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Towards the bioproduction of methyl methacrylate: solving the problem of product toxicity

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Abstract

Methyl methacrylate (MMA) and its derivatives are currently produced using methods which rely on crude oil resources and natural gas as feedstocks. A novel and sustainable route to the precursor methacrylic acid (MAA), has recently been developed. This method involves the production of MAA from renewable feedstocks *via* microbial fermentation. Unfortunately, the toxicity of MAA is a significant issue, inhibiting the growth of potential host biocatalysts at concentrations as low as 10mM. This ultimately limits production titres and reduces the economic viability of the process. Therefore, *in situ* product removal (ISPR) *via* liquid-liquid extraction has been investigated in order to reduce the concentration of MAA in the aqueous phase, preventing inhibition of the biocatalyst during its production.

Twenty-two water-immiscible organic solvents were tested for biocompatibility with *Escherichia coli* MG1655 and *Saccharomyces cerevisiae* DSM70449, the majority of which were toxic towards both organisms. Surprisingly, *E. coli* demonstrated a significantly higher tolerance towards the solvents, particularly when grown in minimal medium. Unfortunately, the biocompatible solvents demonstrated extremely poor extraction efficiencies of between 9 – 50 % MAA, from aqueous systems. Ionic liquids (ILs) were therefore investigated as potential replacement solvents for the ISPR of MAA. Twenty-two ILs were synthesised and tested for biocompatibility with *E. coli* and *S. cerevisiae*. Seventeen biocompatible ILs were identified and their physicochemical properties and extraction capabilities were evaluated. The ILs demonstrated extraction efficiencies superior to those of traditional organic solvents, extracting up to 93 % MAA from aqueous systems.

Alternatively, the direct bioproduction of alkyl methacrylate esters offers the added benefit of phase separation above a critical concentration, removing the need for an extraction solvent, and therefore their toxicity was assessed. However, the esters were found to have a higher toxicity than MAA towards *E. coli* and *S. cerevisiae*. Unexpectedly, an n-butyl methacrylate (BMA) tolerant *E. coli* mutant was discovered during toxicity measurements. Five BMA resistant strains were isolated and upon whole genome sequence analysis, mutations were found in three genes, *soxR, acrR* and *ybcO.* An improved BMA tolerance was found in the mutants containing both the *soxR* and *acrR* mutations. These findings offer the potential for the development of these strains to create a sustainable route to BMA.

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This thesis is dedicated to my late Grandparents

My Grandad's breadth of knowledge never ceased to amaze me

My Nana never stopped partying

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List of Abbreviations

μ	Specific growth rate of microorganisms
ρ	Density
η	Dynamic viscosity
ABC	ATP-binding cassette
ACH	Acetone cyanohydrin
ACN	Acetonitrile
ATP	Adenosine Triphosphate
BMA	Butyl methacrylate
CoA	Coenzyme A
COSMO-RS	Conductor like Screening Model for Real Solvents
D	Distribution ratio
E(%)	Percentage extraction efficiency
EDTA	Ethylenediaminetetraacetic acid
EMA	Ethyl methacrylate
ESI	Electrospray ionisation
gDNA	Genomic deoxyribonucleic acid
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HPLC	High performance liquid chromatography
HSP	Heat shock protein
IC	Ion chromatography
IC_{50}	Inhibitory concentration (50%)
ID	Internal diameter
IL	Ionic liquid
iPMA	Isopropyl methacrylate
ISPR	*In situ* product removal
KF	Karl Fischer method for water content analysis
LB	Luria Bertani medium

$LogP_{o/w}$	n-Octanol-water partition coefficient
Lpps	Lipoproteins
LPS	Lipopolysaccharide
Lrp	Leucine-responsive regulatory protein
MAA	Methacrylic acid
MaxOD	Maximum optical density
MDR	Multiple drug resistance protein
MIC	Minimum inhibitory concentration
MMA	Methyl methacrylate
MOPS	3-(*N*-morpholino)propanesulfonic acid
MS	Mass spectrometry
MSX	Minimal substrate medium
NMR	Nuclear magnetic resonance
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NP	Non-polar
OD	Optical density
pMMA	Poly(methyl methacrylate)
RID	Refractive index detector
RND	Resistance-Nodulation-Division
RTIL	Room temperature ionic liquid
SCFs	Supercritical fluids
scCO2	Supercritical carbon dioxide
SGR	Specific growth rate
TSIL	Task-specific ionic liquid
VOC	Volatile organic compound
WT	Wild type
YEPD	Yeast Extract Peptone Dextrose

1 Introduction

The biocatalytic production of industrially relevant chemicals from renewable substrates reduces the dependency on petrochemical feedstocks, from which many chemicals are currently produced.[1] Microbial fermentations, in particular, enable highly selective and atom efficient production of biofuels and other valuable chemicals from renewable feedstocks, using natural or engineered metabolic pathways.[2] By manipulating enzymes and microbial cells to carry out a particular function, desirable chemicals can be produced in a more economical way.[2–4] Several sustainable routes towards the bioproduction of methyl methacrylate (MMA) have recently been undergoing development.[5–7] These methods have focused on the production of methacrylic acid (MAA) and short chain methacrylate esters from renewable feedstocks *via* microbial fermentation. Unfortunately, accumulation of these products during fermentation can be toxic to the biocatalysts, inhibiting cell growth or resulting in cell death.[8] Inhibition from such chemicals typically limits product titres, affects fermentation performance and operational procedures, and profoundly impacts process economics.[9] The toxic products therefore need to be removed from the aqueous system before they can cause detrimental effects to the cells.

Perhaps the most appealing and simple way to remove toxic products from a microbial system would be the design of biphasic systems in which the toxic materials are extracted *in situ* into a water immiscible layer by means of liquid-liquid extraction.[10–12] Ultimately, production of the desired compound in the aqueous phase creates a shift in the chemical equilibrium of the system. However, the presence of a second phase enables the product to be transferred from the aqueous phase and thus the equilibrium is regenerated. Liquid-liquid extraction therefore presents higher capacity and increased selectivity, whilst allowing integration between recovery and purification.[13] In order for this to be a sustainable separation method, solvents which are biocompatible with the biocatalyst must be identified and implemented.

Conventional solvents have so far been focused on in order to create these two-phase systems, due to their high extraction capacities and ability to dissolve many compounds.[14,15] However, these are unfortunately considered highly toxic themselves, resulting in low production yields due to inhibition of the biocatalyst, making ideas for alternative extraction medium a necessity.[16–19]

Ionic liquids (ILs) have been suggested as promising alternatives for this process as they offer a number of desirable solvent properties including negligible vapour pressures and high solvating abilities.[20,21] The physicochemical properties of ILs can be tuned specifically to suit almost any biological system, whilst also providing reduced toxicities towards microorganisms and improved product recovery.[22] Therefore, the development of ILs as solvents for the *in situ* recovery of MAA has been evaluated. The aim was to explore the scope to prevent inhibition of the biocatalyst during MAA production. This would increase the possibility of improving future production titres and the overall economic viability of the process. A number of biocompatible ILs were identified and demonstrated significantly higher MAA extraction efficiencies compared to traditional organic solvents. The low toxicity and high extraction efficiency of many ILs provided proof of concept that ILs perform exceptionally compared to traditional solvents in this particular process. However, further development of the *Escherichia coli* host strain is necessary to produce higher titres of MAA in order for the process to be considered as economically viable.[6]

An alternative method to improve the sustainability of this process is to improve resistance of the biocatalyst to MMA and its analogues. The bioproduction of methacrylate esters, including methyl methacrylate (MMA), ethyl methacrylate (EMA), isopropyl methacrylate (iPMA) and n-butyl methacrylate (BMA), offer the benefit of phase separation above a critical concentration, removing the need for an extraction solvent. However, the biocatalysts must be able to tolerate high concentrations of the esters, if an economically viable process is to be produced. Therefore, the toxicities of alkyl

methacrylate esters towards *Escherichia coli* MG1655 and *Saccharomyces cerevisiae* DSM70449 were investigated. The esters were found to inhibit growth of the organisms at much lower concentrations than MAA. Surprisingly, during these tests a BMA resistant *E. coli* mutant was discovered and isolated. The mutant tolerated extremely high concentrations of BMA, which were previously completely inhibitory to *E. coli*. Therefore, the discovery and development of three BMA tolerant *E. coli* mutant strains has created the opportunity to develop a novel and sustainable route to BMA, which allows for simple product recovery through phase separation of the BMA. A patent is currently in preparation for this mutant strain and the BMA tolerance is being further improved.

2 Literature Review

2.1 Bio-based production of chemicals

The production of chemicals using sustainable biosynthetic chemistry, based on renewable resources, has been continually gaining interest throughout the chemical industry.[1] The majority of chemicals are currently produced from fossil-fuel derived resources, which has ultimately raised concerns with regards to environmental pollution and the depleting levels of crude oil.[1] Microorganisms can be used to produce high value chemicals *via* fermentation, rather than by chemical synthesis.[23] Recombinant DNA technology and synthetic metabolic engineering of organisms has allowed for the reconstruction of metabolic pathways towards the production of specific chemicals.[1] *Corynebacterium glutamicum* (*C.glutamicum*), *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) in particular have been highlighted as major industrial production organisms.[1] Production of compounds such as antibiotics, L-amino acids and short-chain alkanes at industrial scale *via* microbial fermentation are already developed processes.[24,25] More recently, bacteria as bio-production platforms have become a reliable and cost-competitive alternative for large scale industrial production of many organic acids, including lactic, acetic, itaconic and succinic acid (Figure 1).[23,26,27]

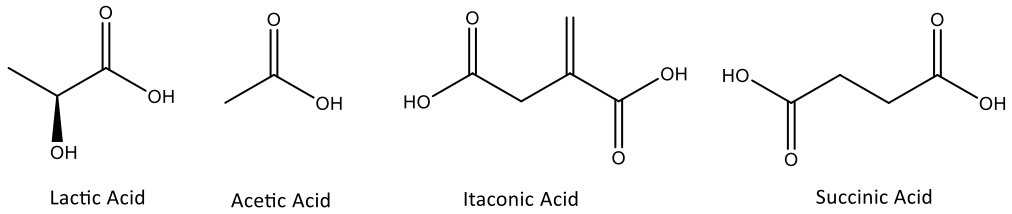


Figure 1. The chemical structures of industrially produced organic acids.

However, limitations have been known to include slow growth, high nutritional requirements, toxicity of metabolic intermediates and products, as

well as low product yields.[1] Therefore, although these methods offer many benefits over petrochemical production, the viability of each individual biosynthetic process must be assessed and these limitations minimized wherever possible.

2.2 The production of methyl methacrylate and methacrylic acid

2.2.1 Current industrial manufacture of methyl methacrylate and methacrylic acid

Methyl methacrylate (MMA, Figure 2) is a key monomer used in the production of polymethyl methacrylate (pMMA), more commonly known as Perspex®, Plexiglas® or Lucite®. The global consumption of pMMA is estimated at approximately 2.1 million tonnes per annum and current production is solely through chemical means, from unsustainable petrochemical sources.[28]

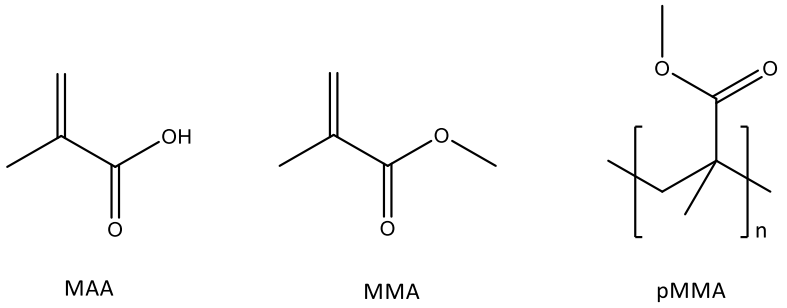
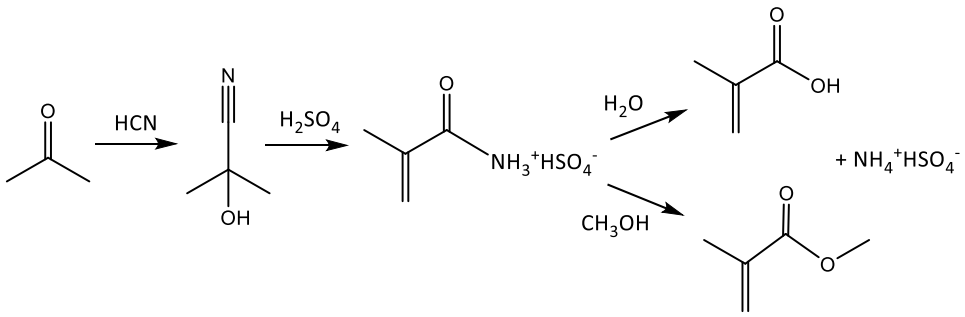


Figure 2. The chemical structures of methacrylic acid (MAA), methyl methacrylate (MMA) and poly(methyl methacrylate) (pMMA)

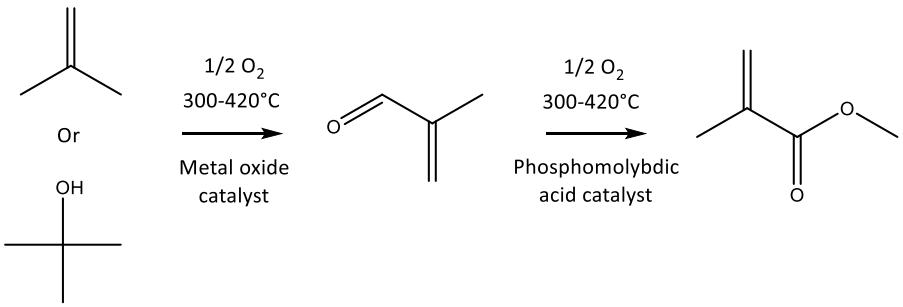
Current methods for the production of MMA include the acetone cyanohydrin (ACH) route and C_4 route.[29] The first commercial route to MMA and MAA was the ACH process (Scheme 1). This involves a reaction between acetone and hydrogen cyanide (HCN) in the presence of an excess of sulfuric acid to form methacrylamide sulfate. This intermediate is then treated with methanol to produce MMA and an equimolar quantity of ammonium bisulfate as a waste by-product. The process is dependent on the availability of HCN which, besides

being extremely toxic, is produced catalytically from ammonia, natural gas and air, and therefore increase in the cost of natural gas can cause this process to become uneconomical.[30] In addition to this, the process also requires large volumes of highly corrosive acid to obtain high yields of product, whilst also producing large amounts of industrial waste in the form of ammonium bisulfate. Sulfuric acid can be regenerated from this, however it is a costly process.[29]



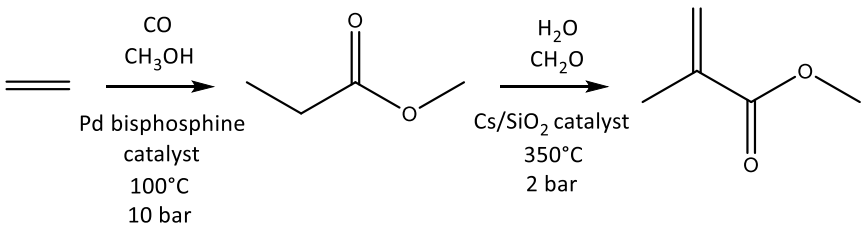
Scheme 1. The chemical steps involved in the production of MMA and MAA *via* the ACH process.

An alternative method of MAA production was developed in the 1980s and is known as the C_4 route (Scheme 2).[31] This process involves multi-step oxidation, using either isobutylene or tert-butyl alcohol as the feedstock, as, under the conditions of the first oxidation step, tert-butyl alcohol is readily converted to isobutylene. Isobutylene is then oxidised to methacrolein at high temperatures in the presence of a molybdenum, iron and bismuth catalyst.[31] A second oxidation step then follows, in the presence of a different catalyst typically composed of heavy transition metals such as vanadium and tungsten, whereby methacrolein is converted to MAA.[31] A number of complex catalysts are required for this process, which typically have limited lifetimes and poor selectivity.[31] This leads to the production of waste and unwanted byproducts. Also, the C4 route requires vast quantities of water which becomes contaminated with MAA, which ultimately effects the economic viability of the process.[31]



Scheme 2. The chemical steps and conditions involved in the production of MAA *via* the C4 route.

The Alpha process (Scheme 3), developed and patented by Lucite International, aimed to reduce the use of the previously used toxic materials, lower energy costs and remove scale limitations, which were a problem in the ACH and C4 routes.[29,32] The process involves the carbonylation and esterification of ethylene with carbon monoxide and methanol, to produce methyl propionate, with 100% selectivity, in the presence of a palladium bisphosphine catalyst. Methyl propionate is then further reacted with formaldehyde under anhydrous conditions in the presence of a silica supported caesium oxide catalyst to afford MMA.



Scheme 3. The chemical steps and conditions involved in the production of MMA *via* the Alpha process.

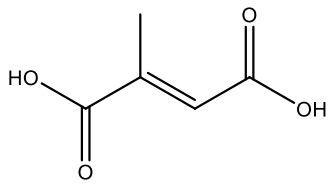
This process offers many advantages such as a reduction in the use of hazardous chemicals, much higher product selectivity, meaning lower by-product formation and waste, and a reduced reliance on crude oil derived feedstocks.[32] However, an issue remains in that the pricing of the feedstock is

still linked to the cost of gasoline. It is due to these factors that an alternative route to the production of MMA and its derivatives would be beneficial, the ideal being biological production from a sustainable feedstock using metabolically engineered microorganisms.

2.2.2 Towards the bioproduction of MAA, MMA and derivatives

Biotechnology and biocatalysis offer many advantages over chemical production, including the opportunity to produce chemicals from renewable feedstocks.[33] It can also minimize hazardous waste production, allow for processes to be carried out under significantly milder conditions, and lower the overall energy consumption of the process.[23] As there were no known natural metabolic pathways or enzymes that produce MAA, this has been the focal point of Lucite International. Their aim was to create a process in which MAA, MMA or other short-chain methacrylate esters would be produced by a microorganism during fermentation, leading to the development of a bio-synthetic route to its production. However, for the process to be economically sustainable, it is vital that the process is highly efficient, producing high concentrations of the bioproducts.

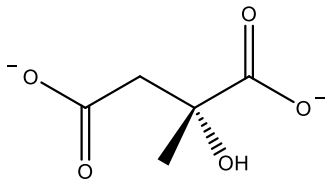
Lucite International have been exploring several different routes towards the bioproduction of MMA, which has been attempted *via* four different approaches.[5–7] The first involved the direct production of MAA within a microorganism through the decarboxylation of metabolic intermediates such as mesaconic acid, however this route was found to be unsuccessful and was therefore rejected.[5]



Mesaconic Acid

Figure 3. The chemical structure of Mesaconic acid.

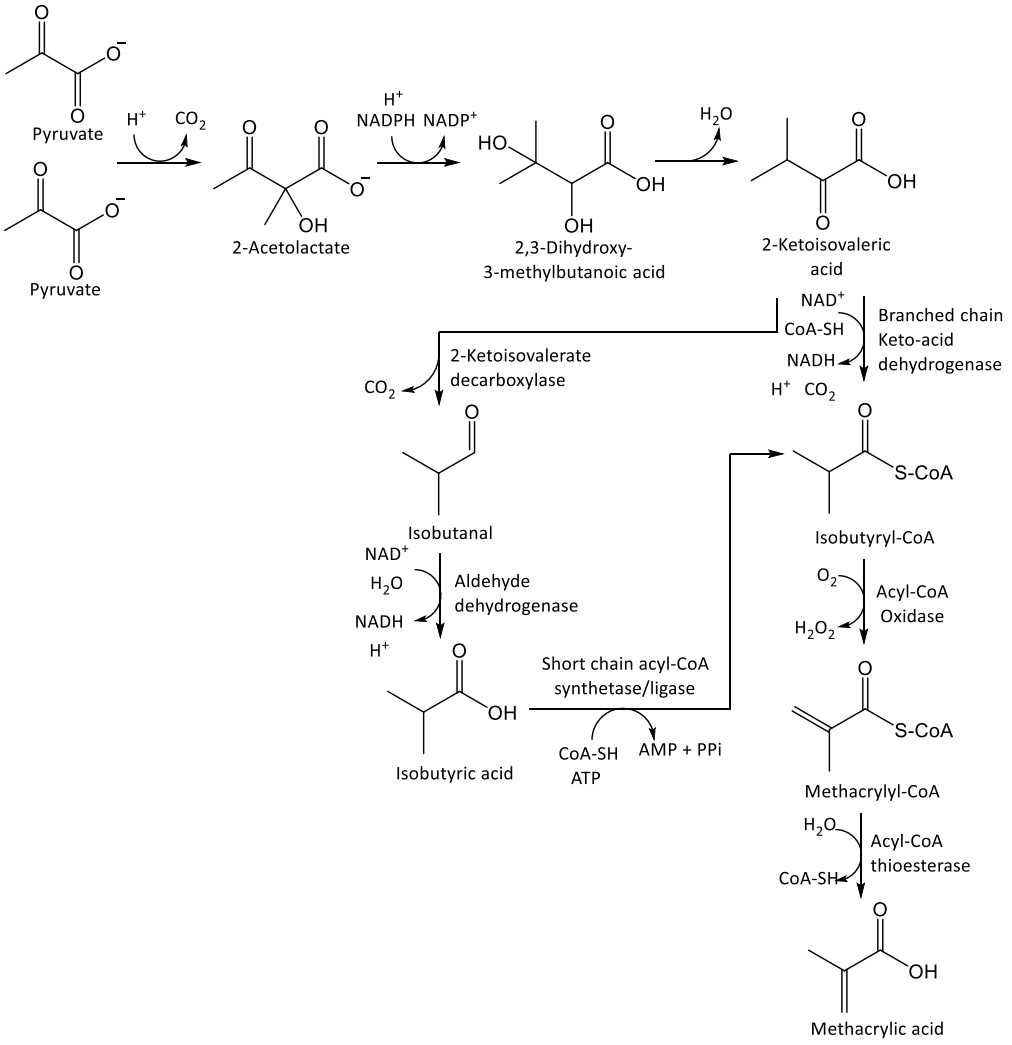
The second route involved the bioproduction of precursor molecules, using *E. coli* as the biocatalyst, which could be further converted to MAA in existing chemical processes. It focused on combined bio- and chemo-catalytic processes by isolating a pathway or enzyme capable of converting a renewable feedstock into *(R)*-citramalate (Figure 4).[7] However, this route produced increasing concentrations of citramalic acid, showing a conversion yield of just 70% and many unwanted side products. This process was therefore considered as uneconomical and was also rejected.[7]



(*R*)-citramalate

Figure 4. The chemical structure of (*R*)-citramalate.

