

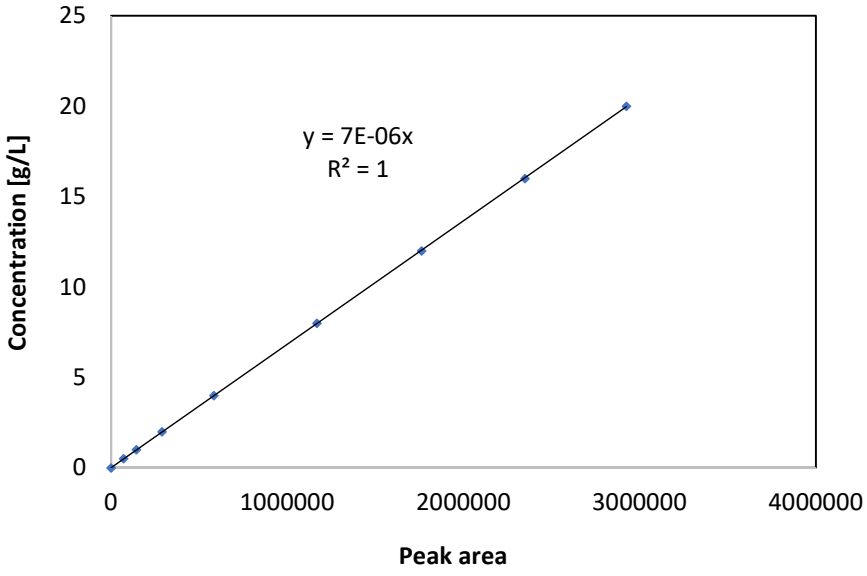


Figure 67: Pyruvate calibration curve.

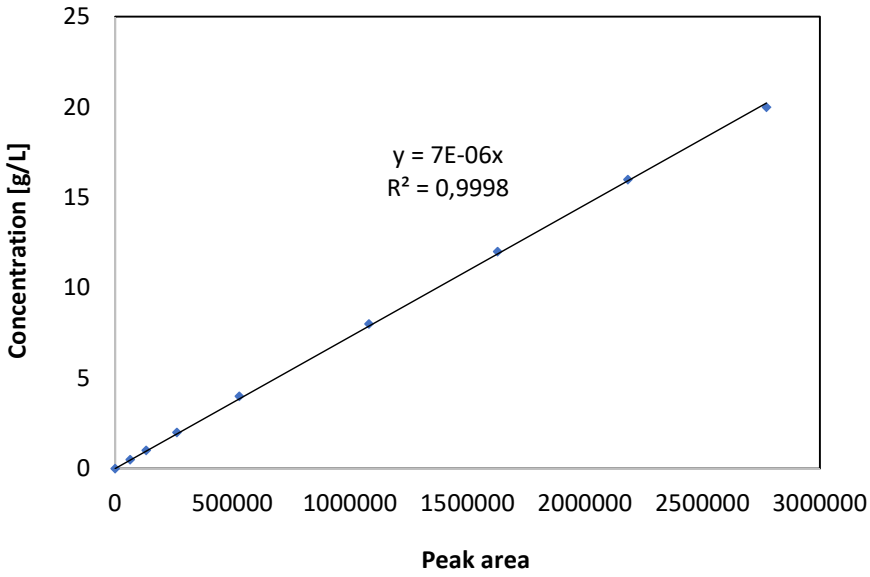


Figure 68: Lactate calibration curve.