The remaining two routes assessed the possibility of the direct production of MAA and BMA in *E. coli*. The MAA route focused on the whole cell biocatalytic production of MAA directly from glucose.[6] The pathway involved the production of pyruvate from glucose, followed by the conversion of 2-ketoisovaleric acid, a widely available intermediate in *E. coli*, to methacrylic acid, using a thioesterase to catalyse the hydrolysis of methacrylyl-CoA as the terminal metabolic step (Scheme 4).[6] This route was successful, using *E. coli* as the host cell, however the concentrations produced were fairly low. MAA was produced to titres of 0.37mM in the biotransformation of isobutyric acid, and 0.17mM directly from glucose.[6] The unproductive consumption of isobutyric acid as an intermediate was observed, which could be tackled *via* optimisation of enzyme expression levels.[6] However, the low concentrations produced also suggested that there were bottlenecks in the pathway which could potentially be due to the toxicity of MAA, highlighting the need to tackle the issue of MAA toxicity in order to improve the sustainability of the process.

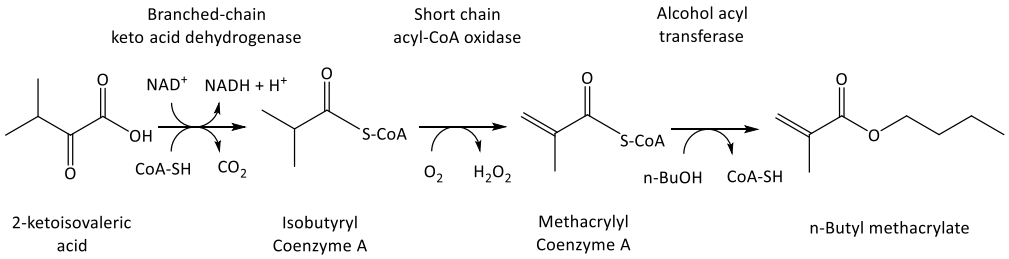


Scheme 4. The successful pathway for the production of MAA from glucose in *E.coli*

MAA toxicity is a significant problem, showing complete cell inhibition to *E. coli* strains at only 20mM concentrations, in previous studies.[7] This suggests that, for a viable microbial fermentation process focusing on the production of MAA, the acid must be removed from the system before inhibition of growth and productivity occurs. This may help improve the current production titres in the newly developed process.[6] It is therefore of great interest to develop continuous *in situ* liquid-liquid extraction systems in which the MAA will be removed into an organic extractant, as it is produced.

The final route investigated was the direct production of BMA using *E. coli* as the host microorganism. A novel, unpublished pathway for the direct

production of BMA has recently been developed, involving a 3-step transformation of 2-ketoisovaleric acid to produce n-BMA, using *E. coli* as the host microorganism (Scheme 5).[34]



Scheme 5. Unpublished pathway for the bioproduction of BMA in *E.coli*

The production of alkyl methacrylate esters, such as BMA, offers the advantage of phase separation from the fermentation broth at high concentrations, removing the need for an extraction solvent. Therefore, the toxicity of methacrylate esters needs to be established in order to evaluate whether the host biocatalyst will be able to tolerate a high enough concentration to create an economically viable process. Therefore, evaluation of these sustainable routes towards MAA and methacrylate esters is essential to understand which would be optimal for further development. The limitations of these processes, such as product toxicity, must be determined in order to assess the ways in which they can be overcome.

2.3 Toxicity of organic acids and solvents towards microorganisms

It is widely known that the accumulation of organic acids as fermentation products can be detrimental to the structure and function of many microorganisms proposed to serve as biorefining platform hosts.[4,35–37] In this *thesis,* two microorganisms in particular have been focused on with regards to their potential as biocatalysts for the production of MAA. The current pathway was developed in *E. coli* and therefore *E. coli* was the primary organism of

choice for the development of this process. However, as previously mentioned, *E. coli* has shown to be inhibited by MAA at low concentrations.[7] Therefore, it has been recently speculated that a more robust organism, such as *S. cerevisiae*, could offer the benefits of a higher tolerance towards low pH environments and potential bioproducts. Therefore, both *E. coli* and *S. cerevisiae* were chosen to be investigated as potential hosts for the bioproduction of MAA, MMA and derivatives.

2.3.1 Cell wall structure

2.3.1.1 Cell wall structure of *E. coli*

The many varieties of bacteria can be divided into two categories; Gram positive and Gram negative, as defined by an empirical staining method.[38] The key features which separate these two classes are their cell membrane structures. Gram negative bacteria are considered to have much higher resistance to biocides, as they are chemically more complex.[39] *E. coli* is a well-known example of a Gram negative bacterium. The vital inner components of these cells are protected from hostile environments by a cell wall which is composed of an outer membrane and a peptidoglycan layer that surrounds the inner cytoplasmic membrane. A schematic diagram of a Gram negative cell wall is shown in Figure 5.[40]

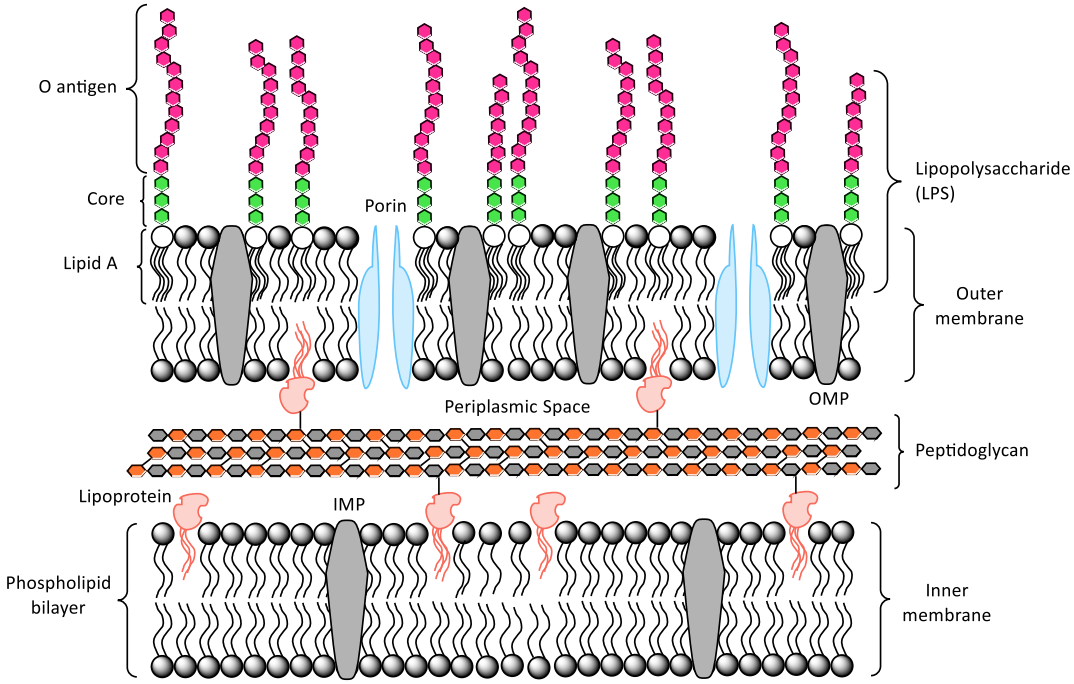


Figure 5. Schematic diagram of a Gram negative cell wall.

The figure was redrawn from information published by Needham and Trent.[40]

The outer membrane of Gram negative bacteria contains lipopolysaccharides (LPS), bound to the membrane by the LPS, lipid A, and several major structural proteins, known as lipoproteins (Lpps). A portion of the total lipoprotein present in the outer membrane is covalently bound to the peptidoglycan layer. The peptidoglycan layer is located in the periplasmic space between the inner and outer membranes of the cell. It is formed from alternating sugars of N-acetylglucosamine and N-acetylmuramic acid, connected by a β-(1,4)-glycosidic bond (Figure 6). Attached to each N-acetylmuramic acid molecule is a peptide chain composed of the amino acids L-alanine, D-alanine, D-glutamic acid and either lysine or diaminopimelic acid. Together, these molecules form sheet like structures, and each sheet is cross-linked *via* interpeptide bonds between the amino acid chains within the structure. The strong covalent linkage of the peptidoglycan layer provides mechanical strength to the cell envelope and regulates diffusion of molecules into cells.[41] It also acts to counteract the high osmotic pressure of the bacterial protoplast. If the

integrity of peptidoglycan is compromised, cells undergo immediate osmotic lysis.[41]

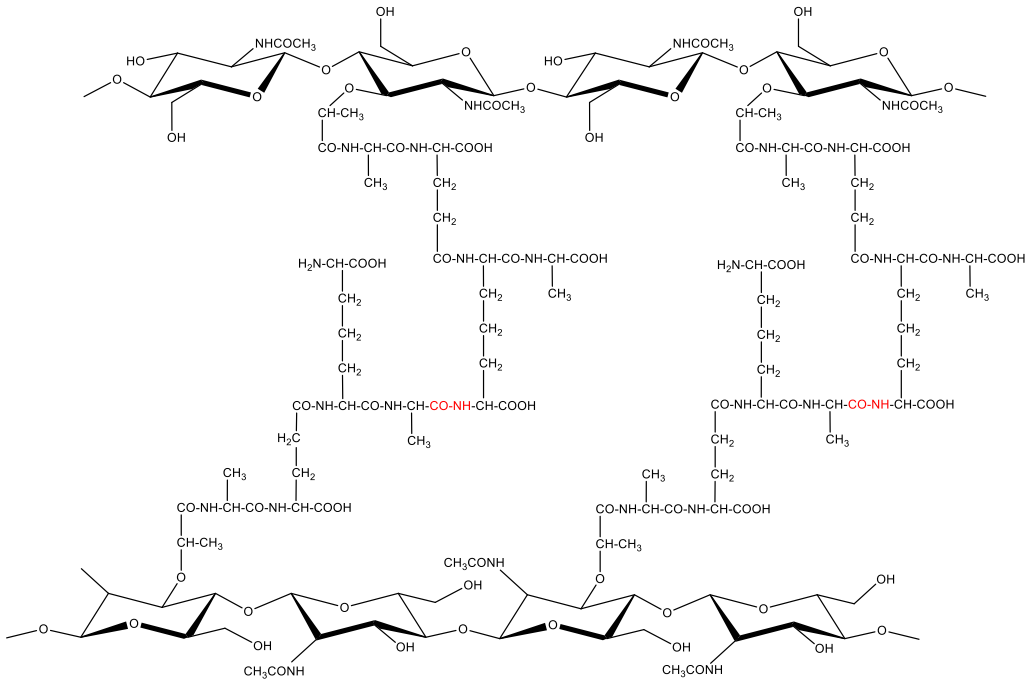


Figure 6. Chemical structure of the cross-linked peptidoglycan layer in *E. coli* cell walls, highlighting the interpeptide bond between diaminopimelic acid and D-alanine.

The inner cytoplasmic membrane is composed of phospholipid and protein (Figure 5). The phospholipid component of the membrane has self-organizational properties. Each phospholipid molecule has a polar hydrophilic head group attached to two hydrophobic fatty acid chains. This structure results in the formation of a bilayer composed of many phospholipid molecules arranged with the hydrophobic chains pointing inwards towards each other, whilst the hydrophilic head groups are exposed on each edge of the bilayer.[40] This orientation prevents polar solutes such as amino acids, carbohydrates and ions from diffusing across the membrane.

The second component of the inner membrane are membrane transport proteins. Integral proteins are responsible for the passage of water soluble molecule in and out of the cell, whereas peripheral proteins act as cell recognition sites.[40] The integral membrane transport proteins are associated

with either simple transport, group translocation or the ABC (ATP-binding cassette) system, the latter of which requires energy from ATP.[42] Integrity of the membrane is crucial to the survival of the cell and damage to the inner membrane can result in cell death.[17]

2.3.1.2 Cell wall structure of *S. cerevisiae*

The structure of a yeast cell wall is highly dynamic, changing constantly during cell division, growth and morphogenesis. It is composed of an outer layer, containing mannoproteins, and an inner layer, consisting of a network of polysaccharides including β-1,3-glucan, β-1,6-glucan and chitin.[43] Mannoproteins and β-glucan contribute to over 80% of the cell wall composition, whereas chitin represents less than 2%.[44] These components are covalently linked to one another, forming a strong network which prevents leakage of the mannoproteins from the cell and also protects the inner components of the cell from external stresses.[44] Mannoproteins are connected to β-1,6-glucan *via* a glycosylphosphatidylinositol anchor and, therefore, although β-1,6-glucan is a minor component of the cell wall, it plays an extremely important role in cellular homeostasis.[44] Glucan creates a supporting matrix for the mannoproteins, while the mannoproteins provide structural rigidity to the cell wall.[45] The β-1,3-glucan network spans the majority of the cell surface due to its branched structure, with all three components of β-glucan and chitin arranged in a crosslinked network. β-1,6-glucan and chitin are attached to the ends of β-1,3-glucan, and therefore chitin and β-1,6-glucan are attached *via* a β-1,3-linked glucose branch to the non-reducing terminal glucose of the β-1,3-glucan polysaccharide.[45] A schematic diagram of a yeast cell wall is shown in Figure 7.[46]

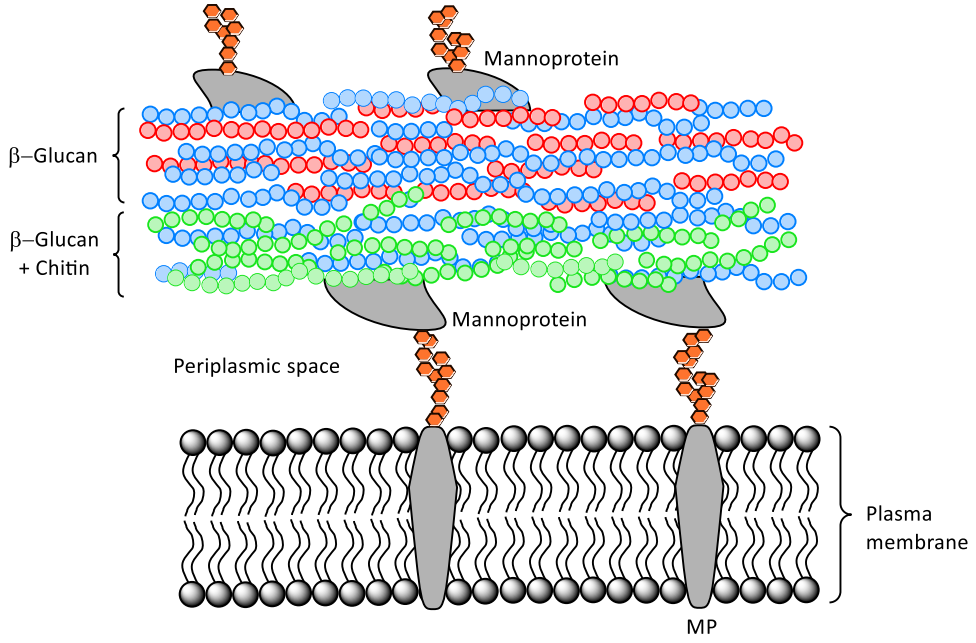


Figure 7. Schematic diagram of a yeast cell wall.

The figure was redrawn from information published by Cabib and Arroyo.[46]

Mannoproteins are glycoproteins containing 15-90 % mannose by weight. Their structure consists of a hydrophobic protein chain and hydrophilic mannose subunits. Cell wall mannoproteins include proteins, which do not have enzymatic activity.[47] Of the enzymatic mannoproteins, some are involved in cell wall construction and remodelling (hydrolase and *trans*-glycolase activity), and others are structural proteins.[48,49] The other component of the *S. cerevisiae* outer wall is chitin. Chitin is a long chain polymer of *N*-acetylglucosamine and is essential for cell survival due to its role in cell division, whilst also offering strength and support to the cell wall.[46]

The inner section of the yeast cell wall is a phospholipid membrane, which was previously described for Gram-negative bacterium. Similarly to an *E. coli* plasma membrane, it contains a mixture of polar lipids and proteins, which governs the membrane structure *via* the subsequent interactions. An *S. cerevisiae* membrane is enriched with three major lipid classes include glycerophospholipids, sphingolipids and sterols.[17] The sterol component of *S. cerevisiae* consists mainly of ergosterol. Ergosterol is a compact, rigid,

hydrophobic molecule which creates stability and strength within the cell membrane and is a major and vital lipid component.[17] Furthermore, the sphingolipid components are believed to protect the cell surface from harmful environmental factors. Sterols are not present in bacterial membranes; hence it is believed that yeast cell walls may be more resistant towards solvents due to the presence of these rigid, hydrophobic membrane components.[50]

2.3.2 Organic acid toxicity

The pH of an MAA solution determines whether the acid (undissociated) or the conjugate base (dissociated) is present. The two species will interact with cells *via* different mechanisms, hence changing the pH of the solution may change the toxicity of the MAA (pKa 4.66) product. In a low pH solution (pH < pKa), the majority of the acid will be in its undissociated form. Undissociated acids are able to pass freely through the outer and plasma membranes of *E. coli* and other organisms.[35] Upon entry into the alkaline cytoplasm, the acids dissociate into a proton and the conjugate base, in this case the methacrylate anion.[35] The decrease in internal pH (pH_i) and the presence of the conjugate base inhibit various aspects of metabolism, leading to impaired cellular growth.[51,52] Higher concentrations of the anionic conjugate base results in an increase in the transport of K^+ ions into the cell, which subsequently increases the turgor pressure.[53] In order to maintain a constant turgor pressure and cell volume, glutamate is transported out of the cells.[54] Glutamate is the most dominant anionic species in *E. coli*.[54] It is a vital amino acid used in the biosynthesis of proteins in cells, therefore its displacement from the cell results in severe growth inhibition. A reduction of glutamate levels in *E. coli* also inhibits the glutamate-dependant decarboxylase system, which acts to consume excess protons in the cytoplasm.[55] Therefore, this system would be defective, thus, cell death may occur due if the pH_i became too low. This response, in combination with the disruption of other vital cellular processes, make organic acids highly toxic towards microorganisms.

At neutral pH the majority of organic acids dissociate into their conjugate bases, depending on the pKa of the acid. Anionic species, such as the dissociated methacrylate anions, are only taken into cells by secondary transport.[35] This mechanism is known to involve H^+/monocarboxylic acid symporters, however, the specific mechanism is unknown. The accumulation of excess protons within the cytoplasm can also damage both RNA and DNA, whilst simultaneously altering enzymatic activities.[56] Therefore, production of the acid at a neutral pH would be beneficial as the toxicity of the dissociated acid would, in theory, be much lower.

Although the effects of anions on the growth of microorganisms are not well characterized, a small number of reports have been published discussing their specific toxicities. The enzymes involved in protein synthesis are sensitive to a combination of two unrelated mechanisms; the decrease in pH_i and the formation of an anionic pool.[35,37,56] During the fermentative production of acetate and formate by *E. coli*, a number of proteins were either induced or repressed, however very few proteins responded in the same way to both acids.[57] For example, RpoS regulon, DNA binding proteins and extreme-acid periplasmic chaperones exhibited increased expression in the presence of extracellular acetate. However, in the presence of formate, the expression of the proteins was repressed. This pattern of responses indicated that the major effects of acetate or formate were specific to each anion. Thus, a metabolic switch converting the cell between two different growth states was suggested, one preferred in the presence of each organic acid. The inhibition of specific intracellular enzyme activities by specific acid anions has also been reported, where the inhibition of phosphofructokinase by benzoic acid was observed.[58] In a similar study, acetic acid inhibited enolase in *S. cerevisiae* cells, further supporting the theory that organic acids can involve multiple mechanisms other than classical membrane uncoupling.[59] Specific inhibitory effects due to the methacrylate anion have been reported.[60] MAA, as a Micheal acceptor, is toxic to cells due to its ability to undergo Michael addition reactions with thiols, including proteins and glutathione, across the double bond of the MAA

(Figure 8).[60] This process inhibits enzymes, particularly DNA polymerase, whilst also disrupting other cellular mechanisms, such as the oxidative stress response, which is essential for organisms dealing with toxic substrates.[61]

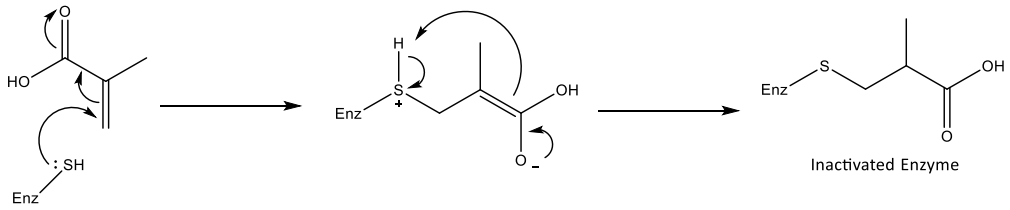


Figure 8. The mechanism of inactivation of an enzyme *via* Michael addition across the double bond of MAA.

Clearly the inhibitory effects of MAA on microorganisms will cause problems during its production, particularly if the undissociated acid is to be produced. Removal of MAA from the fermentation broth would be essential to minimize inhibition of the biocatalyst, ultimately increasing production. Therefore, liquid-liquid extraction using organic solvents offers the simplest method for ISPR of MAA.

2.3.3 Organic solvent toxicity

In biotechnology, solvents generally enable and facilitate product recovery from aqueous medium, and therefore are an essential component of many bioprocesses. An ideal solvent for biphasic whole-cell transformations should neither interfere with the bacterial cell functions nor inhibit growth. Unfortunately, many organic solvents do have detrimental effects on cell function and integrity, in some cases, inhibiting growth entirely.[62]

Solvents have been reported to affect cells in a number of ways. Generally, solvents accumulate in and disrupt the bacterial cell membrane, affecting the structural and functional integrity of the cells.[63] They impart biophysical changes to cell membranes, thus affecting vital membrane processes such as energy generation and transport.[17] Furthermore, solvents can damage and

denature biological molecules. These can include unfolding of proteins, DNA and lipid damage by oxidative mechanisms and RNA unfolding and degradation.[17] These effects may elicit severe stress responses and, in many cases, cell death.[17] However, this degree of sensitivity to a particular solvent is highly dependent on the bacterial cell structure.[64,65] Gram positive bacteria tend to be more solvent sensitive than Gram negative owing to a much different, and less complex, cell surface composition, as was discussed previously.[65,66] *E. coli* has been a focal point with respect to the choice of bacteria for toxicity testing, as it is frequently used as a host strain for the expression of industrial enzymes and also in metabolic engineering.[67,68] Therefore, results can be rapidly implemented into industrial biocatalysis making it a viable choice for this particular area of research.

Many studies have been carried out in order to determine the mechanism of toxicity solvents when in contact with various microorganisms.[63,65,69] These compounds having increased lipophilic character, and therefore interactions with hydrophobic regions of cells plays an important role in the mechanism of toxicity.[65] Lipophilicity is a physicochemical feature of a compound which is dependent on a number of characteristics such as polarity, molecular surface area, volume and structure.[70] Generally, in the presence of hydrophobic organic solvents, bacteria show rapid cell death and loss of selective permeability.[65] Alcohols provide a strong insight into the interactions of solvents with lipid membranes showing the disruption of lipoprotein membranes with the release of water-soluble proteins.[71] They penetrate and accumulate in the hydrocarbon region of phospholipid membranes and disorganise the lipid structures.[72] This results in expansion of the membrane, in a similar way to anaesthetics, causing the cells to lyse.[72] This is generally accepted to be the mode of toxicity for the majority of organic solvents.

Many studies have shown that bacterial sensitivity to solvents is highly dependent on the solvents $\log P_{o/w}$ value.[65,73,74] This parameter is defined as the logarithm of the partition coefficient of a particular solvent between a standardized 1:1 mixture of 1- octanol and water and is directly related with

the possible accumulation of a chemical within a microorganism as water-saturated 1-octanol is considered as a realistic model of the physicochemical environment in living organisms. In general, with increasing $\log P_{o/w}$ value there is a decrease in the resultant toxicity, most likely due to the reduced concentration of solvent which would be dissolved in the aqueous layer.[69] Although organic solvents with $\log P_{o/w}$ values ranging from 1-4 (e.g. toluene and n-heptane) would be ideal with respect to their use as extractants for MAA, they are unfortunately toxic towards the majority of microorganisms involved in biocatalytic processes.[19,65] Generally, solvents with $\log P_{o/w}$ values of less than 1 are hydrophilic in character which results in their prevention from entering cell membranes. Whereas, compounds with $\log P_{o/w}$ values of over 4 exhibit extremely poor water solubility and are therefore not considered as bio-available, essentially resulting in little or no toxic effects.[75] Within this range however, solvents are generally toxic. Lipophilic solvents are known to accumulate in the cytoplasmic membrane of bacteria, disrupting the cell membrane structure.[62] They do this by expanding and disorganising the phospholipid layer, essentially allowing the leakage of cytoplasmic components from within the cell, generally resulting in cell lysis and death.[65] It has been found that solvent toxicity depends on both the inherent toxicity of the compound and the intrinsic tolerance of the bacterial strains.[76] For example, particular strains of *E. coli* are tolerant to cyclohexane, whereas others are sensitive.[76]

The strength of growth inhibition of a given solvent is generally correlated with its polarity; however, it is not determined by the solvent polarity alone.[75] One study reported that solvents with $\log P_{o/w}$ values greater than 4.9 did not affect the growth of *E. coli* or *P. putida*, but those with $\log P_{o/w}$ value of less than 4.5 lowered the overall relative amount of growth.[75] Some solvents, such as ethyl benzoate and octene have been reported as being extremely toxic to bacteria irrespective of their $\log P_{o/w}$ values.[66] Therefore, toxic effects can be attributed to various other factors besides the partition coefficient of the solvents; most likely structural variations and specific functionalities.[66] One study compared

the growth of *E. coli* in the presence of methylcyclohexane and octene, which both maintain a $\log P_{o/w}$ value of 3.7. Interestingly, methylcyclohexane allowed full growth whereas octene did not.[66] This suggested that it is not the just the polarity of the organic solvent that determine the bacterial cell growth, but also the chemical characteristics.[77–79]

2.3.4 Solvent tolerance mechanisms

Cells respond in a number of ways towards solvent effects. These responses include; the induction of stress responses, which engage specific components of the stress response system within the organism, and induction of detoxification mechanisms in order to metabolize the toxic chemical, or transport it out of the cell *via* molecular pumps.[79,80] Longer term adaptive responses include the alteration of biosynthetic and morphogenetic systems, which counteract solvent toxicity, such as alterations in cell membrane composition.[64,78]

2.3.4.1 Changes in cell membrane composition

In response to organic acid exposure, the changes in membrane fluidity are counteracted primarily through alteration of the phospholipid bilayer.[64] Cells are able to alter the lipid composition of their membranes to counteract the detrimental effects, which occur as a result of exposure to organic solvents between a particular $\log P_{o/w}$ range.[65,78,81] The known mechanisms involving modification of the cell envelope to increase cell membrane rigidity and decrease permeability include; *cis-trans* isomerisation of membrane fatty acids by *cis-trans* isomerase, decreased cell surface hydrophobicity, and changes in chemical composition/ratios of membrane lipids and proteins.[63,82]

Upon initial exposure, in order to counteract the increase in membrane fluidity, *P.putida* cells express the gene *cis-to-trans* isomerase, which is responsible for the isomerisation of *cis-to-trans* unsaturated fatty acids.[64,78] This conversion of the phospholipid structure from *cis* to *trans* produces a denser packing structure of the membrane.[83] Longer term adaptations, which

are typically seen as a response to elevated temperatures, have been reported in *E. coli* cells. An increase in the unsaturated fatty acid concentration of the cell membrane on exposure to ethanol, propanol and butanol, has been observed.[84] This same effect has also been seen in the presence of aromatic compounds.[81] In contrast, exposure to longer chain alcohols results in an increase in the saturated fatty acid content within the membrane, which increases the transition temperature of the membrane, thus reducing the overall fluidity.[84] *S. cerevisiae* has been reported to respond in a similar way to *E. coli* cells that had been exposed to short chain alcohols. Upon exposure to ethanol, *S. cerevisiae* cells increase the unsaturated fatty acids in the lipid composition.[85] The proposed model is that by increasing the concentration of unsaturated fatty acids to minimize solvent effects on the membrane, or by increasing the saturated fatty acids to restore order in the membrane, the membrane functions will be protected.[17]

Other cell membrane modifications include decreased cell surface hydrophobicity, and changes in chemical composition/ratios of membrane lipids and proteins.[82] The cell surface properties of solvent-tolerant mutants of *E. coli* K12, with regards to the alteration of membrane hydrophobicity, were studied.[82] A decrease in the hydrophobicity of the solvent tolerant cells, when compared to the parent strain, was observed, suggesting that this was likely due to an increase in LPS content.[82] The effect of ethanol on adapted *E. coli* cultures was also studied and it was found that the membranes of these cells were much more rigid to those of the parent strain. The ethanol adapted cells had a higher protein to lipid ratio in the cell membrane, which gave rise to the subsequent rigidity, resulting in improved solvent tolerance.[82]

S. cerevisiae cells are also known to alter the composition of fatty acids in the membrane in order to counteract detrimental solvent effects on membrane fluidity and function.[17] Yeast cell walls are considerably stronger and more complex than bacterial cell walls, and their ability to alter their cell wall composition has been reported.[50] The literature is much more limited for *S. cerevisiae*, with regards to the changes in membrane composition due to

solvent exposure. However, due to their strong cell walls consisting of a thick peptidoglycan layer and many structurally supportive sterols, they are thought to have a much greater tolerance to solvent stress.[17]

2.3.4.2 Efflux pumps

Efflux pumps are active transporters localised in the cytoplasm of many types of cells and are responsible for the extrusion of toxic substances from within the cell.[86] They consist of three components which span both inner and outer membranes. These include an inner membrane transporter which utilizes ATP to enable export, a periplasmic membrane fusion protein which generates a channel, and an outer membrane protein which facilitates efflux out of the cell.[17]

Gram negative organisms, such as *E. coli*, contain proteins of the multidrug efflux pump family (MDR), along with various other transporters, which present an important role in solvent tolerance.[87] Transporters such as these utilize energy (ATP or electrochemical (H^+/Na^+)) to transport chemicals against an external concentration gradient in an antiport mechanism. Ramos *et al.* have reported that the resistance cell nodulation family (RND) have the greatest involvement in solvent extrusion in *E. coli* cells, an example of which would be the AcrAB-TolC efflux pump.[64]

The AcrAB-TolC system consists of the inner membrane transporter, AcrB, which acts as a peristaltic pump, a membrane fusion protein spanning the periplasmic space, AcrA, and an outer membrane channel, TolC.[88] Tsukagoshi and Aono investigated the solvent resistance of Δ*acrAB* and Δ*tolC E. coli* mutants.[89] Both mutants were observed to have hypersensitivity to solvents including linear alkanes of chain length C_6-C_{10}. They suggested that the AcrAB-TolC complex likely extrudes solvents with a logPo/w in the range of 3.4 to 6.0 through a first-order reaction. The expression of genes which regulate the proteins composing the AcrAB-TolC efflux pump have been reported in a number of solvent tolerant *E. coli* mutants.[90] Transcriptional regulators *marR*, *marA*, *soxR* and *soxS* modulate the expression of *acrAB*. Aono *et al.* observed

an increase in tolerance towards cyclohexane due to overexpression of *marA* and *soxS*, thus increasing the production of AcrA and TolC and therefore the activity of the efflux pump.[91] Furthermore, White *et al.* observed a reduction in tolerance to hexane and cyclohexane on deletion of *acrAB*, further supporting the relationship between the *acrAB* operon and solvent tolerance.[92] Additionally, numerous publications have reported *marA* overexpression to improve solvent tolerance in *E. coli*.[90,92]

Other notable efflux pumps include the AcrEF and TolC efflux pump in *E. coli* and the Pdr12p transporter in *S. cerevisiae*. Pdr12p is a carboxylate ATP-binding cassette (ABC) transporter localized in the plasma membrane, which has been shown to be involved in resistance to several weak organic acids.[93] ABC transporters are proteins which utilize energy from ATP hydrolysis to transport substrates across cell membranes. It has been reported that this ABC transporter, which is localised in the membrane, has shown to be involved in the resistance of *S. cerevisiae* towards several organic acids.[94] The involvement of Pdr12p in C1-C7 monocarboxylic-acid resistance in *S. cerevisiae* cells has been reported.[93] Similarly, a relationship between Pdr12p and resistance of *S. cerevisiae* cells to organic acids obtained from the metabolism of various amino acids, including leucine, phenylalanine and tryptophan.[95] Overexpression of Pdr12 increases the tolerance of the cells to acids with longer C chain length, such as propionic acid (pKa 4.87), which has the same length chain as MAA, and levulinic acid (pKa 4.62), which also has a very similar pKa to that of MAA (pKa 4.66).[94] The activity of this transporter led to exhaustion of intracellular ATP. Therefore, the involvement of the Pdr12 transporter could offer an increased tolerance of *S. cerevisiae* towards MAA.

2.3.4.3 Chaperones (HSPs)

Molecular chaperones, also known as heat-shock proteins, are involved in processes including the synthesis, transport and folding of proteins, and their expression is known to be induced by stress factors such as toxic solvents and chemicals.[96,97] HSP's act alongside other proteins in order to respond to and

tolerate chemical stresses, however the mechanisms in which they work are not entirely understood. Under stress conditions, the role of major molecular chaperones is to prevent aggregation and also facilitate in the refolding of damaged proteins, generally using energy from ATP hydrolysis, although some HSPs' actions are ATP independent. An example of a chaperone which is upregulated in *E. coli* under solvent stress conditions where there is a lack of intracellular ATP is Hsp33. This chaperone acts to minimize protein aggregation, which under normal conditions would be the role of DnaK, however the lack of ATP due to oxidative stress causes it to become inactive.[98,99]

Cells can engage specialized proteins that initiate RNA synthesis, known as sigma factors, to upregulate a group of genes which are responsible for the expression of a particular protein acting as a repressor or activator, to help counteract cell damage due to solvent stress. The major regulator of general stress response in *E. coli* is sigma factor RpoS which has been investigated in great detail by Weber *et al*.[100] RpoS is responsible for the transcription of over 10% of the genes in *E. coli* and overlaps with many major regulons, including cAMP which is used for intracellular signal transduction, activates protein kinases and also binds to and regulates the function of ion channels.[55] RpoS also interacts with the transcription factor Lrp (Leucine-responsive regulatory protein), which is responsible for modulating a variety of metabolic functions, and therefore significantly impacts cell physiology under stress conditions.[100]

The stress response in *S. cerevisiae* is regulated by the heat-shock factor *hsf1*, which includes chaperones that are involved in solvent tolerance.[101] Quan *et al.* observed an accumulation of Hsp70 (DnaK) when cells were exposed to high concentrations of ethanol, suggesting that for solvent tolerance, protein repair is a necessary requirement.[102] The plasma membrane chaperone, Hsp30, and lipid binding protein Hsp12 have also shown to be induced under solvent, heat and oxidative stresses.[103,104] However, the exact mechanism of tolerance in *S. cerevisiae* is not yet known.[101]

There are increasing reports that glycerol, proline and trehalose, which are generally used as a means of protection against osmotic stress, could be involved in protection against solvents.[105] Trehalose biosynthesis in *E. coli* is controlled by the RpoS sigma factor and therefore it may also be linked to solvent tolerance. Ding *et al.*[101] reviewed their role in protecting yeasts against ethanol and other stresses and included the work of Shima and Takagi who observed an accumulation of trehalose and proline in yeast cells under solvent stress.[105] However, they remain to be fully investigated in the context of solvent toxicity.

Ultimately, despite the effective response mechanisms that organisms can use to tolerate and adapt to solvent exposure, solvent toxicity is likely to be an issue during the ISPR of MAA. Therefore, it is vital that we investigate alternative solvents in order to prevent inhibition of the biocatalyst during MAA production.

2.4 Ionic Liquids

2.4.1 Chemical Structure

Ionic liquids (ILs) are a class of non-molecular compound of which the majority are in the liquid state below a predefined temperature, usually accepted to be 100˚C. They consist of bulky, non-symmetrical organic cations combined with large, weakly coordinating anions, some common ones of which are shown in Figure 9. Asymmetry and large steric bulk reduces the coordinating ability of the two ionic components. These structural features, often coupled with charge delocalisation throughout the individual ions, act to reduce the lattice enthalpies (ΔH) of ILs. Such properties prevent efficient crystal packing of the ions, ensuring that the IL remains in the liquid state at low temperatures, as opposed to traditional salts which have melting temperatures of well over 600˚C. Room Temperature ILs (RTILs) are therefore considered as interesting candidates for a range of applications.

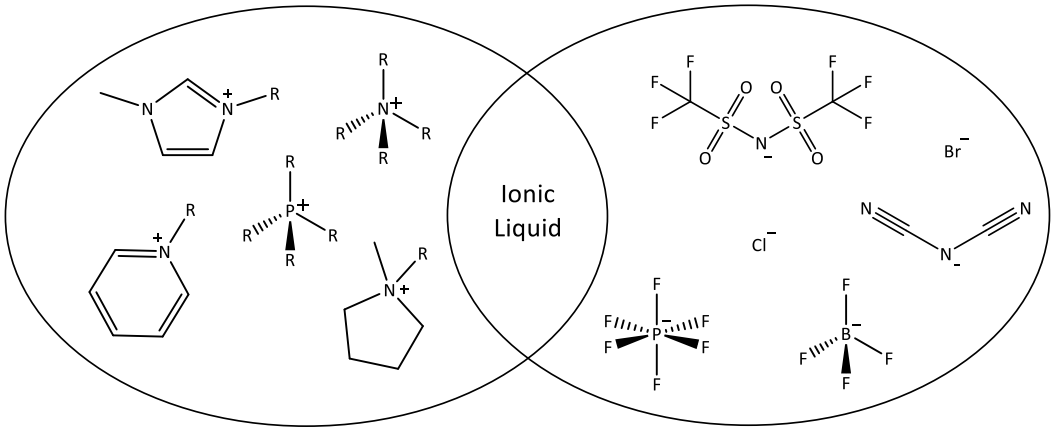
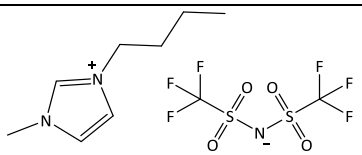
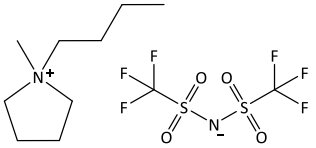
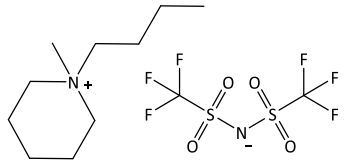
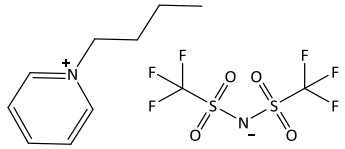
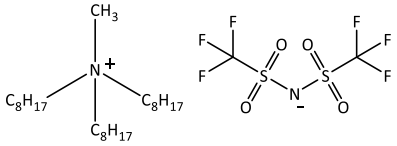
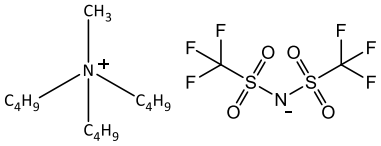
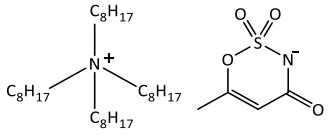
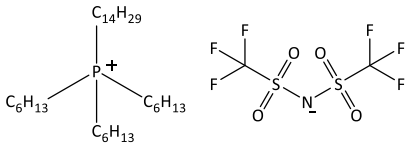
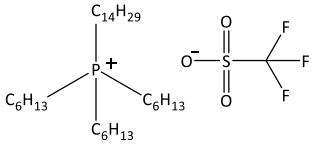
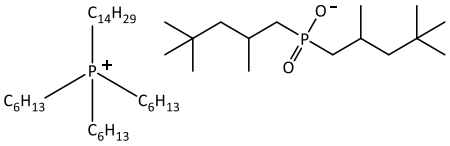
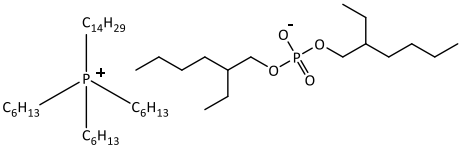


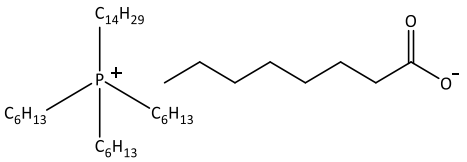
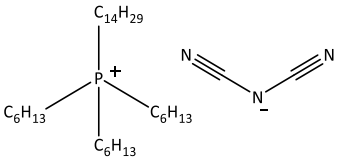
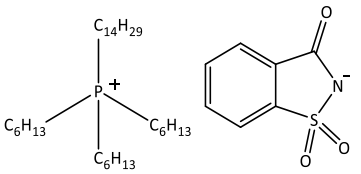
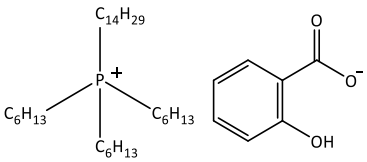
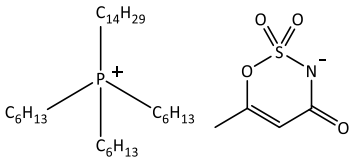
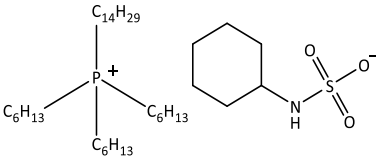
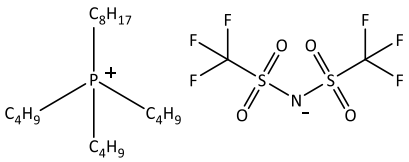
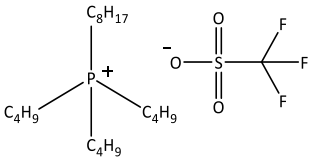
Figure 9. Chemical structures of a selection of common cation and anions in ionic liquids.