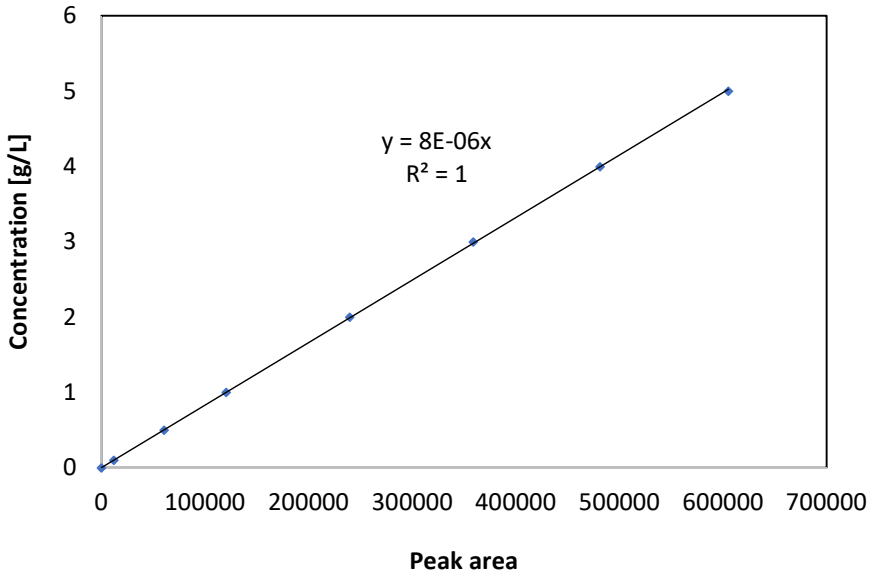


Figure 69: Formate calibration curve.

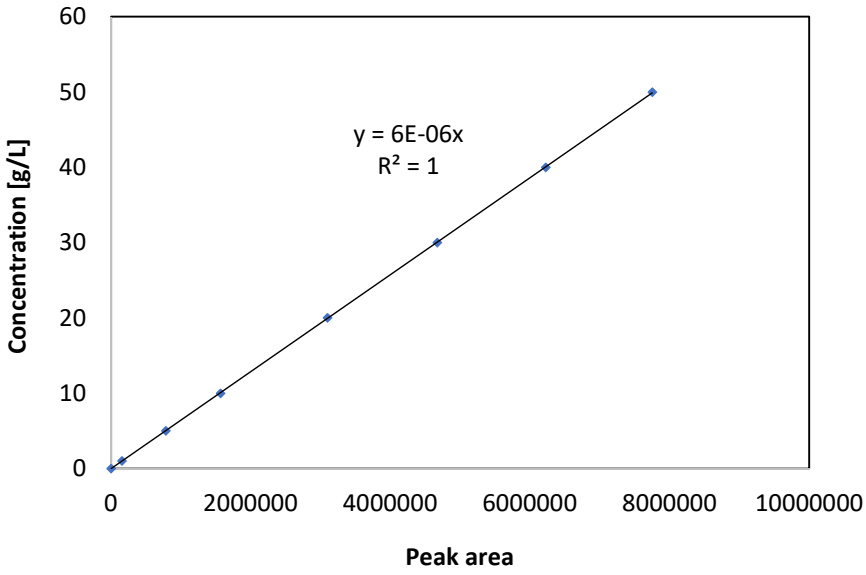


Figure 70: Acetate calibration curve.

8.7 Improving the analytical method for styrene analysis

Table 18: The obtained peak areas of ionic liquid solution with different styrene concentrations (1 – 10 mM) and *t*-butyl benzene (10 mM) when analysed directly by GC-FID.

Styrene [mM]	[Styrene]/[IS]	Area Styrene [pA×s]	Area IS [pA×s]
10	1	45.1	45.3
		41.0	39.9
		41.1	40.3
8	0.8	33.5	41.8
		34.3	42.3
		29.6	32.8
6	0.6	22.5	22.4
		20.1	17.9
		19.4	17.9
4	0.4	13.9	31.3
		12.4	24.5
		12.8	28.8
2	0.2	10.2	40.2
		10.1	39.7
		10.0	38.4

Table 19: The obtained peak areas of ionic liquid solution with different styrene concentrations (1 – 10 mM) and *t*-butyl benzene (10 mM) when previously mix with ethyl acetate and later analysed by GC-FID.

Styrene [mM]	[Styrene]/[IS]	Area Styrene [pA×s]	Area IS [pA×s]
10	1	144.5	135.5
		143.9	138.0
		143.4	135.5
8	0.8	102.5	134.9
		100.5	135.6
		100.9	133.8
6	0.6	83.0	135.8
		81.5	134.2
		81.4	135.8
4	0.4	66.1	133.5
		65.7	133.4
		63.6	134.4
2	0.2	37.8	132.1
		37.3	130.7
		38.6	133.7

8.8 Fed-batch fermentation set-up and safe operating procedures and instructions

8.8.1 Equipment details

The main equipment and tools used are identified and indicated in Figures 50–54, such as:

- New Brunswick™ BioFlo®/CelliGen® 115 control system
- 3L glass bioreactor vessel
- Tubing- silicone, butyl rubber, PTFE flexible tubing for water and air connections
- Glass feed vessels
- Air filters
- DO probe
- pH probe
- Absorption column (height: 20 cm; diameter: 3 cm. Figure 51)

The process operation conditions were:

- Air flow: 1 L/min
- Agitation: 400 – 1200 rpm
- Culture temperature: 37 °C

8.8.2 Preparing the bioreactor vessel for use

Refer to the manufacturers operating instructions throughout. Full training must be completed before using this equipment.