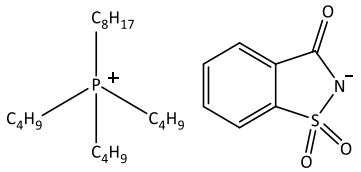
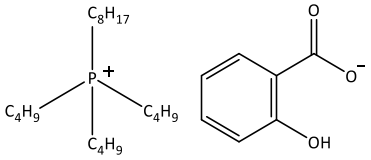
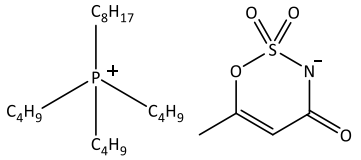
The reduced environmental impact of a number of ILs has lead them to be deemed as more environmentally benign than most conventional solvents, with potential benefits for sustainable chemistry, allowing them to be introduced into an ever expanding range of areas within the chemical industry.[106,107] In this thesis, a variety of ILs have been investigated, however there has been particular focus on phosphonium ILs, the structures of which are displayed below (Table 1).

Table 1. Synthesized IL structures and their common abbreviations.

Name	Abbreviation	Structure
1-n-Butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide	[BMIm][NTf_2]	
1-n-Butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide	[BMPyrr][NTf_2]	

1-n-Butyl-1-methylpiperidinium bis(trifluoromethane)sulfonimide	[BMPip][NTf_2]	
1-n-Butylpyridinium bis(trifluoromethane)sulfonimide	[BPyr][NTf_2]	
Trioctyl(methyl)ammonium bis(trifluoromethane)sulfonimide	[N_{8881}][NTf_2]	
Tributyl(methyl)ammonium bis(trifluoromethane)sulfonimide	[N_{4441}][NTf_2]	
Tetraoctylammonium Acesulfame	[N_{8888}][Ace]	
Trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide	[P_{66614}][NTf_2]	
Trihexyl(tetradecyl)phosphonium trifluoromethanesulfonate	[P_{66614}][OTf]	
Trihexyl(tetradecyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate	[P_{66614}][($^{i}C_8$)$_2PO_2$]	
Trihexyl(tetradecyl)phosphonium bis(2-ethylhexyl)phosphate	[P_{66614}][($^{i}C_8O$)$_2PO_2$]	

Trihexyl(tetradecyl)phosphonium octanoate	$[P_{66614}][C_7COO]$	
Trihexyl(tetradecyl)phosphonium dicyanamide	$[P_{66614}][DCA]$	
Trihexyl(tetradecyl)phosphonium saccharinate	$[P_{66614}][Sacch]$	
Trihexyl(tetradecyl)phosphonium salicylate	$[P_{66614}][Sal]$	
Trihexyl(tetradecyl)phosphonium acesulfame	$[P_{66614}][Ace]$	
Trihexyl(tetradecyl)phosphonium cyclamate	$[P_{66614}][Cyc]$	
Tributyl(octyl)phosphonium bis(trifluoromethane)sulfonimide	$[P_{4448}][NTf_2]$	
Tributyl(octyl)phosphonium trifluoromethanesulfonate	$[P_{4448}][OTf]$	

Tributyl(octyl)phosphonium saccharinate	$[P_{4448}][Sacch]$	
Tributyl(octyl)phosphonium salicylate	$[P_{4448}][Sal]$	
Tributyl(octyl)phosphonium acesulfame	$[P_{4448}][Ace]$	

2.4.2 Physicochemical properties

ILs offer a number of attractive physicochemical properties, including high thermal stability, wide liquid ranges, electrical conductivity, highly solvating and non-coordinating.[108] These properties have brought them attention with regards to their potential use as solvents, additives, electrolytes and lubricants.[109–111] The cationic and anionic moieties interact predominantly by strong coulombic interactions that are responsible for the low vapour pressures exhibited by ILs. ILs remain liquid, whereas VOCs vaporise, at high temperatures and therefore ILs are, in certain respects, considered in certain respects more suitable and potentially less hazardous than the highly flammable VOCs.

There has been considerable interest in using ILs as replacements for VOCs in large scale industrial processes.[112,113] ILs are known to be highly solvating, which has resulted in them playing an increasingly important role in various industrial applications, such as organic synthesis, biphasic catalysis, and liquid-liquid extraction.[114–117] Perhaps the most impressive property that ILs possess, which has earned them the accolade 'designer solvents', is their ability to be tuned very specifically in order to exhibit different physical properties, and

therefore, solvating abilities. By altering the combination of cation and anion or the functional groups within the ions, the hydrophobic/hydrophilic nature of the liquid can be manipulated for specific purposes.[114,117] Therefore, due to the large number of possible combinations, estimated at around 10^{18}, it is possible for ILs to be manipulated for use in an extensive variety of organic reaction, as both solvent and extraction medium.[21,118–120]

The potential for ILs to be tailored to possess unique physicochemical properties has sparked interest in their use in biocatalytic extraction processes.[22,121] The viability of replacing traditional solvents with ILs in multiphase extraction processes is dependent upon two main physicochemical characteristics; viscosity and miscibility with solvents, in particular water, which is necessary for the solubilisation of a range of chemicals and substrates required for biotransformations.

2.4.2.1 Viscosity

The viscosity of a liquid is caused by its internal friction, and it manifests itself externally as the resistance of the fluid to flow. To date, ILs have been treated under the class of Newtonian fluids, thus they have a constant viscosity irrespective of strain rate.[114] There are two types of viscosity; dynamic and kinematic. Dynamic viscosity (η) is defined as a measure of internal resistance and is measured in Pascal-seconds (Pa·s). Kinematic viscosity is based on the ratio of dynamic viscosity to the density of the liquid and is measured in Stokes (St).[122] When compared with more traditional solvents, ILs generally exhibit much higher viscosities, ranging between 10 and 1000 mPa·s.[114] For comparison purposes, at room temperature water and toluene maintain a viscosity of 1 mPa·s and 0.590 mPa·s, respectively.

The viscosity of ILs is strongly influenced by the chemical structure of the cation and anion.[114] For example, an IL composed of a cation coordinated with a large bulky anion, such as $[NTf_2]^-$, would greatly reduce the viscosity of the IL, when compared with a compact, high charge density anion such as $[Cl]^-$. The trend in increasing viscosity has been reported to not exactly correlate with a

decrease in anion size.[114] Previous work reported that the order of increasing viscosity, with respect to the anion was bis(trifluoromethylsulfonyl)amide, tetrafluoroborate, trifluoroacetate, triflate, heptafluoroethylsulfonate, heptafluoropropyl acetate, methyl acetate, mesylate, nonafluorobutylsulfonate. The importance of the effect of other anion properties which affect viscosity was suggested, such as their ability to form weak hydrogen bonds with the cation.[114]

With regards to the cation structure, highly asymmetric substitution within the cationic component has been identified as important to obtain low viscosities.[123] Furthermore, upon extension of the alkyl chain within the cationic moiety, a significant increase in viscosity is typically observed, which can be explained by the increase in Van der Waals interactions within the hydrophobic domains of the IL.[124]

The viscosity of ILs is known to be highly influenced by temperature, whereby an increase in temperature reduced the viscosity of the liquid. For example, the viscosity of [BMIm][PF_6] increases by 27% with a 5 K decrease in temperature from 298 to 293 K.[125] Impurities from improper purification techniques and poor synthetic procedures can have a significant influence of the viscosity of an IL. Seddon *et al.* reported how a residual halide content of between 1.5 - 6 wt.% raised the observed viscosity of the IL between 30 and 600%.[126] The influence of the addition of solutes and co-solvents on IL viscosity has also been investigated. Perry *et al.* observed a reduction in absolute viscosity by 50% on the addition of 5 wt.% acetonitrile or 15 wt.% benzene.[127,128] It should also be noted that a large number of ILs are hygroscopic and therefore absorb water from the atmosphere. This affects the interactions within the ILs, which influences their subsequent viscosities, even at very low concentrations of water.[126]

Ultimately, in two-phase extraction processes, IL viscosity is an issue. Low viscosity ILs must be utilized to ensure mass transfer of the solute is not limited and mixing and process costs are low. However, the presence of water in the

system will reduce the viscosity of ILs, which would help reduce these problems associated with IL viscosity in extraction processes.

2.4.2.2 Miscibility with Solvents

In order to determine phase behaviour in ILs, a knowledge of cohesive energies is essential. The strength or disruption of intramolecular forces determines whether the molecules stick together or move freely, which in turn affects the viscosity of the fluid.[129] It is believed that the more common intramolecular forces involved in ILs are coulombic interactions, Van der Waals forces and hydrogen bonding.[130,131]

Modifying the chemical structure of ILs can alter their miscibility with water and other solvents. Both the cationic and anionic moieties have a significant influence on the polarity of an IL, and therefore its miscibility with particular solvents. ILs are considered as highly polar, yet often weakly coordinating solvents. They are generally immiscible with nonpolar solvents, such as toluene, and miscible with polar, aprotic solvents such as DMSO, ACN and short-chain alcohols.[132–134] An IL can be designed to be either hydrophobic or hydrophilic in nature, simply by changing the combination of cation and anion. For example, an IL composed of a short chain imidazolium cation coordinated with $[EtOSO_3]^-$ would create an IL which was miscible with water and other polar solvents. However, by changing the anion to $[NTf_2]^-$, an entirely water immiscible IL is produced. Similarly, lipophilicity of the IL can be modified by the degree of cation substitution. By utilizing a large, bulky cation with long hydrophobic alkyl chains, such as $[P_{66614}]^+$, allows the coordination of the anion with more polar cations such as $[DCA]^-$ or $[EtOSO_3]^-$, whilst still producing a water immiscible IL. The role of water in ILs is complex and depends on the molecular structure of the IL. For example, water can modify the self-organizational patterns of ILs. Cammarata *et al*. found that water molecules bind to ILs *via* hydrogen bonding and they can form aggregates with anions of strong basicity such as $[NO_3]^-$ and $[CF_3CO_2]^-$. They stated that the strength

between water and anion increases in the order $[PF_6]^-$ < $[BF_4]^-$ < $[NTf_2]^-$ < $[CF_3SO_3]^-$ < $[NO_3]^-$ < $[CF_3CO_2]^-$.[135]

The main solvent features of ILs are their H-donor ability from the cation to polar solutes, H-bond accepting functionality of the anion, and π-π or C-H···π interactions, which subsequently enhance aromatic solubility.[114] The unique solvation properties of ILs have encouraged the investigation and development of them as alternatives to traditional organic solvents in liquid/liquid extractions.[110,136,137] Many reports have highlighted the use of hydrophobic ILs as possible replacements for VOCs in aqueous/IL biphasic separations.[138,139] Therefore, the knowledge of the mutual solubilities between water and ILs is of major significance prior to their consideration for extractive applications and concerning their practical point of view.

2.4.3 Application of ILs in bioprocessing

The potential application of ILs in biocatalytic processing as replacements for conventional solvents first gained interest in the 1990s.[140] The devastating impact of organic solvents and man-made chemicals on the environment, in terms of chemical processing and high energy demands, began to become apparent across industry and academia. New synthetic strategies which allowed for improved atom efficiency, reduced generation of waste materials and byproducts, and required less hazardous conditions, began to receive attention. ILs, however, were not the only next-generation solvents to gain interest; fluorous solvents, supercritical fluids (SCFs) and liquid polymers also offered beneficial properties over conventional solvents. However, these solvents have limitations with regards to their implementation in biocatalytic processes. Supercritical CO_2 ($scCO_2$) is the most widely used SCF in industry, as it is cheap and readily available. However, it is known to reduce or destroy the catalytic activity of an enzyme *via* the formation of carbamates on the enzyme surface, or the formation of carbonic acid in the presence of water.[141] Whereas, biocatalysis in fluorous solvents generally poses the problem of insolubility of enzymes and enzyme substrates within the solvent.[141]

Therefore, ILs in particular have gained an increasing amount interest over the past decade with regards to their potential use in the biochemical manufacturing industries. Their unique properties have caused them to be considered as greener alternatives to conventional solvents, gaining them attention as potential replacements as solvents in biocatalysis and biotransformations.[22,121,142]

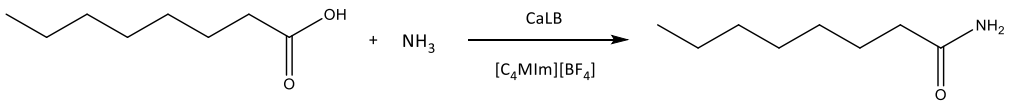
ILs possess a number of attractive physicochemical properties, as discussed previously, including low flammability and negligible vapour pressure, the latter of which creates a significant reduction in evaporation of the solvent into the atmosphere, when compared to conventional solvents. This lowers the risk of solvent leaching and subsequently reduces air pollution, creating safer working conditions. ILs also offer a wide liquid range which is particularly attractive in catalysis where a range of temperatures may be required. Additionally, ILs have the ability to dissolve a wide range of organic substances and can be specifically designed to carry out a particular task, thus are commonly known as task-specific ILs (TSILs). Due to the large number of potential cation and anion combinations, estimated at around 10^{18} possible combinations, ILs can be designed to possess unique physicochemical properties and toxicological profiles. These properties combined open up a huge range of possibilities for the utilization of ILs in bioprocessing. By combining biocatalysts and ILs to replace current chemical processes employing organic solvents, there is potential for improved yields, due to high selectivity and reduced number of reaction steps provided by microorganisms, and beneficial product recovery involving lower toxicity solvents with reduced environmental impact.

2.4.3.1 Enzymatic catalysis

Previous studies which found that enzymes performed superiorly in the absence of an aqueous environment and could catalyse particular reactions in solvents, prompted the investigation of their compatibility with ILs in chemical production processes. Enzymes are Nature's catalysts, offering low toxicity,

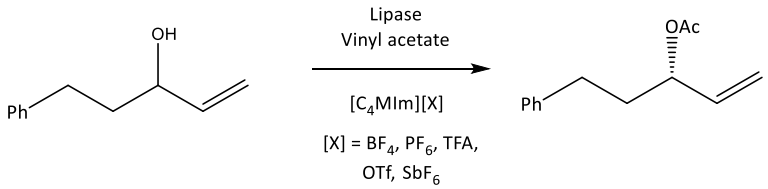
high biodegradability, improved stereo-selective and chemo-selective properties, and an ability to operate under mild conditions. Therefore, the combination of enzyme and IL to create a viable bioprocess became an appealing concept.

The first reported enzyme-catalysed reactions in a pure IL was reported by Lau *et al*.[143] They carried out the successful lipase-catalysed alcoholysis, ammoniolysis and perhydrolysis reactions in [BMIm][BF_4] and [BMIm][PF_6] using immobilized *Candida antarctica* lipase B (CaLB). The reaction rates were found to be comparable, faster or slower than those in organic solvents, whilst demonstrating conversions of 56-81 %, highlighting the potential of ILs as solvents in enzyme catalysis.



Scheme 6. The successful *Candida antarctica* lipase B catalysed ammoniolysis of octanoic acid in a pure ionic liquid.

Itoh *et al.* also used *Candida antarctica* lipase B in order to demonstrate the use of ILs in enantioselective reactions using enzymes.[144] Their work involved anchoring the enzyme to imidazolium based ILs, then carrying out the enantioselective acylation of allylic alcohols, which highlighted the potential recyclability of these systems.



Scheme 7. Lipase-catalysed transesterification in an ionic liquid solvent system.

Many different enzymes have shown potential biocompatibility with ILs. The stability of lipases, esterases and dehydrogenases have all been observed to have higher stabilities and activities in ILs as opposed to aqueous medium.[145] Yang *et al.* carried out further studies on enzymatic biocatalysis in ILs involving transesterification, perhydrolysis and ammonolysis reactions, which all showed an increase in the activity and stability of enzymes.[146] It is important to note that ILs have not always shown an improvement on enzymatic biocatalysis, as the high viscosities of ILs can create mass transfer limitations.[146]

Numerous reports have suggested the use of ILs in biphasic systems with $scCO_2$, utilizing $scCO_2$ to deliver substrates and extract products from the IL during enzymatic reactions. Lonzano *et al.* developed the continuous production of short-chain esters by the transesterification of alcohols and vinyl esters using a $[BMIm][NTf_2]/scCO_2$ system, catalysed by *Candida antarctica* lipase B.[147] They reported an increase in synthetic efficiency when using this system and also highlighted the significance with regards to ease of product recovery and catalyst recycling. ILs have been shown to extend the solvent range for biocatalysis in biphasic, as well as monophasic systems. The publications highlight the significant impact on selectivity, stability and activity by utilizing ILs and highlight a potential in many biological applications.

2.4.3.2 Whole-cell biocatalysis

The application of ILs in bioprocessing is a promising concept and many studies have reported successful improvements in yield, stability and activity in processes involving enzymatic catalysis, as previously discussed. However, the employment of isolated enzymes in organic synthesis is a costly process and requires stoichiometric amounts of cofactors such as NADH and NADPH.[148] Therefore, chemical transformations can be catalysed using whole-cells in an attempt to avoid this issue, as the cofactors are regenerated within the cells. However, an issue with the production of chemicals in whole-cell biocatalysis is poor substrate and product solubility in the aqueous medium, which a large

number of biocatalysts require for cell survival. Additionally, accumulation of the product in the aqueous phase can have significant detrimental effects on cell growth and maintenance.[149] In order to overcome these challenges, multiphase solvent extraction systems have been developed throughout industry and academia, using water immiscible organic solvents. The utilization of multiphase systems allows the removal of toxic products as they are produced, which is essential in order to minimize cell inhibition during production. Organic solvents are currently used as extraction media in bioprocesses, including liquid-liquid extraction of commercially valuable products such as antibiotics, and two-phase biotransformation reactions. As discussed previously, the use of these solvents is limited due to their high flammability and detrimental effects on cellular membranes, ultimately reducing cell viability. Therefore, hydrophobic ILs have also been suggested as alternatives to solvents in biocatalytic extraction processes, in an attempt to overcome these issues.[20,22,142,150]

The first known system which employed ILs in a biphasic system involving whole-cell biocatalysts was reported by Cull *et al*.[142] They demonstrated the use of [BMIm][PF_6] as a direct replacement for toluene in the process for the production of 3-cyanobenzamide by *Rhodococcus* R312. [BMIm][PF_6] successfully acted as a reservoir for the substrate 1,3-dicyanobenzene and as an extraction solvent for the product 3-cyanobenzamide, simultaneously. They observed an improvement of yield and selectivity on utilization of the IL, but also suggested an additional advantage of [BMIm][PF_6] in downstream processing due to the reduced cell aggregation at the phase interface, essentially making product recovery and solvent recycling easier. In a similar study, Sendovski *et al.* demonstrated the use of [BMIm][NTf_2] in the extractive fermentation of 2-phenylethanol from *S. cerevisiae* cultures.[151] The microbial metabolism of *S. cerevisiae* is usually inhibited by the presence of 2-phenylethanol and therefore productivities are generally extremely low. However, in the presence of the IL, Sendovski *et al.* reported a five times increase in production of 2-phenylethanol due to the *in situ* removal of the

product from the aqueous phase. Bräutigam *et al.* identified 12 ILs as suitable in the biphasic biocatalytic asymmetric reductions of 2-octanone and 4-chloroacetophenone, in recombinant *E. coli*.[150] They stated that $[PF_6]^-$ and $[NTf_2]^-$ ILs were particularly successful at acting as substrate reservoirs and for the *in situ* extraction of the products, resulting in yields of up to 95%. In a similar study, Dennewald *et al.* investigated the asymmetric reduction of 2-octanone to (*R*)-2-octanol in recombinant *E. coli* in biphasic/IL water systems.[148] They observed a conversation of 98.5% and an enantiomeric excess of 99.5% (*R*) in the presence of $[HMPyrr][NTf_2]$. They also reported that the IL could be reused 25 times without loss of productivity and without degradation of the solvent, highlighting the potential recyclability of ILs in biphasic fermentation systems.

Many studies have focused heavily on the *in situ* extractive fermentation of lactic acid.[152–154] Matsumoto *et al.* investigated the feasibility of replacing VOCs with imidazolium ILs for lactic acid extraction from an aqueous fermentation broth.[153] Their studies included both extraction ability and toxicity of the ILs towards the bacteria *Lactobacillus rhamnosus*, stating that alkyl chain length had little influence on both the extractability of the organic acids and the survival of the cells. They also observed extremely low extraction efficiencies by $[C_4MIm][PF_6]$, $[C_6MIm][PF_6]$ and $[C_8MIm][PF_6]$ in the absence of the extractant tri-*n*-butylphosphate, however, in its presence the efficiencies were comparable to those of toluene and hexane. Oliveira *et al.* investigated the extraction of L-lactic, L-malic and L-succinic acid, in an attempt to demonstrate the potential use of ILs for the extraction of bioproducts from fermentation broths.[155] They found that phosphonium-based hydrophobic ILs, in particular $[P_{66614}][(^iC_8)PO_2]$, had much higher extraction capacities for the organic acids when compared to traditional organic solvents.

To conclude, the application of ILs in biotechnological processes is an emerging technology. The extraction capabilities of ILs is most likely a combination of electrostatic and hydrophobic interactions between the product and the IL,

depending on the nature of the IL forming the two-phase system, and on the chemical properties of the target molecule.[156,157] The tunability of ILs is a highly promising characteristic of this class of solvents, offering them as having many advantages over traditional organic solvents in multiphase bioprocesses. However, IL toxicity towards biocatalysts presents a key limitation for implementation of ILs in these systems and it is essential that their individual toxicities are considered in order to design a viable biphasic whole-cell bioprocess.

2.4.3.3 Toxicity

By utilizing ILs as extraction solvents in order to minimize inhibition of the biocatalyst due to the accumulation of toxic substrates and products, the efficiency of the process can be vastly improved. However, the toxicity of the IL itself is important to consider. The toxicity of ILs is an important factor in their use in whole-cell biotransformations, as in order to recover product from the aqueous phase the IL will likely come into direct contact with the cells during mixing. Therefore, their toxicity profiles are important to consider when designing or selecting an IL to be used as an extraction solvent in a bioprocess.

ILs appear to close the gap between the polar and non-polar solvents that are suitable for microbial cells as, being composed of two parts, there is synthetic flexibility that is not available for a single component molecular solvent. This helps ensure that these natural catalysts can now be used over the entire polarity scale in order to increase the efficiency of many large-scale industrial fermentation processes.[124] ILs have the potential to be tailored for a specific purpose or to exhibit particular physical properties.[114] This includes the ability to alter the cation and anion combinations in order to reduce the toxicity of the IL.[67] This can be done by incorporating linkages, functional groups and altering the length of alkyl chains within the ions in order to create a more biocompatible solvent.[67,158,159] Unfortunately, the high biological and chemical stability of ILs mean that they possess a high potential for environmental persistence and bioaccumulation.[160] The need to implement them into

industrial processes has catalysed the need for further understanding of the toxicities of these compounds, particularly in environmental and biochemical companies which are continuously looking for low toxicity alternatives to conventional solvents.[161] Accurate prediction of toxicity depends on having a much better understanding of why particular IL structures are toxic or not. To date, extensive studies have been carried out looking into the environmental fate of ILs including factors such as biodegradability, bioaccumulation and eco-toxicity.[160,162,163] Many of these studies include investigation into the oxidative and thermal degradation of ILs in aqueous media in order to assess their persistence in the environment after use, prior to disposal.[162] Stepnowski and Zaleska and Morawski *et al*. showed that chemical degradation of imidazolium ILs can be achieved using a combination of UV light and a catalytic oxidant such as hydrogen peroxide.[164,165] It is generally in agreement that ILs containing longer alkyl chains are more readily degradable, however this usually leads to an increase in toxicity.[166,167] Therefore, further studies are necessary to assess the overall sustainability of ILs and find an appropriate balance between their toxicity and biodegradability.

Research on IL toxicity has been shown to be largely driven by both alkyl length and branching and also the hydrophobicity of the cation.[163,168,169] There has been substantial research carried out on the effects of imidazolium and pyridinium based ILs, the latter having previously shown to have significant toxic effects on a range of bacteria and fungi.[170] It has been suggested that these ILs, particularly structures with long exposed hydrophobic aliphatic chains, may interact and disrupt the phospholipid cell membranes, as demonstrated by the increased membrane/ water partition coefficients.[171] Similarly to VOCs, most toxicology studies have correlated the lipophilicity of IL ion-pairs with their biological effects, suggesting that the disruption of the plasma membrane does play a major role in IL toxicity.[171] It should however be noted that this opposes the trend of decreased toxicity with increased $\log P_{o/w}$ value shown by organic solvents, suggesting an alternative mechanism of toxicity.

The generally accepted mechanism of toxicity of ILs involves an interaction between the positively charged cationic moiety with the negatively charged phospholipid membrane. The exposed hydrophobic alkyl chain penetrates into the outer membrane of the cell, disrupting vital membrane function and results in cell lysis.[162] Investigations comparing monocationic ILs with their dicationic equivalents have recently been published, the research having been carried out with the anticipation that by blocking the hydrophobic chain, the toxicity of the IL would be significantly reduced. On insertion of a new dicationic head group, biocompatibility of ILs is significantly increased.[172] However, despite the reduction in toxicity of a dicationic IL, the specific chemical structure of the anion coordinated with these cations has been shown to induce a toxic effect.[173] A series of dicationic imidazolium ILs with a range of anions were investigated. The structural features that conferred hydrophobicity to ILs decreased the minimum inhibitive concentration (MIC) and IC_{50}, simultaneously.[173] Therefore, ILs of greater hydrophobicity, as a result of the chosen anion, had significant toxic effects towards cells.[173]

Despite the overly reported correlation between an increase in alkyl chain length and increase in toxicity, in very lipophilic compounds of over 14 carbon atoms in length, a cut-off in toxicity has been reported with various bacteria, suggesting a relationship between steric effects and a slow uptake of the substance by the cells.[174] This, however, could be questioned as to whether the compound is still liquid above 14 carbon atoms in length and if so are the ions acting individually as opposed to as a true IL. Anion effects on toxicity appear to vary substantially,[175] however it has been reported that highly fluorinated anions such as $[PF_6]^-$ and $[BF_4]^-$ exhibit much higher toxic effects due to hydrolytic instability.[171] In other cases, in studies involving imidazolium cations, anion effects have been shown to have very minimal to no effect at all on the antimicrobial activity.[168,171] Recent reports have shown pyrrolidinium cations to exhibit much lower toxic effects on bacteria than the well-studied aromatic imidazolium and pyridinium cations.[67] This could show a promising area of ionic liquids to look into when searching for biocompatible solvents as

generally these non-aromatic head groups have been found to be less toxic than their aromatic analogues.[171] The effect of aromaticity on the toxicity of ILs towards *Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna* has been reported.[176] The results suggested the possibility of designing ILs with enhanced hydrophobic character and lower toxicity, by elimination of their aromatic nature.[176] The toxic effects of phosphonium based ILs coordinated with various anions have also been investigated. Phosphonium halides were found to be the most toxic, but when exchanged with a different anion they caused fairly low bio-activity.[177]

Alternative investigations on IL toxicity to microorganisms have highlighted the effect of different functionalities within both the cation and anion. Wood *et al.* carried out an extensive study, testing over ninety ILs from diverse structural classes against *E. coli* in order to gain information on the inhibitory effects on the bacterial growth when in the presence of ILs.[67] They observed similar trends as already discussed with respect to alkyl chain length and aromaticity, however they also discovered that for water-miscible ILs, with the implementation of oxygen functionality into the cationic head group, forming ethers and alcohols, came a significant decrease in toxicity. Interestingly however, the opposite effect was observed for water-immiscible ILs, in that on the introduction of an alcohol group the toxicity significantly increased. They attributed this effect to the ability of a hydrophilic cation being combined with a toxic, hydrophobic anion, resulting in the anion essentially being "pulled" into the aqueous layer, effectively increasing the concentration of the anion in the medium creating a greater toxic effect.

Whole cells have been reported to tolerate water immiscible ILs to a greater extent than water miscible ones, however the effects have shown to be varied. Ganske and Bornscheuer observed that a second phase of [BMIm][PF_6] was entirely biocompatible with *Pichia pastoris*, inhibited *Bacillus cereus* by approximately 50% and completely inhibited the growth of *E. coli*.[178] Studies focusing on toxicity of ILs in whole-cell bioprocesses have been carried out on various microorganisms during the production of chemicals. Pfruender *et al.*

investigated the toxicity of [BMIm][PF_6], [BMIm][NTf_2] and [OMA][NTf_2] on whole cells of *Lactobacillus kefir* during the asymmetric reduction of chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol.[179] They observed an improvement in yield and enantioselectivity when using ILs as a second phase and membrane integrity was 10 fold higher in their presence when compared with several organic solvents, suggesting that the ILs did not partition into membranes like traditional solvents. They carried out further experiments with *S. cerevisiae* FasB and His6 and *E. coli* K12, both of which also demonstrated a higher membrane integrity in biphasic IL systems.[139]

Overall, the main conclusions drawn to date are that the toxicity of ILs is mainly governed by the cationic component, with aromatics corresponding to an increase in toxicological effects when compared with a non-aromatic equivalent. An additional increase in toxicity is also observed on extension of the substituted alkyl chain; however, the addition of ether or alcohol groups along the chain generally can reduce this effect. Furthermore, an aromatic ring with multiple nitrogen atoms leads to higher toxicity, however, in the case of imidazolium, methylation at the C2 position of the ring significantly decreases it.[180] The role of the anion in toxicity appears to have a more varied effect which may be due to its chemical structure, hydrophilicity and is typically organism specific.[177,181–183] Therefore, it is clear that the toxicity of each individual IL must be investigated in order to determine if they are appropriate as replacement solvents in a bioprocess, dependant on the biocatalyst.

3 Project Aims

This work forms part of a project developing a sustainable biosynthetic route to methyl methacrylate (MMA). The aim of this project was to solve the problems associated with product toxicity during the bioproduction of precursors and analogues of MMA. The first objective was to determine the toxicity of a range of potential bioproducts towards two biocatalysts of interest, *Escherichia coli* MG1655 and *Saccharomyces cerevisiae* DSM70449. The products of interest included methacrylic acid (MAA), methyl methacrylate (MMA), ethyl methacrylate (EMA), isopropyl methacrylate (iPMA) and *n*-butyl methacrylate (BMA).

Solving the toxicity issues associated with the bioproduction of MAA focused on *in situ* product removal (ISPR) via liquid-liquid extraction, in an attempt to reduce the concentration of MAA in the aqueous phase, preventing inhibition of the catalyst. Therefore, the aim was to test the viability of using organic solvents as potential extractants. Hence, the second objective was to assess the toxicities and MAA extraction capabilities of a variety of water-immiscible solvents. As there are many issues surrounding the sustainability of using large quantities of organic solvents in industrial processes, the next objective was to investigate ILs as alternative solvents for the extraction of MAA. This part of the project involved the synthesis of a range of water-immiscible ILs and testing their toxicities towards the biocatalysts with the aim of identifying a number of biocompatible ILs. Following this, the physicochemical properties, which may affect the extraction process, and the ability of the ILs to extract MAA from aqueous systems, was investigated. Thus, the overall aim was to evaluate their potential as alternative solvents for the bioproduction of MAA, with regards to replacing traditional solvents, to create a more sustainable process.

Solving the toxicity issues associated with production of the alkyl methacrylate esters, however, was focused on the improvement of solvent resistance of the biocatalysts. As the alkyl methacrylates offer desirable phase separating

properties at reasonably low concentrations, their bioproduction was also of interest. Therefore, the final objective was to determine which alkyl methacrylate was the least inhibitive towards the biocatalysts, and therefore has the potential for future development towards its bioproduction.

4 Materials and Methods

4.1 Analytical Methods

4.1.1 Biomass concentration

Optical density (OD) measurements were made at 600 nm with an Agilent 8453 spectrophotometer using polystyrene cuvettes (10 mm path length). Samples (1 mL) to be analysed were transferred to cuvettes and the OD measured. When the reading was outside of a range from 0-0.8, samples were diluted 1 in 10 in dH_2O until they were within the specified range.

4.1.2 DNA concentration

Prior to the submission of the BMA-tolerant mutant strains of *E. coli* for genomic sequencing, the extracted DNA concentration of each strain sample was determined using a Nanodrop ND1000 Spectrophotometer. All samples were confirmed at a concentration of > 50 ng/µL.

4.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse and purify DNA fragments isolated from the five mutant *E. coli* strains, prior to submission for genomic sequencing. A Green Range™ Horizontal Gel Electrophoresis Unit (Scie-Plas) was used and the gel was prepared with Tris-Acetate-EDTA (TAE) buffer (Bio-Rad) containing agarose (1 %). TAE buffer (1X) was prepared by adding 50X TAE buffer stock solution (20 mL) to distilled water (980 mL), and mixing. 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) in distilled water. Each component was added to the distilled water in the order stated.

Agarose-TAE gels were prepared by adding agarose (1 g) to TAE buffer (100 mL) and microwaving until the agarose was fully dissolved. The agarose-TAE was allowed to cool for 15 min then adding ethidium bromide (7 µL) before the solution began to set. The solution was thoroughly mixed and the gel was

cast in a gel-mould with a well-comb in place. The gel was allowed to set for 1h before being submerged in TAE buffer in the electrophoresis tank, and the well-comb was removed. DNA samples were mixed with 6X loading dye in a 5:1 volumetric ratio, and loaded into the wells. The agarose gel electrophoresis analyses were performed at 50-80 mV for 40-60 min. Gene Ruler^{TM} 1 Kb Plus DNA Ladder was also added as standard for each analysis and the DNA was visualised on the gel with a UV transilluminator.

4.1.4 Nuclear Magnetic Resonance

All ILs were analysed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy to identify common organic impurities, unreacted starting materials or the formation of unwanted byproducts (if >1 mol%). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker DPX 300 spectrometer, resonant frequencies 300.1, 75.5 and 121 MHz respectively, and all spectra were recorded at room temperature, 293 K. Chemical shifts (δ) are reported in parts per million (ppm) with reference to residual traces of protic solvents found in commercially available deuterated solvents. NMR solvents, protonated chloroform (δH 7.27) and (δC 77.00) and protonated dimethylsulfoxide (δH 2.50) and (δC 39.51). ^{31}P NMR spectra were referenced to an external H_3PO_4 signal (0.00 ppm). Coupling constants (*J*) are given in Hz.

4.1.5 Mass Spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) was recorded on a Bruker microTOF mass spectrometer with both positive and negative ionisation sources. Solutions were made using a sample (3 – 5 mg), serially diluted several times using analytical grade acetonitrile or methanol, dependent on solubility.

4.1.6 High Performance Liquid Chromatography

Methacrylic acid was detected and quantified using an Agilent 1200 Series HPLC System. The method was optimised starting from protocols already used within the group. The mobile phase was 100 % H_2SO_4 (0.01 M, 0.7 mL/min) and components were separated using an Aminex HPX-874 column (Bio-Rad)

at 60 °C. Detection was performed at 210 nm using a G1314B VWD (Variable Wavelength Detector) and by Refractive Index using a G1362A Detector at 35 °C. Samples (1 mL) were prepared by diluting aliquots taken from the MSX phase of the extraction systems with pure MSX, until the concentration of MAA fell within the calibrated concentration range. Aqueous samples (20 µL) were injected into the apparatus using an auto sampler, and data analysis was performed with ChemStation Software. Calibration for the analysis and quantification of MAA was performed using authentic standards at a concentration range between 0.1 and 20 mM (Appendix 12.2.1). Each sample was run in triplicate and MAA concentrations were calculated using the calibration curve. Each value is typically reported as an average of three experiments and injections, along with the standard deviations.

4.1.7 Ion chromatography

IC was used to measure halide content (detection limit ≈ 100ppm) left after IL synthesis. IC detection of anions was measured using a Dionex ICS-3000 equipped with a RFIL IonPac AS-20 (2 x 250 mm) analytical column and an AG-20 guard column (2 x 50 mm). Anion solutions were run using an isocratic elution solution consisting of 60% ultrapure water, 25 % MeCN and 15 % NaOH (100 mM) solution at a flow rate of 0.25 mL/min. Solutions for analysis were prepared by dissolving the sample (3 – 5 mg) in ultrapure H_2O (10 mL). Solutions (0.5 mL) were injected manually, using a 2 mL glass syringe. As the majority of the ILs tested were hydrophobic, samples were made up using an aqueous solution of HPLC grade ACN:H_2O (1:4 v/v). All ILs synthesised via metathesis reactions were determined as containing < 1.00 wt.% Cl^- or Br^-.

4.1.8 Karl-Fischer Analysis

Coulometric Karl-Fischer (KF) titration was measured using a Mitsubishi CA-100 moisture meter. The electrolytic solutions were provided by AQUAMICRON®. The anode solution was AQUAMICRON® AKX and the cathode solution was AQUAMICRON®CXU. Calibration was achieved using NIST traceable water standards. ILs were dried *in vacuo* (5 x 10^{-2} mbar) overnight at

40 – 60°C and the residual water content was determined as an average of three sequential titrations from a sample size between 0.05 and 0.20 g.

The water content of each IL was also determined after full saturation of the IL with MSX medium, containing no glucose. Saturation was achieved by rapidly stirring (500rpm) a biphasic mixture of the IL and MSX medium in a 40 mL Teflon-sealed glass vial for 24 h, at either 30 or 37°C. The ratio of IL/MSX was 20 %v/v, equating to volumes of MSX (8 mL) and IL (2 mL). The biphasic system was then allowed a 21-day separation period, at the respective temperature, in order to achieve full phase separation. The IL layer was then sampled (0.5 mL) using a glass syringe. The exact mass was calculated by weighing of the IL filled syringe, before and after injection, and the mass (g) was inputted into the KF immediately prior to the titration in order for the correct water concentration to be calculated for each IL at the respective temperatures.

4.1.9 Viscometry

Viscosities of ILs were measured using an Anton Paar AMVn falling ball viscometer. Viscosities were measured at both 30 and 37 °C using 3.0 mm and 4.0 mm internal diameter (ID) quartz tubes, with viscosity ranges of 20 – 230 mPa·s (3.0 mm) and 80 – 2500 mPa·s (4.0 mm). The viscosity was determined at an 80° angle, by taking six measurements at each temperature, and the dynamic viscosity was calculated as an average of these six readings. Each viscosity measurement was taken after full saturation of the IL with MSX medium, containing no glucose. Saturation was achieved by rapidly stirring (500 rpm) a biphasic mixture of the IL and MSX medium in a 40 mL Teflon-sealed glass vial for 24 h at either 30 or 37 °C. The ratio of IL/MSX was 20 %v/v, equating to volumes of MSX (8 mL) and IL (2 mL). The biphasic system was then allowed a 21-day separation period at the respective temperature, in order to achieve full phase separation. An aliquot of the IL layer was then drawn up into the relevant tube and its viscosity determined.

4.2 Reagents

All reagent chemicals and solvents were purchased from either Sigma-Aldrich, VWR International or Fisher Scientific and used without further purification. Methacrylic acid (MAA) and *n*-butyl methacrylate (BMA) were donated by Lucite International. Purity of all chemicals was between 96 and > 99 %.

4.3 Synthesis and Purification of ILs

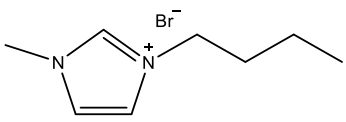
4.3.1 General

All IL synthetic procedures were carried out under an inert atmosphere of argon. All synthesised compounds were determined >99 % pure with regards to starting material remnants and solvent impurities before further use, as characterized by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Any residual solvents or reactants were removed *in vacuo* (5×10^{-2} mbar) prior to their use in further experiments. Ion Chromatography (IC) was carried out on all ILs to ensure complete anion exchange had occurred and no residual halide was present. All ILs contained < 1.00 wt.% halide (Cl^- or Br^-) as confirmed by IC. All ILs were prepared using modified literature procedures.