1. The vessel will normally be assembled in its metal frame with stainless steel top plate in place when you come to use it (Figure 52).
2. Unscrew the 6 thumb nuts which secure the top plate.
3. Remove to plate and set down carefully and securely making sure not to bend the cooling loop or impellers.
4. Prepare the required volume of batch medium in a suitable vessel and then transfer to the bioreactor vessel. Some components may be added later after filter sterilisation.
5. Place the top plate back on the vessel and screw down the 6 thumb screws, only moderate hand pressure is required.
6. Install all the accessories required into the top plate and ensure that:
   a) All O rings are in place.
   b) All O rings and port screw threads are lightly greased.
7. Seal the O rings by tightening (only minimum finger pressure required).
8. Insert the DO and pH probes by removing port screw, ensuring O ring is in place and then carefully inserting the probes down into the vessel and screwing into position, attach the screw lids to the tops of probes.
9. Attach tubing to all necessary ports, *e.g.* anti-foam, acid addition, base addition, air inlet and secure with cable ties (Figure 53).
10. Attach the condenser unit and tighten the grub screw using suitable alun key.
11. Attach the tubing and feed bottles (filled) to the correct ports.
12. Attach the gas exhaust with suitable tubing and a directional air filter.
13. Attach clamps to the following tubing close to the vessel:
    a) Anti-foam addition
    b) Acid addition
    c) Base addition
    d) Sampling line
    e) Innoculation/Feed lines
    f) Air inlet tube
14. It is important to ensure the vessel is ventilated to prevent pressure build up, therefore DO NOT CLAMP OFF THE GAS EXHAUST TUBING.

Instead secure it to the top plate making sure there are no kinks in the tubing.

15. The free end of any tubing except the gas exhaust should be plugged with non-adsorbent cotton wool and covered with foil.
16. Cover the impeller motor shaft with foil.
17. Neatly curl the condenser and cooling water tubing up and place neatly on to plate. Make sure the chiller (Figure 54) is off when performing this step or water will leak on the benches.
18. Empty water jacket (about half of it) to avoid breaking during autoclavation.
19. Transfer the bioreactor to the autoclave using the trolley.
20. Ensure the gas exhaust is open and if preferred loosen the 6 thumb screws of the top plate.
21. Loosen the tops of all feed bottles.
22. Push the trolley into the autoclave chamber and then ask a trained person to operate the autoclave.
23. After autoclaving and after allowing the vessel to cool, place the vessel on the bench and attach all the appropriate cables:
    a) Antifoam addition
    b) Acid addition
    c) Base addition
    d) Sampling line
    e) Inoculation/Feed lines
    f) Air inlet tube
    g) Condenser/Air exhaust
    h) Heating/Cooling system
24. Inoculate microbial strain and start fermentation.

8.8.3 During the run

The fermentation is likely to last for several days continuously, meaning an EXPERIMENT IN PROGRESS FORM must be completed and on display at all times. If the correct procedure is followed, only minimal supervision of the fermentor is necessary when in use. When examining the set-up, the following must be checked:

- Make sure all vent filters are clear and there are no signs of plugging.
- Make sure foaming is kept to a minimum.
- Make sure all water supply connections are secure.
- Make sure the pressure on the air supply regulator is set to 0.5 bar (Max.).

    NOTE: If oxygen-enriched air is used (*via* the oxygen gas line), make sure that there is no leak in the tubing, that a snorkel is placed on top of the exhaust line and that an oxygen detector

is switch on in the room where the fermentation is being performed.

- In case of fed batch process, when pumping Feed, make sure total volume never goes over maximum reactor working volume.

- Sampling is likely to be required, which should be done in the following way:
    1. Attach a sterile universal bottle to the sampling device.
    2. Attach a syringe to the free end of tubing on the sampling device.
    3. Remove the clamp on the sample line and draw liquid into the universal bottle by pulling the syringe.
    4. Re-attach the clamp to the sample line and remove the universal bottle containing culture.