4.3.2 Synthesis and purification

4.3.2.1 Procedure for the synthesis of bromide ILs

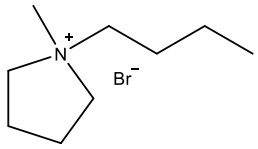
4.3.2.1.1 Synthesis of 1-n-butyl-3-methylimidazolium bromide[123]



A two-neck 100 mL round bottom flask equipped with reflux condenser and addition funnel was charged with 1-methylimidazole (15.0 g, 0.0183 mol) in toluene (30 mL). To the stirred solution, 1.2 molar equivalents of 1-n-bromobutane (30.0 g, 0.219 mol) in toluene (20 mL) was added dropwise. The

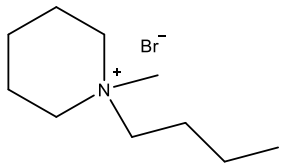
resulting mixture was heated and stirred at 40 °C for 24 h. The IL phase was separated and the crude product purified by recrystallisation in acetonitrile/ethyl acetate (≈1:3 v:v) and dried *in vacuo* (5 x 10^{-2} mbar) to afford 1-n-butyl-3-methylimidazolium bromide (26.5 g, 66 %) as a white crystalline solid. M.p.: 73 - 75 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δH (ppm) 9.41 (s, 1H) 7.91 - 7.78 (m, 2H) 4.20 (t, *J*=7.2 Hz, 2H) 3.88 (s, 3H) 1.82 - 1.68 (m, 2H) 1.30 - 1.15 (m, 2H) 0.91 - 0.80 (m, 3H) ^{13}C-NMR (75.4 MHz, DMSO-d_6) δC (ppm) 136.5, 128.2, 123.5, 122.2, 48.3, 35.7, 31.3, 18.7, 31.2. MS (+ve) m/z 139.12 [100% $C_8H_{15}N_2^+$] (calcd: 139.12).

4.3.2.1.2 Synthesis of 1-n-butyl-1-methylpyrrolidinium bromide[184]



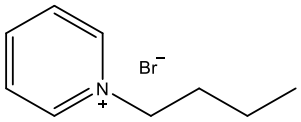
The same procedure used to prepare 1-n-butyl-3-methylimizadolium bromide was used to prepare 1-butylmethylpyrrolidinium bromide from 1-methylpyrrolidine (23.4 mL, 0.225 mol) and 1-n-bromobutane (29.1 mL, 0.27 mol) to afford a white crystalline solid (29.4 g, 66 %). M.p.: 214-215 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δH (ppm) 3.56 - 3.39 (m, 4H) 3.37 - 3.29 (m, 2H) 3.00 (s, 3H) 2.07 (s, 4H) 1.74 - 1.60 (m, 2H) 1.31 (dq, *J*=14.8, 7.38 Hz, 2H) 0.97 - 0.87 (m, 3H). ^{13}C-NMR (75.4 MHz, DMSO-d_6) δC (ppm) 63.3, 62.7, 47.4, 24.9, 21.0, 19.3, 13.5. MS (+ve) m/z 142.15 [100% $C_9H_{20}N^+$] (calcd: 142.16).

4.3.2.1.3 Synthesis of 1-n-butyl-1-methylpiperidinium bromide[184]



The same procedure used to prepare 1-n-butyl-3-methylimizadolium bromide was used to prepare 1-n-butyl-1-methylpiperidinium bromide from 1-methylpiperidine (25.7 mL, 0.212 mol) and 1-n-bromobutane (25.1 mL, 0.233 mol) to afford a white solid (29.7 g, 60 %). M.p.: 230 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δH (ppm) 3.38 - 3.25 (m, 6H) 3.00 (s, 3H) 1.85 - 1.71 (m, 4H) 1.70 - 1.45 (m, 4H) 1.31 (dq, *J*=14.7, 7.4 Hz, 2H) 0.98 - 0.87 (m, 3H). ^{13}C-NMR (75.4 MHz,$CDCl_3$) δC (ppm) 152.36, 64.07, 61.33, 47.48, 23.63, 20.62, 19.90, 19.47, 13.37. MS (+ve) m/z 156.17 [100% $C_{10}H_{22}N^+$] (calcd: 156.17).

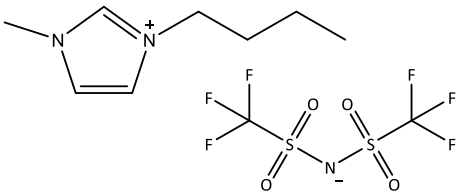
4.3.2.1.4 Synthesis of 1-n-butylpyridinium bromide[185]



A two-neck 250 mL round bottom flask equipped with reflux condenser was charged with 1.5 molar equivalents of 1-n-bromobutane (37.4 mL, 0.347 mol) in toluene (50 mL). To the stirred solution, pyridine (18.7 mL, 0.231 mol) was added dropwise *via* needle syringe. The resulting mixture was heated and stirred at 50 °C for 48 h. The pure product was isolated by vacuum filtration and dried *in vacuo* (5 x 10^{-2} mbar) to afford 1-n-butylpyridinium bromide (12.4 g, 25 %) as a white crystalline solid. M.p.: 104 °C. ^{1}H-NMR (300 MHz, ACN-d_3) δH (ppm) 8.72 (d, *J*=4.5 Hz, 2H), 8.50 (t, *J*=6.4 Hz, 2H), 8.03 (t, *J*=6.0 Hz, 1H), 4.52 (t, *J*=7.3 Hz, 2H), 2.01-1.86 (m, 2H), 1.45–1.27 (m, 2H), 0.98–0.90 ppm (t, *J*=7.5 Hz, 3H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 145.6, 144.8, 128.6, 62.3, 33.5, 19.3, 13.4. MS (+ve) m/z 136.11 [100% $C_9H_{14}N^+$] (calcd: 136.11).

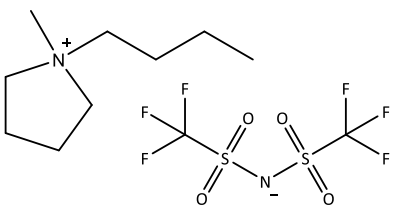
4.3.2.2 Procedure for the synthesis of bis(trifluoromethane)sulfonimide ILs

4.3.2.2.1 Synthesis of 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide[134]



A single neck 250 mL round bottom flask was charged with 1-n-butyl-3-methylimidazolium bromide (26.5 g, 0.121 mol) in water (50 mL). To the stirred solution, 1.1 molar equivalents of lithium bis(trifluoromethane)sulfonimide (38 g, 0.133 mol) in water (30 mL) was added dropwise. The resulting mixture was heated and stirred at 40 °C for 24 h. The IL phase was separated and washed with water (3 x 50 mL) and dried *in vacuo* (5 x 10^{-2} mbar) to afford 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (45.1 g, 87 %) as a pale clear orange liquid. ^{1}H-NMR (300 MHz, DMSO-d_6) δH (ppm) 9.10 (s, 1H) 7.71 (m, 2H) 4.16 (t, *J*=7.2 Hz, 2H) 3.85 (s, 3H) 1.85 - 1.68 (m, 2H) 1.36 - 1.18 (m, 2H) 0.95 - 0.83 (m, 3H) ^{13}C-NMR (75.4 MHz, DMSO-d_6) δC (ppm) 136.5, 123.6, 122.2, 121.6, 117.4, 48.5, 35.7, 31.4, 18.8, 13.2. MS (+ve) m/z 139.12 [100% $C_8H_{15}N_2^+$] (calcd: 139.12). Ion chromatography: Br^- : 0.08 wt.%.

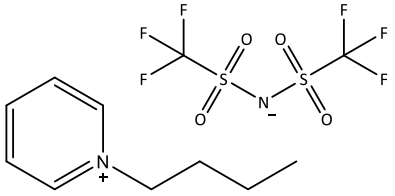
4.3.2.2.2 Synthesis of 1-n-butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide[184]



The same procedure used to prepare 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide was used to prepare 1-n-butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide from 1-n-butyl-1-

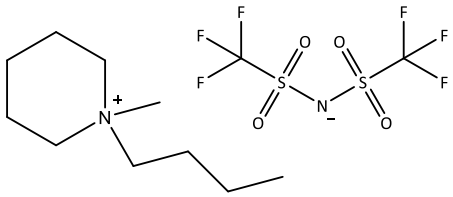
methylpyrrolidinium bromide (29.4 g, 0.132 mol) and lithium bis(trifluoromethane)sulfonimide (41.6 g, 0.145 mol) to afford a clear colourless liquid (53.5 g, 95 %). ^{1}H-NMR (300 MHz, DMSO-d_6) δH (ppm) 3.54 - 3.35 (m, 4H) 3.34 - 3.23 (m, 2H) 2.98 (s, 3H) 2.08 (br. s., 4H) 1.76 - 1.61 (m, 2H) 1.32 (dq, *J*=14.7, 7.39 Hz, 2H) 0.97 - 0.88 (m, 3H) ^{13}C-NMR (75.4 MHz, DMSO-d_6) δC (ppm) 121.6, 117.4, 63.5, 62.9, 47.5, 24.9, 21.1, 19.3, 13.4. MS (+ve) m/z 142.16 [100% $C_9H_{20}N^+$] (calcd: 142.27). Ion chromatography: Br^- : 0.12 wt.%.

4.3.2.2.3 Synthesis of 1-n-butylpyridinium bis(trifluoromethane)sulfonimide[186]



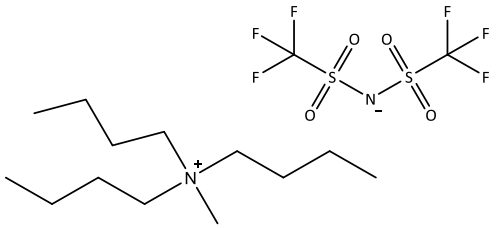
The same procedure used to prepare 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide was used to prepare 1-n-butylpyridinium bis(trifluoromethane)sulfonimide from 1-n-butyl-1-methylpyrrolidinium bromide (12.5 g, 0.057 mol) and lithium bis(trifluoromethane)sulfonimide (18.4 g, 0.064 mol) to afford a clear colourless liquid (19.3 g, 80 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 8.98 (d, J=4.5 Hz, 2H), 8.46 (t, J=6.4 Hz, 1H), 8.06 (t, J=6.0 Hz, 2H), 4.69 (t, J=7.3 Hz, 2H), 1.96 (m, 2H), 1.37 (m, 2H), 0.95 (t, J=7.5 Hz, 3H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 145.6, 144.8, 128.6, 62.3, 33.5, 19.3, 13.4. MS (+ve) m/z 136.11 [100% $C_9H_{14}N^+$] (calcd: 136.11). Ion chromatography: Br^- : 0.09 wt.%.

4.3.2.2.4 Synthesis of 1-n-butyl-1-methylpiperidinium bis(trifluoromethane)sulfonimide[187]



The same procedure used to prepare 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide was used to prepare 1-n-butyl-1-methylpiperidinium bis(trifluoromethane)sulfonimide from 1-n-butyl-1-methylpiperidinium bromide (20.0 g, 0.085 mol) and lithium bis(trifluoromethane)sulfonimide (29.3 g, 0.102 mol) at 60 °C to afford a clear yellow liquid (31.2 g, 84 %). ^{1}H-NMR (300 MHz, $CDCl_3$ δH (ppm) 3.39 - 3.22 (m, 6H) 3.03 (s, 3H) 1.96 - 1.81 (m, 4H) 1.80 - 1.62 (m, 4H) 1.41 (dq, *J*=14.9, 7.4 Hz, 2H) 1.04 – 0.95 (m, 3H). ^{13}C-NMR (75.4 MHz,$CDCl_3$) δC (ppm) 152.36, 64.07, 61.33, 47.48, 23.63, 20.62, 19.90, 19.47, 13.37. MS (+ve) m/z 156.17 [100% $C_{10}H_{22}N^{+}$] (calcd: 156.17). Ion chromatography: Br^{-} : 0.31 wt.%.

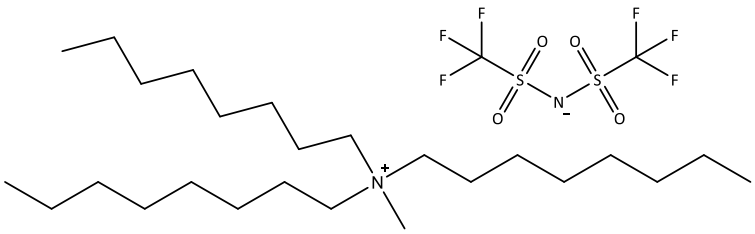
4.3.2.2.5 Synthesis of tributyl(methyl)ammonium bis(trifluoromethane)sulfonimide[133]



The same procedure used to prepare 1-n-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide was used to prepare tributyl(methyl)ammonium bis(trifluoromethane)sulfonimide from tributyl(methyl)ammonium chloride (9.81 g, 0.049 mol) and lithium bis(trifluoromethane)sulfonimide (14.3 g, 0.049 mol) to afford a clear colourless liquid (16.6 g, 83 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 3.28 (m, 9H), 1.73 (m, 6H), 1.31 (m, 6H), 0.90 (m, 9H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC

(ppm) 62.2, 50.4, 23.2, 19.0, 13.8. MS (+ve) m/z 200.24 [100% $C_{13}H_{30}N^+$] (calcd: 200.24). Ion chromatography: Cl^- : 0.26 wt.%.

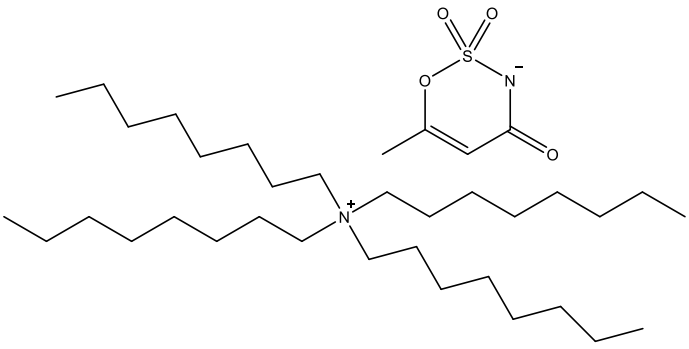
4.3.2.2.6 Synthesis of trioctyl(methyl)ammonium bis(trifluoromethane)sulfonimide[188]



A single neck 250 mL round bottom flask was charged with trioctyl(methyl)ammonium chloride (15 g, 0.037 mol) in chloroform (80 mL). To the stirred solution, 1.3 molar equivalents of lithium bis(trifluoromethane)sulfonimide (13.8 g, 0.048 mol) in water (80 mL) was added dropwise. The resulting mixture was was stirred at room temperature for 72 h. The IL phase was separated and washed with water (4 x 100 mL) and dried *in vacuo* (5 x 10^{-2} mbar) to afford trioctyl(methyl)ammonium bis(trifluoromethane)sulfonimide (17.7 g, 74 %) as a clear yellow liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 3.28 (m, 9H), 1.55 (m, 36H), 0.90 (m, 9H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 121.0, 63.9, 62.9, 49.4, 33.9, 32.9, 32.7, 30.0, 27.2, 26.9, 23.7, 23.6, 23.3, 15.1. MS (+ve) m/z 368.43 [100% $C_{25}H_{54}N^+$] (calcd: 368.43). Ion chromatography: Cl^- : 0.13 wt.%.

4.3.2.3 Procedure for synthesis of tetraoctylammonium ILs

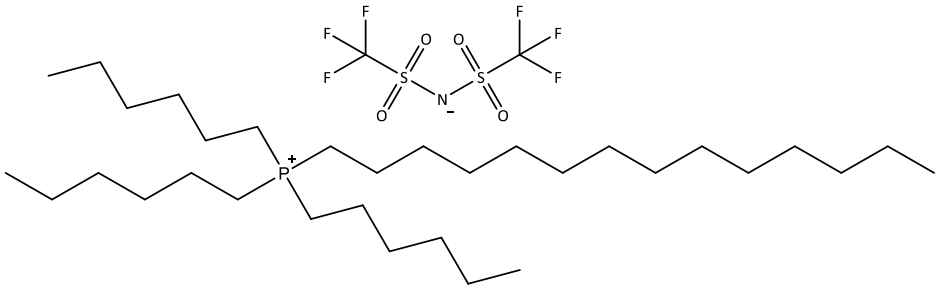
4.3.2.3.1 Synthesis of tetraoctylammonium acesulfame



A single neck 100 mL round bottom flask was charged with tetraoctylammonium bromide (13.7 g, 0.025 mol) in chloroform (50 mL). To the stirred solution, 1.2 molar equivalents of potassium acesulfame (6.03 g, 0.03 mol) in water (50 mL) was added dropwise. The resulting mixture was stirred at room temperature for 48 h. Chloroform (50 mL) and water (50 mL) were added to the mixture and the IL phase was separated, washed with water (4 x 50 mL) and dried *in vacuo* (5 x 10^{-2} mbar) to afford tetraoctylammonium acesulfame (12.4 g, 80 %) as a clear colourless semi-solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 3.29 (m, 8H), 1.56 (m, 44H), 0.88 (m, 9H). MS (+ve) m/z 466.53 [100% $C_{32}H_{68}N^{+}$] (calcd: 466.53). Ion chromatography: Br^{-} : 0.26 wt.%.

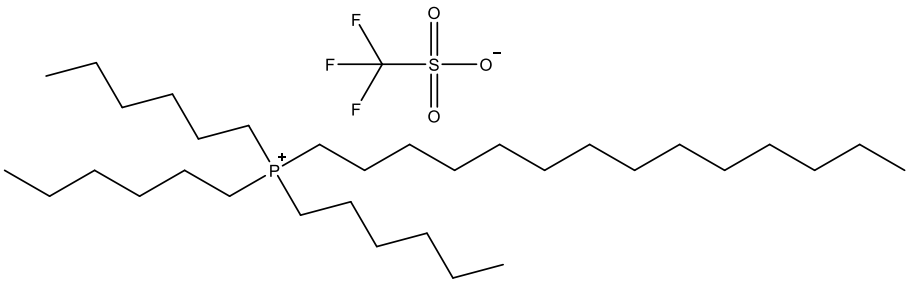
4.3.2.4 Procedure for the synthesis of tetraalkylphosphonium ILs

4.3.2.4.1 Synthesis of trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide[177]



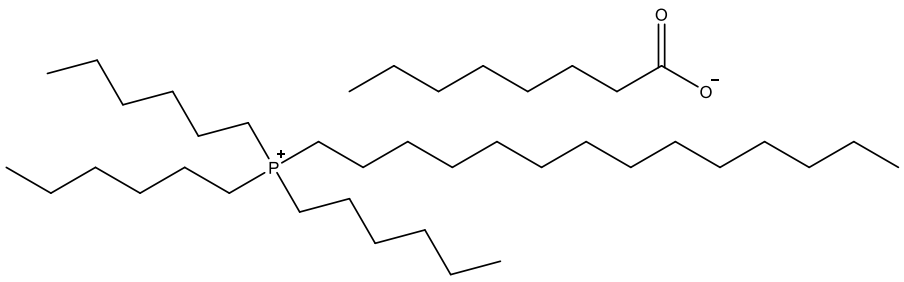
A single neck 250 mL round bottom flask was charged with trihexyl(tetradecyl)phosphonium chloride (16.9 g, 0.0327 mol) in chloroform (40 mL). To the stirred solution, 1.2 molar equivalents of lithium bis(trifluoromethane)sulfonimide (11.3 g, 0.0392 mol) in water (40 mL) was added dropwise. The resulting mixture was stirred at room temperature for 48 h. Chloroform (50 mL) and water (50 mL) were added to the mixture and the IL phase was separated, washed with water (3 x 60 mL) and dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide (23.9 g, 96 %) as a clear colourless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.35(m, 8H), 1.70(m, 8H), 1.40(m, 40H), 1.00(m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 321.5, 120.5, 32.3, 31.2, 31.0, 30.6, 30.5, 30.0, 29.9, 29.6, 29.2, 23.0, 22.6, 21.9, 21.8, 19.3, 18.7, 14.5, 14.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.39. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^{+}$] (calcd: 483.51). MS (-ve) m/z 279.92 [100% $C_2F_6O_4S_2N^{-}$] (calcd: 279.92). Ion chromatography: Cl^{-} : 0.04 wt.%.

4.3.2.4.2 Synthesis of trihexyl(tetradecyl)phosphonium trifluoromethanesulfonate[189]



The same procedure used to prepare trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide was used to prepare trihexyl(tetradecyl)phosphonium trifluoromethanesulfonate from trihexyl(tetradecyl)phosphonium chloride (16.9 g, 0.0327 mol) and lithium trifluoromethanesulfonate (5.84 g, 0.0392 mol) to afford a clear colourless liquid (18.9 g, 95 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.35(m, 8H), 1.70(m, 8H), 1.40(m, 40H), 1.00(m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 321.9, 121.2, 32.2, 31.2, 30.8, 30.7, 30.5, 30.0, 29.8, 29.7, 29.2, 23.0, 22.6, 21.9, 21.8, 19.3, 18.7, 14.4, 14.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.39. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 148.95 [100% $CF_3O_3S^-$] (calcd: 148.95). Ion chromatography: Cl^- : 0.10 wt.%.

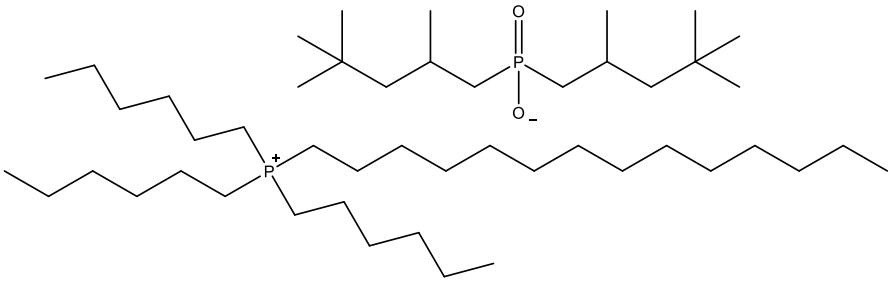
4.3.2.4.3 Synthesis of trihexyl(tetradecyl)phosphonium octanoate[190]



The same procedure used to prepare trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide was used to prepare trihexyl(tetradecyl)phosphonium octanoate from trihexyl(tetradecyl)phosphonium chloride (11.0 g, 0.021 mol) and sodium

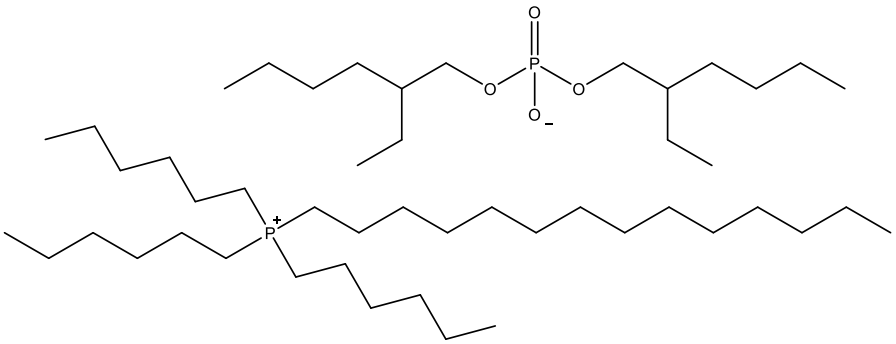
octanoate (4.22 g, 0.025 mol) to afford a clear yellow oil (11.3 g, 86 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.44(m, 8H), 2.14(t, 2H), 1.7-1.4(m, 18H), 1.4-1.1(m, 40H), 0.88(m, 16H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 178.7, 38.6, 31.2, 30.8, 30.7, 30.5, 30.0, 29.8, 29.7, 29.2, 23.0, 22.6, 21.9, 21.8, 19.3, 18.7, 14.4, 14.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.39. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 143.00 [100% C_7COO^-] (calcd: 143.11). Ion chromatography: Cl^- : 0.49 wt.%.

4.3.2.4.4 Synthesis of trihexyl(tetradecyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate[191]



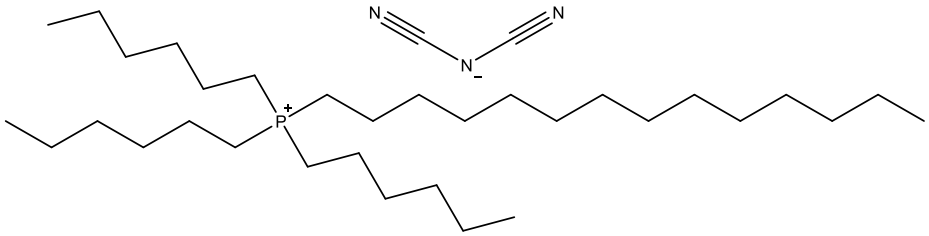
A two-neck 250 mL round bottom flask equipped with reflux condenser was charged with trihexyl(tetradecyl)phosphonium chloride (13.5 g, 0.026 mol) in water (30 mL). To the stirred solution bis(2,4,4-trimethylpentyl)phosphinic acid (7.55 g, 0.026 mol) in water (30 mL)was added dropwise. Sodium hydroxide (0.026 mol; 25 % aqueous solution) was added over a period of 30 min at 55 °C, and the reaction mixture was stirred for a further hour at 55 °C. The IL phase was separated and washed with water (3 x 100 mL) and during each wash cycle, the phases were agitated for 1 h at 55 °C. The product was then dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate (17.2 g, 86 %) as a clear orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.6(m, 8H), 1.7 (m, 8H), 1.4(m, 40H), 1.15(m, 4H), 1.0 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 43.3, 32.2, 31.5, 30.7, 30.6, 30.4, 29.9, 29.8, 29.6, 29.3, 25.9, 24.8, 22.9, 22.2, 19.5, 18.9, 14.4, 14.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.99, 0.40. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 289.23 [100% $C_{16}H_{34}O_2P^-$] (calcd: 289.23). Ion chromatography: Cl^- : 0.38 wt.%.

4.3.2.4.5 Synthesis of trihexyl(tetradecyl)phosphonium bis(2-ethyl hexyl)phosphate[191]



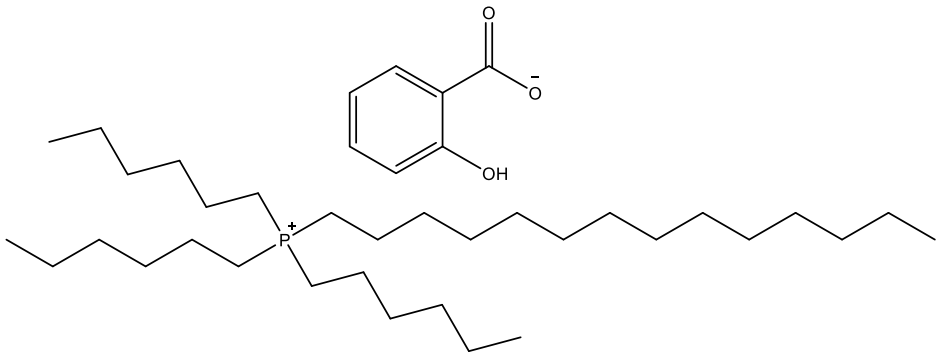
A two-neck 250 mL round bottom flask fitted with reflux condenser was charged with trihexyl(tetradecyl)phosphonium chloride (29.7 g, 0.057 mol) in hexane (70 mL). To the stirred solution, bis(2-ethyl hexyl)phosphate (18.4 g, 0.057 mol) was added. A solution of KOH (3.2 g, 0.057 mol) and water (60 mL) was added dropwise to the rapidly stirred reaction and the resulting mixture was stirred at room temperature for 24 h. The IL phase was washed with water (3 x 100 mL), separated and dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium bis(2-ethyl hexyl)phosphate (43.1 g, 94 %) as a pale yellow clear liquid. ^{1}H-NMR (400 MHz, $CDCl_3$) δH (ppm) 3.76-3.72 (m, 4H), 2.46-2.39 (m, 8H), 1.54-1.21 (m, 66H), 0.91-0.85 (m, 24H). ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.85, 0.37. MS (+ve) m/z 483.4 [100% $C_{32}H_{68}P^{+}$] (calcd: 483.51). MS (-ve) m/z 321.3 [100% $C_{16}H_{34}O_2PO_2^{-}$] (calcd: 321.22). Ion chromatography: Cl^{-} : 0.16 wt.%.

4.3.2.4.6 Synthesis of trihexyl(tetradecyl)phosphonium dicyanamide[192]



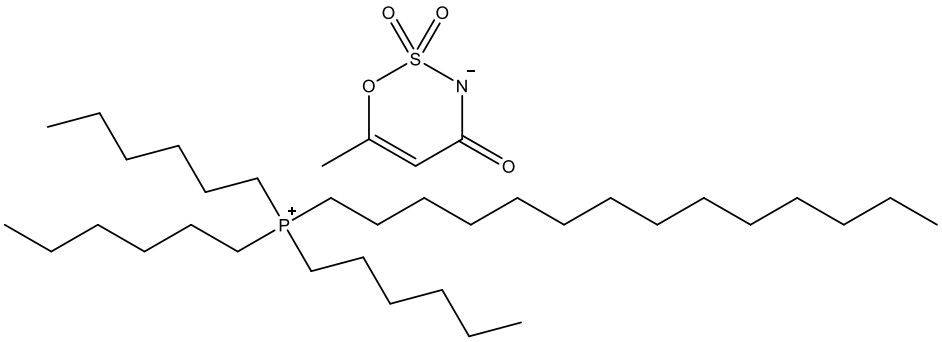
A solution of sodium dicyanamide (3.12 g; 0.035 mol) in water (80 mL) was added to a solution of trihexyl(tetradecyl)phosphonium chloride (15.0 g; 0.029 mol) in EtOH (40 mL) and left to stir at room temperature for 48 h. The mixture was then concentrated under vacuum and washed with water (4 × 100 mL). The IL layer was then eluted with DCM in a column containing celite, decolourizing charcoal and SiO_2 gel. The solvent was then dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium dicyanamide (12.1 g; 76 %) as a clear pale yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δH (ppm) 2.13 (m, 8H) 1.47 (m, 8H), 1.27 (m, 40H), 0.84 (m, 12H) ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 119.70, 31.64, 30.65, 30.51, 30.37, 30.17, 30.02, 29.37, 29.24, 29.08, 28.58, 22.41, 22.05, 21.31, 21.27, 18.84, 18.37, 13.85, 13.66. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.39. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 66.00 [100% $C_2N_3^-$] (calcd: 66.01). Ion chromatography: Cl^- : 0.20 wt.%.

4.3.2.4.7 Synthesis of trihexyl(tetradecyl)phosphonium salicylate[193]



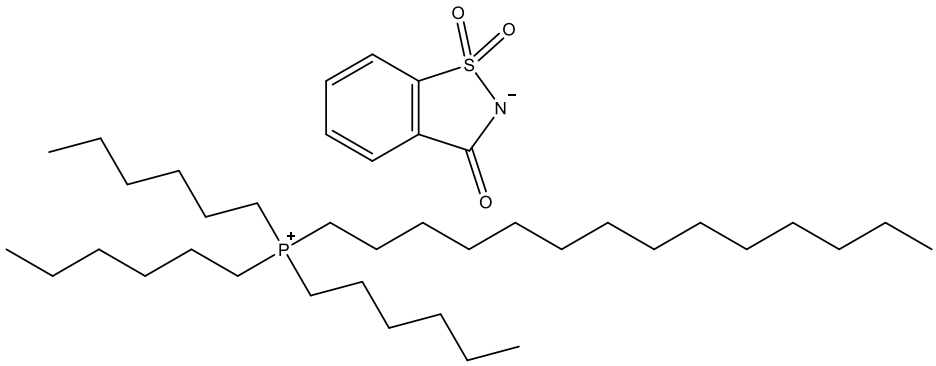
A single neck 250 mL round bottom flask was charged with trihexyl(tetradecyl)phosphonium chloride (51.9 g, 0.100 mol) in chloroform (100 mL). To the stirred solution sodium salicylate (22.42 g, 0.140 mol) was added. The resulting mixture was stirred at room temperature for 24 h. To the pink solution, water (100 mL) was added, immediately turning the solution orange. The IL phase was washed with water (8 x 100 mL) allowing the phases at least 2 hours to separate after each wash. The product was then dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium salicylate (52.1 g, 84 %) as a clear yellow liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 7.93 (dd, *J*=7.6, 1.8 Hz, 1H) 7.21 (dd, *J*=8.1, 7.2, 1.9 Hz, 1H) 6.81 (dd, *J*=8.1, 1.1 Hz, 1H) 6.73 - 6.66 (m, 1H) 2.31 - 2.16 (m, 8H) 1.53 - 1.35 (m, 16H) 1.34 - 1.17 (m, 32H) 0.93 - 0.80 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 218.2, 131.2, 130.6, 122.8, 31.5, 30.6, 30.2, 30.1, 29.9, 29.2, 28.9, 28.8, 28.5, 22.2, 21.9, 21.4, 21.3, 18.9, 18.3, 13.7, 13.5. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.76. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 137.02 [100% $C_7H_5O_3^-$] (calcd: 137.02). Ion chromatography: Cl^- : 0.49 wt.%.

4.3.2.4.8 Synthesis of trihexyl(tetradecyl)phosphonium acesulfame[194]



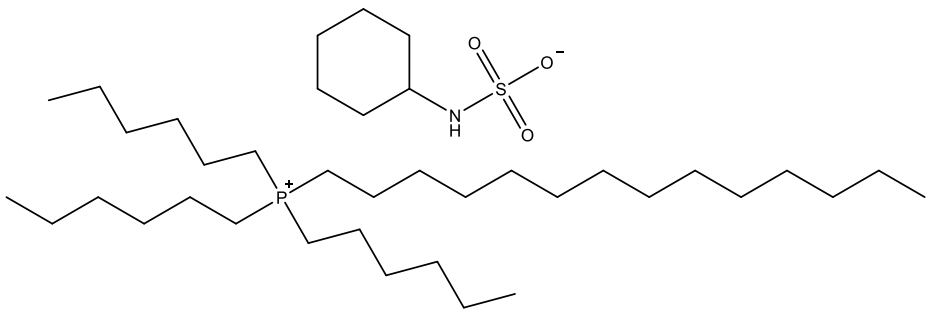
A single neck 250 mL round bottom flask was charged with trihexyl(tetradecyl)phosphonium chloride (25.9 g, 0.050 mol) in chloroform (40 mL). To the stirred solution potassium acesulfame (12.1 g, 0.060 mol) in water (40 mL) was added. The resulting mixture was stirred at room temperature for 24h. The IL phase was washed with water (8 x 100 mL) allowing the phases at least 2 hours to separate after each wash. The product was then dried *in vacuo* (5 x 10^{-2} mbar) to afford trihexyl(tetradecyl)phosphonium acesulfame (27.2 g, 84 %) as a clear colourless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 5.43 (s, 1H) 2.35 - 2.16 (m, 8H) 1.98 (s, 3H) 1.62 - 1.38 (m, 16H) 1.35 - 1.09 (m, 32H) 0.96 - 0.79 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 169.6, 160.6, 102.1, 31.8, 30.9, 30.7, 30.6, 30.4, 30.2, 29.6, 29.5, 29.2, 28.9, 22.6, 22.3, 21.7, 21.6, 19.9, 19.2, 18.6, 14.0, 13.9. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.78. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^+$] (calcd: 483.51). MS (-ve) m/z 161.99 [100% $C_4H_4O_4SN^-$] (calcd: 161.99). Ion chromatography: Cl^- : 0.50 wt.%.

4.3.2.4.9 Synthesis of trihexyl(tetradecyl)phosphonium saccharinate



The same procedure used to prepare trihexyl(tetradecyl)phosphonium acesulfame was used to prepare trihexyl(tetradecyl)phosphonium saccharinate from trihexyl(tetradecyl)phosphonium chloride (25.9 g, 0.050 mol) and sodium saccharinate (12.3 g, 0.060 mol) to afford a clear yellow liquid (26.82 g, 81 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 7.85 - 7.67 (m, 2H) 7.58 - 7.43 (m, 2H) 2.47 - 2.19 (m, 8H) 1.68 - 1.37 (m, 16H) 1.37 - 1.15 (m, 32H) 0.95 - 0.75 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 218.2, 131.2, 130.6, 122.8, 31.5, 30.6, 21.2, 28.9, 28.5, 22.2, 21.9, 21.3, 18.3, 13.5. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.74. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^{+}$] (calcd: 483.51). MS (-ve) m/z 181.99 [100% $C_7H_4O_3SN^{-}$] (calcd: 181.99). Ion chromatography: Cl^{-} : 0.44 wt.%.

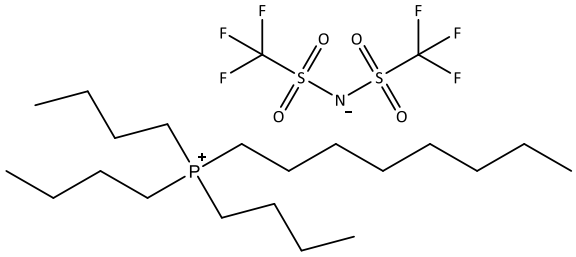
4.3.2.4.10 Synthesis of trihexyl(tetradecyl)phosphonium cyclamate



The same procedure used to prepare trihexyl(tetradecyl)phosphonium acesulfame was used to prepare trihexyl(tetradecyl)phosphonium cyclamate

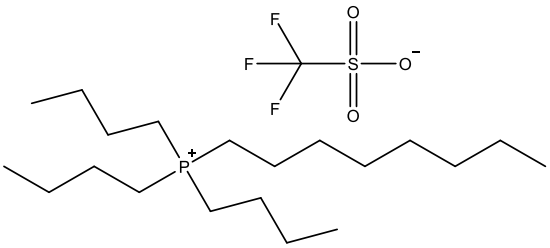
from trihexyl(tetradecyl)phosphonium chloride (25.9 g, 0.050 mol) and sodium cyclamate (12.1 g, 0.060 mol) to afford a clear colourless liquid (21.1 g, 64 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 3.8-3.6(m, 2H), 3.4-3.2(m, 1H), 2.3-2.2(m, 8H), 2.1(m, 2H), 1.6(m, 2H), 1.5-1.4 (1m, 16H), 1.3-1.2 (m, 32H), 0.85-0.79 (m, 12H). ^{13}C-NMR (300 MHz, $CDCl_3$) δC (ppm) 193.6, 53.4, 31.9, 31.3, 31.1, 30.9, 30.8, 30.7, 30.5, 30.4, 29.6-29.7 29.5, 29.3, 29.0, 22.7, 22.5, 22.4, 22.0 ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.07. MS (+ve) m/z 483.51 [100% $C_{32}H_{68}P^{+}$] (calcd: 483.51). MS (-ve) m/z 178.02 [100% $C_6H_{12}O_3SN^{-}$] (calcd: 178.05). Ion chromatography: Cl^{-} : 0.70 wt.%.

4.3.2.4.11 Synthesis of tributyl(octyl)phosphonium bis(trifluoromethane)sulfonimide[195]



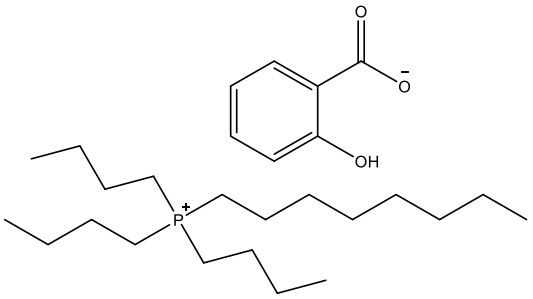
The same procedure used to prepare trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide was used to prepare tributyl(octyl)phosphonium bis(trifluoromethane)sulfonimide from tributyl(octyl)phosphonium chloride (15.1 g, 0.0429 mol) and lithium bis(trifluoromethane)sulfonimide (14.8 g, 0.0516 mol) to afford a clear colourless liquid (24.4 g, 95 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.19 (m, 8H) 1.5-1.1 (m, 24H) 0.88 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 117.7, 31.6, 30.4, 28.8, 28.7, 23.8, 23.4, 22.5, 21.5, 21.4, 18.7, 18.1, 13.9, 13.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.07. MS (+ve) m/z 315.32 [100% $C_{20}H_{44}P^{+}$] (calcd: 315.32). MS (-ve) m/z 279.92 [100% $C_2F_6O_4S_2N^{-}$] (calcd: 279.92). Ion chromatography: Cl^{-} : 0.08 wt.%.

4.3.2.4.12 Synthesis of tributyl(octyl)phosphonium trifluoromethanesulfonate[195]



The same procedure used to prepare trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide was used to prepare tributyl(octyl)phosphonium trifluoromethanesulfonate from tributyl(octyl)phosphonium chloride (15.1 g, 0.043 mol) and lithium trifluoromethanesulfonate (8.05 g, 0.052 mol) to afford a clear colourless liquid (17.6 g, 88 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 2.19 (m, 8H) 1.5-1.1 (m, 24H) 0.88 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 117.7, 31.6, 30.4, 28.8, 28.7, 23.8, 23.4, 22.5, 21.5, 21.4, 18.7, 18.1, 13.9, 13.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 33.07. MS (+ve) m/z 315.32 [100% $C_{20}H_{44}P^+$] (calcd: 315.32). MS (-ve) m/z 148.95 [100% $CF_3O_3S^-$] (calcd: 148.95). Ion chromatography: Cl^- : 0.18 wt.%.

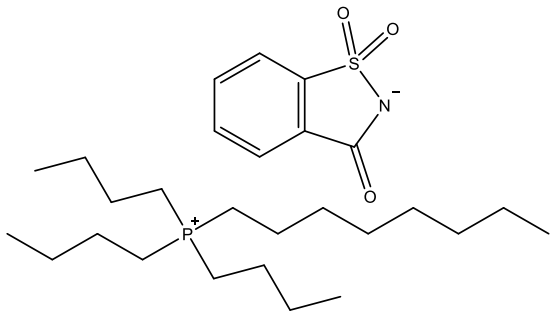
4.3.2.4.13 Synthesis of tributyl(octyl)phosphonium salicylate



A single neck 250 mL round bottom flask was charged with tributyl(octyl)phosphonium chloride (15 g, 0.043 mol) in chloroform (80 mL). To the stirred solution sodium salicylate (8.26 g, 0.052 mol) was added. The resulting mixture was stirred at room temperature for 72 h. To the resulting solution, water (100 mL) was added immediately turning the solution orange.