8.8.4 After the run

After the fermentation experiment has been completed, the system modules (pH, DO, agitation, etc.), can be switched off, providing all data has been collected. Then:

1. Switch off cooling water supply and disconnect from vessel.
2. Switch off air/gas supply and disconnect from vessel.
3. Disconnect all other connections – acid, base, antifoam, feed bottles, etc.
4. Remove agitator motor from vessel top plate.
5. Disconnect all leads from probes and clean with small amounts of 70% isopropanol.
6. Transport vessel to autoclave trolley and loosen nuts to top plate.
7. Autoclave the vessel on Fluid Discard cycle.
8. Discard liquid into drains after cooling

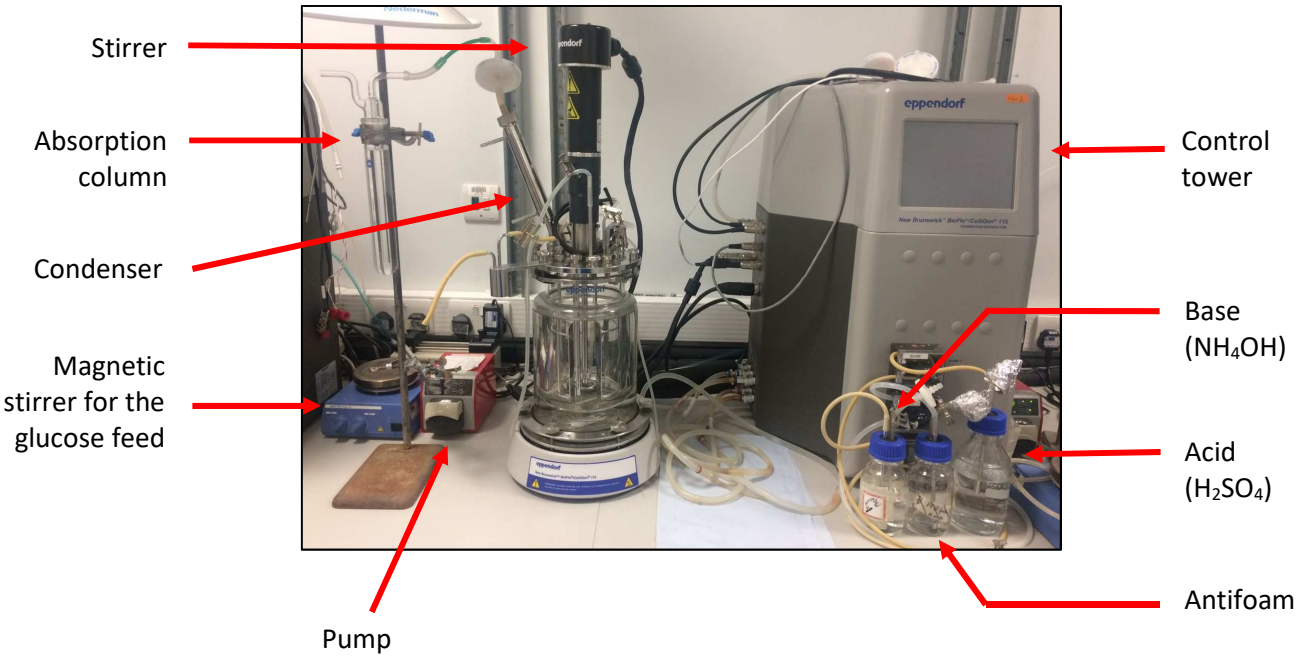


Figure 71: Description of the fed-batch fermentation devices and set-up.

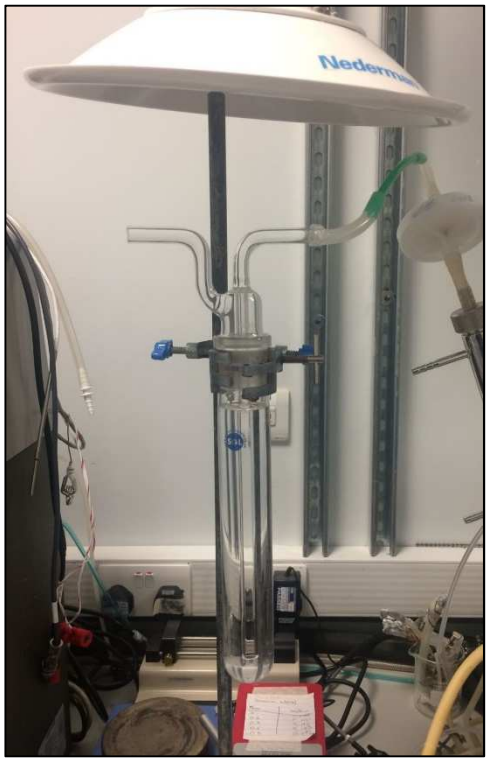


Figure 72: Dodecane absorption column.

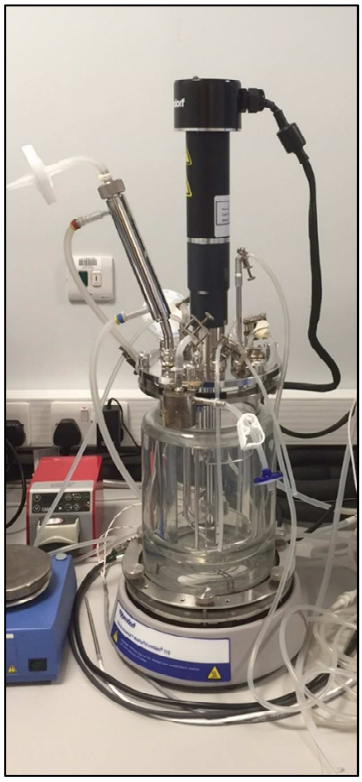


Figure 73: New Brunswick™ BioFlo®/CelliGen® 115 3L in frame.

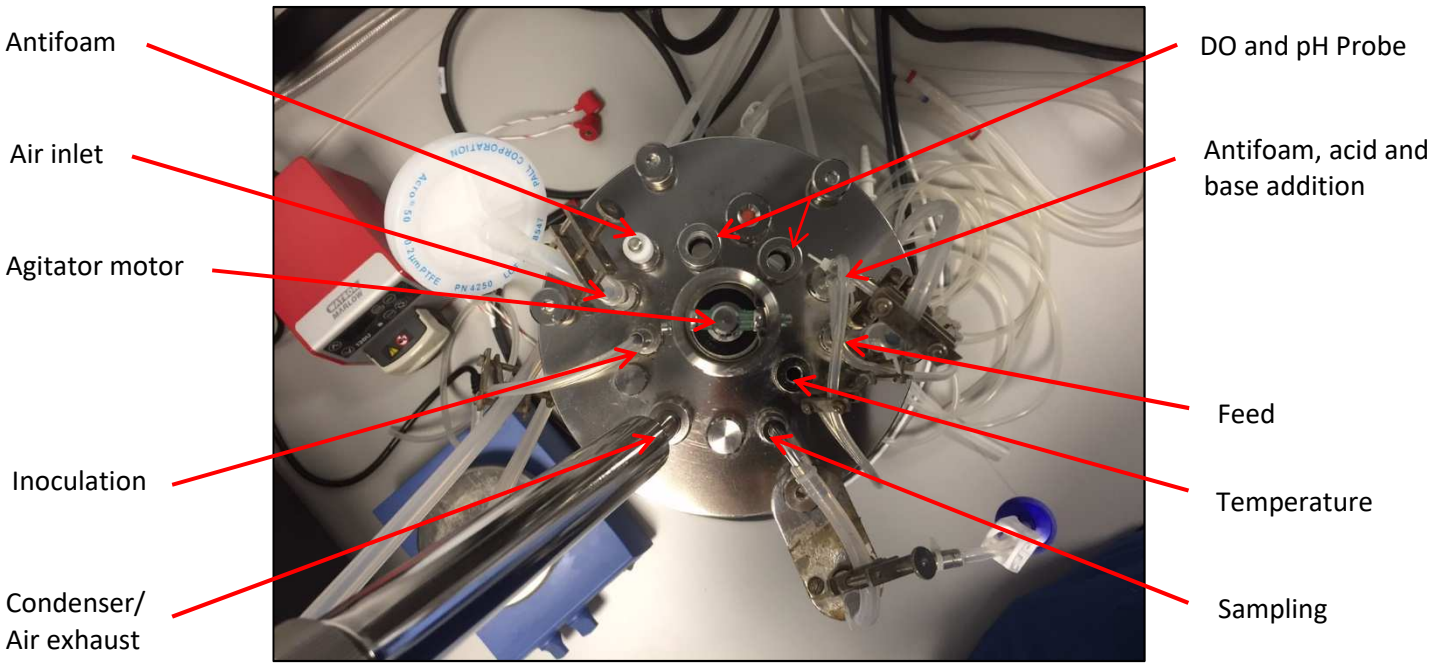


Figure 74: Top plate layout showing ports and lines.



Figure 75: Dedicated chiller unit.