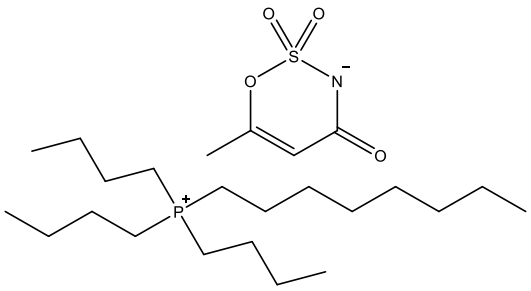
The IL phase was washed with water (5 x 100 mL) allowing the phases at least 2 hours to separate after each wash. The product was then dried *in vacuo* (5 x 10^{-2} mbar) to afford tributyl(octyl)phosphonium salicylate (17.7 g, 91 %) as a clear orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 7.93 (m, 1H) 7.27 (m 1H) 6.80(m, 1H) 6.7 (td, *J*=7.4 Hz, 1H) 2.19 (m, 8H) 1.5-1.1 (m, 24H) 0.88 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 218.2, 131.2, 130.6, 122.8, 31.6, 30.4, 28.8, 28.7, 23.8, 23.4, 22.5, 21.5, 21.4, 18.7, 18.1, 13.9, 13.2. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.96. MS (+ve) m/z 315.32 [100% $C_{20}H_{44}P^+$] (calcd: 315.32). MS (-ve) m/z 137.03 [100% $C_7H_5O_3^-$] (calcd: 137.02). Ion chromatography: Cl^- : 0.98 wt.%.

4.3.2.4.14 Synthesis of tributyl(octyl)phosphonium saccharinate



The same procedure used to prepare tributyl(octyl)phosphonium salicylate was used to prepare tributyl(octyl)phosphonium saccharinate from tributyl(octyl)phosphonium chloride (15 g, 0.043 mol) and sodium saccharinate (10.5 g, 0.051 mol) to afford a clear colourless liquid (26.7 g, 90 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 7.9 (m, 1H) 7.6-7.4 (m 3H) 2.19 (m, 8H) 1.5-1.1 (m, 24H) 0.88 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 218.2, 131.2, 130.6, 122.8, 31.5, 30.6, 30.2, 30.1, 29.9, 29.2, 28.9, 28.8, 28.5, 22.2, 21.9, 21.4, 21.3, 18.9, 18.3, 13.7, 13.5. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.76. MS (+ve) m/z 315.32 [100% $C_{20}H_{44}P^+$] (calcd: 315.32). MS (-ve) m/z 181.99 [100% $C_7H_4O_3SN^-$] (calcd: 181.99). Ion chromatography: Cl^- : 0.07 wt.%.

4.3.2.4.15 Synthesis of tributyl(octyl)phosphonium acesulfame



The same procedure used to prepare tributyl(octyl)phosphonium salicylate was used to prepare tributyl(octyl)phosphonium acesulfame from tributyl(octyl)phosphonium chloride (15 g, 0.043 mol) and potassium acesulfame (10.3 g, 0.051 mol) to afford a clear colourless liquid (15.1 g, 74 %). ^{1}H-NMR (300 MHz, $CDCl_3$) δH (ppm) 5.43 (s, 1H) 2.19 (m, 8H) 1.98 (s, 3H) 1.5-1.1 (m, 24H) 0.88 (m, 12H). ^{13}C-NMR (75.4 MHz, $CDCl_3$) δC (ppm) 169.6, 160.6, 102.1, 31.5, 30.6, 30.2, 30.1, 29.9, 29.2, 28.9, 28.8, 28.5, 22.2, 21.9, 21.4, 21.3, 18.9, 18.3, 13.7, 13.5. ^{31}P-NMR (121 MHz, $CDCl_3$) δP (ppm) 32.78. MS (+ve) m/z 315.32 [100% $C_{20}H_{44}P^{+}$] (calcd: 315.32). MS (-ve) m/z 161.96 [100% $C_4H_4O_4SN^{-}$] (calcd: 161.99). Ion chromatography: Cl^{-} : 0.08 wt.%.

4.4 Preparation of frequently used acids and buffers

4.4.1 Methacrylic acid (1M)

Stock solutions of methacrylic acid (MAA) (1M) were prepared by adding MAA (84.79 mL) (>99 % pure stock solution) to distilled water (800 mL), then making up the solution to 1L with distilled water. The MAA (1M) solution was filter sterilised (0.45 µm pore size) directly into a sterile Duran bottle by aseptic technique, and stored for up to one month at room temperature.

4.4.2 Potassium phosphate buffer (100mM)

Stocks solutions of potassium phosphate buffer (100 mM) were prepared by titrating potassium dihydrogen orthophosphate (KH_2PO_4) (100 mM) into di-potassium hydrogen orthophosphate (K_2HPO_4) (100 mM) until a pH of 7.5 was reached. Potassium phosphate buffer (100 mM) was filter sterilised directly

into 50 mL falcon tubes by aseptic technique, and stored for up to six months at 4 °C.

4.4.3 MOPS buffer (1M)

Stock solutions of 1M 3-morpholinopropane-1-sulfonic acid (MOPS) (1M) were prepared by dissolving MOPS (20.9 g) in distilled water (80 mL), adjusting the pH to 7.5 with NaOH (1M). The volume was then made up to 100 mL with distilled water. MOPS buffer (1M) was filter sterilised directly into 50 mL sterile falcon tubes by aseptic technique, and stored for up to six months at 4 °C.

4.5 Culture growth and maintenance

4.5.1 Microorganisms

Escherichia coli K12 MG1655 and *Saccharomyces cerevisiae* DSM70449 were used in this project. The *E. coli* MG1655 strain was obtained from John Hobman (School of Biosciences, University of Nottingham) and the *S. cerevisiae* DSM70449 strain was obtained from Invista™ (Edinburgh, Scotland). Both strains were stored in 16 % glycerol stock solutions at -80 °C. Working cultures were prepared on agar plates.

4.5.2 Luria Bertani (LB) and Agar Preparation

Luria Bertani high salt medium (Melford) (25 g/L) was used whenever LB medium is referred to throughout this thesis. LB medium was prepared by dissolving peptone from casein digest (10 g), yeast extract (5 g) and sodium chloride (10 g) in 1 litre of water. The mixture was sterilised by autoclaving at 126 °C for 15 min, then left to cool to room temperature. Liquid LB medium was stored in a sealed sterile Duran bottle for up to one week at room temperature. LB agar plates were prepared using LB high salt medium (25 g/L) and agar (20 g/L). The LB and agar mixture was sterilised by autoclaving and allowed to cool in a 50 °C water bath prior to pouring, using aseptic techniques into sterile petri dishes. Plates were stored for up to two weeks at 4 °C.

4.5.3 Yeast extract-Peptone-Dextrose (YEPD) and Agar Preparation

Yeast extract-Peptone-Dextrose (YEPD) medium and agar plates were prepared by dissolving yeast extract (3 g), malt extract (3 g) , peptone from soybeans (5 g) and glucose (10 g) in distilled water (1 L). Agar (15 g) was added if preparing agar plates. The mixture was autoclaved at 126 °C for 15 min and then allowed to cool to 60 °C before pouring the plates using aseptic techniques into sterile petri dishes. Plates were stored for up to two weeks at 4 °C. Liquid YEPD medium was stored for up to one week at room temperature.

4.5.4 Acidic and MAA containing Media Preparation

For pH tests, stock solutions of LB and YEPD medium were adjusted to pH values of 3.0, 4.0, 5.0, 6.0 and 7.0, as indicated using a pH meter, using HCl (1M). HCl was used experimentally to determine the effect of hydrogen ion concentration based on the assumption that HCl itself is not toxic because it dissociates completely in aqueous solution to form hydrogen and chloride ions. For the MAA toxicity tests buffered at pH 7.0, a stock solution of each medium containing MOPS (50 mM) was prepared by combining pre-prepared LB, MSX and YEPD (475 mL) with MOPS (1M, 25 mL). For the MAA toxicity tests buffered at pH 4, stock solutions containing the desired concentration of MAA were prepared using a pH meter to adjust the medium to pH 4 using HCl (1M) and NaOH (1M).

4.5.5 MSX Broth Preparation

Minimal salts (MSX) medium[196] was prepared in three parts; MSA, MSB and Vishniac Trace Elements. Vishniac trace elements solution (1 L) was prepared by combining EDTA disodium salt (50 g) with water (800 mL), dissolved by adding KOH pellets (2-3 at a time). Chemicals were then added in the following order; $ZnSO_4$ (2.2 g), $CaCl_2$ (5.54 g), $MnCl_2.4H_2O$ (5.06 g), $FeSO_4.7H_2O$ (5 g), $(NH_4)_6Mo_7O_{24}.4H_2O$ (1.1 g), $CuSO_4.5H_2O$ (1.57 g) and $CoCl_2.6H_2O$ (1.61 g). The solution was adjusted to pH 6.0 using KOH (1M) then made up to 1 L using

water. The solution was stored for up to six months at 4 °C. MSA was prepared by dissolving KH_2PO_4 (6 g) and Vishniac trace elements (2 mL) in water (660 mL). The solution was adjusted to pH 7.0 using KOH (1M). The solution was then made up to 760 mL with water. MSB was prepared by dissolving NH_4Cl (3 g) and $MgSO_4.7H_2O$ (0.4 g) in water (200 mL). A stock solution of glucose (12.5 %) was also prepared by dissolving D-glucose (12.5 g) in water (100 mL). MSA, MSB and glucose (40 mL) were autoclaved at 126 °C separately for 15 min then combined aseptically once cooled, the final solution having a pH of 6.8. The solution was stored for up to one week at room temperature.

4.5.6 Cultivation of microorganisms in liquid medium

4.5.6.1 General

From glycerol stock solutions, *E. coli* MG1655 and *S. cerevisiae* DSM70449 were streaked onto a sterile agar plate using four disposable inoculation loops. The inoculated plate was placed in an incubator overnight at 37 or 30 °C for *E. coli* and *S. cerevisiae*, respectively. An appropriate amount of sterile LB, YEPD or MSX medium (25 mL of medium in a 100 mL flask and 50 mL of medium in a 250 mL flask) was added to a sterile Erlenmeyer flask, fitted with a polyurethane foam bung and covered with aluminium foil, using aseptic techniques. To inoculate from an agar plate, a well isolated single colony was removed from the plate using a disposable inoculation loop. The colony was transferred to a pre-autoclaved flask containing the appropriate medium. The flask stopper was replaced and the flask incubated in an orbital incubator at 30 or 37 °C, 200 rpm, for the respective microorganism. Once an OD_{600} value of ≈0.8 was reached, an aliquot of inoculum was removed from the overnight culture using a sterile pipette and transferred to a pre-autoclaved flask. The type of 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 initially inoculate all cultures for growth and toxicity tests carried out throughout this thesis.

Specific growth rates of cultures were calculated in the exponential growth phase using the equation $\ln N_t/N_0 = \mu t$, where N_t is the arbitrary light scattering units at time t (h) and μ is the growth rate (h^{-1}). Growth rates varied slightly dependent on type of growth medium and also between different tests, therefore all growth test results are expressed as a percentage value of the control run alongside that particular test.

4.5.6.2 Growth Inhibition Tests

Toxicity tests were carried out using either the shake-flask method or Bioscreen C MBR method. Tests were carried out using media, temperatures, pH values, and additions of organic solvent or ILs, as described in the text. ILs, organic solvents and alkyl methacrylates were determined as self-sterile as there was no growth of organisms when the ILs or solvents were added to sterile uninoculated medium.

4.5.6.2.1 Shake-flask cultures

Organic solvent and methacrylate ester toxicity tests were carried out in sterile 40 mL Teflon sealed glass vials, due to their volatility and ability to degrade the plastic well plates used in the Bioscreen. To each vial the appropriate medium of LB or MSX medium was added for *E. coli*, and YEPD for *S. cerevisiae*, by aseptic technique. The medium was then inoculated with an overnight culture of *E. coli or S. cerevisiae* (100 µL). One vial was then left containing only these components as a control test, and to the remaining three vials was added the desired solvent. The four vials per solvent were then transferred to an orbital incubator set to 30 or 37 °C, 250 rpm, for *E. coli* or *S. cerevisiae*, respectively. The aqueous medium phase was sampled every 30 minutes initially, and every 10 min once the cells entered the exponential growth phase, and again every 30 min once the stationary phase was reached, to produce a growth curve. Samples were taken through the Teflon-sealed lids using a sterile glass syringe and needle, and the OD_{600} values were recorded using a UV/Vis spectrophotometer, diluting samples to the appropriate detection range.

4.5.6.2.2 Bioscreen cultures

The toxicity tests involving MAA and ILs were carried out in a Bioscreen C microplate reader which takes kinetic measurements of turbidity in real time to generate growth curves. Tests were carried out in sterile unique honeycomb micro-plates containing 100 wells (400 µL capacity per well) and a lid, to prevent evaporation of liquid components, using media, temperatures, pH values, MAA concentrations and additions of ILs as described in the text. The Bioscreen C microplate reader was used with a 600 nm filter to measure OD_{600} readings for both microorganisms. The obtained readings were collected using BioScreener™ software, which was later exported to Microsoft excel for analysis. Data was used to plot growth curves to calculate growth rates and $MaxOD_{600}$ values for each organism under the conditions tested. The speed and agitation of the plate during the test was set to maximum and was kept consistent for all tests. Tests were run for between 48-72 h with continuous heat and agitation and OD_{600} readings were taken every 10 min with a 15 s period without agitation prior to the reading. Each test was carried out in triplicate, and each complete experiment was carried out twice to ensure reliability of results.

4.5.7 Preparation of cryostocks

Stock solutions of glycerol (80 %) were prepared in an un-autoclaved flask by dissolving glycerol (65 g) in water (100 mL) and autoclaving at 126 °C for 15 min. Cryostocks were prepared by inoculating growth medium (10 mL), from a single microbial colony. *E. coli* and *S. cerevisiae* cultures were incubated in an orbital shaker at 37 °C, 250 rpm and 30 °C, 250 rpm, respectively. All cultures were incubated until an OD_{600} of between 0.8 and 1.0 had been reached, and were then aliquots (800 µL) were transferred into cryo-vials containing 80 % glycerol (200 µL). The vials containing these glycerol cultures were vortexed to create a 16 % glycerol stock solution, which was then stored at -80 °C until use.

4.6 Molecular Biology

4.6.1 Preparation of genomic DNA extracts from *E. coli* MG1655 mutant

The extraction and isolation of genomic DNA from wild type *E. coli* MG1655 and five mutant strains was carried out using a GenElute^{TM} Bacterial Genomic DNA Kit (Sigma Aldrich). *E. coli* MG1655 and the five mutant strains were grown overnight in fresh LB medium (50 mL) at 37 °C, 200 rpm. A sample of the culture (1 mL) was transferred to a sterile 2 mL Eppendorf and was centrifuged at 12000 x *g* for 2 min, and the supernatant was discarded. The pellet was then resuspended thoroughly in Lysis Solution T (180 µL). As RNA-free genomic DNA was required, RNase A Solution (20 µL) was added, inverting the mixture several times by hand to ensure and even distribution of the solution, and incubated for 2 min at room temperature. Proteinase K Solution (20 µL) was then added to the sample, inverted several times, and incubated at 55 °C for 30 min in a pre-heated water bath. The cells were lysed by the addition of Lysis Solution C (200 µL) and vortexed thoroughly for ~ 15 s to ensure a homogenous mixture. The cells were then incubated at 55 °C for 10 min. To isolate the genomic DNA, elution columns were prepared by adding Column Preparation Solution (500 µL) to each pre-assembled GenElute Miniprep Binding Column fitted into a 2 mL collection tube, and centrifuging at 12000 x *g* for 1 min. To the lysate, ethanol (96 %, 200 µL) was added, and the solution was mixed thoroughly by vortexing for 10 s to create a homogenous mixture. The lysate was then transferred into the binding column using a wide-bore pipette to reduce DNA shearing. The column and lysate were then centrifuged at 6500 x *g* for 1 min. The collection tube containing the eluate was discarded, and the column was placed in a new 2mL collection tube. The DNA was then eluted by pipetting the Elution Solution (200 µL) directly onto the centre of the column and incubating for 5 min at room temperature. The column was then centrifuged for 1 min at 6500 x *g* to elute the DNA. The elution step was repeated twice to improve the yield and the purity of the

samples was determined using agarose gel electrophoresis (4.1.3). The eluate containing the pure genomic DNA was stored at -20 °C, until it was sent for sequencing. Sequencing and bioinformatics was done by Deep Seq (Sunir Malla, Centre for Genetic and Genomics, University of Nottingham). Bioinformatic data was analysed with the help of Luca Rossoni.

5 Toxicity of MAA towards growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM70449 at various pH values

5.1 Effect of pH on the growth of *E. coli* MG1655

It is widely known that product toxicity can be one of the main limiting factors in the bioproduction of chemicals, hindering the biocatalysts effectiveness and the overall viability of the process.[197] Previous research investigated the biosynthetic production of MAA and was proven successful *via* metabolic engineering.[6] The titres, however, are fairly low, possibly due to the toxicity of the MAA itself causing inhibition of the biocatalyst as it is produced.[6,7] A further complication during MAA production is that the acidic product will either be in its dissociated or undissociated form based on the pKa of the acid and the pH value of the system. As discussed previously, the pKa of MAA is 4.66, therefore an external pH value of between 3 and 4 would be ideal in order to produce and extract the undissociated acid species from the fermentation broth. Alternatively, if the acid is to be in its dissociated form, a pH value of 7 would be required. Therefore, if producing and extracting the free acid is to be considered as a viable option, it is important that the microorganism itself can tolerate an acidic environment of pH 3-4.

While *Escherichia coli* cells are known to survive extremely low pH values, they are highly sensitive to pH fluctuations throughout the exponential growth phase, during which time the MAA would be being produced.[198,199] Therefore, the initial aim of this work was to determine whether or not *E. coli* can grow at a pH value of < 4.66, in order to produce the undissociated acid species in excess, potentially creating a more efficient extraction process. The effect of pH on the growth of *E. coli* MG1655 was tested in LB medium, which had been adjusted to various pH values (appendix 12.1.1, page 204) (Table 2).

Table 2. Effect of pH value on the growth of *E. coli* in LB medium

pH	Specific growth rate (μ) (h^{-1})	μ (%)	$MaxOD_{600}$	$MaxOD_{600}$ (%)
7.0	0.92 ± 0.10	100 ± 10	1.76 ± 0.01	100 ± 1
6.0	0.81 ± 0.06	88 ± 6	1.76 ± 0.01	100 ± 0
5.0	0.736 ± 0.06	80 ± 6	1.73 ± 0	98 ± 0
4.0	0.254 ± 0.02	27 ± 2	1.76 ± 0.02	100 ± 1
3.0	-	-	-	-

E. coli was grown in LB medium (300 µL) which was adjusted to the desired pH value using 1M HCl. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Growth rates (µ) and $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures at pH 7.0. $MaxOD_{600}$ values were taken as the highest OD_{600} at each pH value after 30 h, during the stationary phase. Data are the means of five replicates and the standard deviations are shown.

The growth rate of *E. coli* MG1655 decreased with decreasing pH value of the medium. The reduction in growth rate between the values of pH 5.0 – 7.0 is not statistically significant, however, at pH 4 the growth rate decreased to just 27% compared to control cultures. Between the pH values of 4.0 – 7.0, the OD_{600} values were unaffected and the biomass concentrations reached a value consistent with control cultures at pH 7.0. At pH 3.0, however, no growth was observed even after prolonged incubation. Therefore, the optimal growth rate of *E. coli* MG1655 occurred between pH 5.0 – 7.0. Although the growth rate was lower at pH 4.0, the final biomass concentration was unaffected, compared with the cultures grown at high pH values. Therefore, the production of undissociated MAA could be carried out at pH 4.0, without affecting the biomass concentration of *E. coli* cultures. However, this would be dependent on the ability of *E. coli* to tolerate the undissociated MAA itself.

5.2 Effect of pH on the growth of *S. cerevisiae* DSM70449

Although less industrially relevant, *Saccharomyces cerevisiae* is known to be a more chemically robust organism than *E. coli* and has previously shown to tolerate low pH environments of ~ pH 2.[154,200] At pH 3.0, 98 % of the MAA

would be in its undissociated form. Therefore, a greater concentration would likely be extracted, improving the economic viability of the process. Therefore, the effect of pH value on the growth of *S. cerevisiae* was determined in order to establish whether it may be a more promising biocatalyst for use in this process. The effect of pH value was tested by growing *S. cerevisiae* DSM70449 in YEPD medium adjusted to various pH values (appendix 12.1.1, page 204) (Table 3).

Table 3. Effect of pH value on the growth of *S. cerevisiae* in YEPD medium

pH	Specific growth rate (μ) (h^{-1})	μ (%)	$MaxOD_{600}$	$MaxOD_{600}$ (%)
7.0	0.444 ± 0.012	100 ± 3	1.49 ± 0.03	100 ± 2
6.0	0.366 ± 0.02	82 ± 4	1.48 ± 0.03	99 ± 2
5.0	0.248 ± 0.005	56 ± 1	1.45 ± 0.01	98 ± 0
4.0	0.251 ± 0.009	56 ± 2	1.47 ± 0.02	99 ± 1
3.0	0.251 ± 0.019	57 ± 4	1.43 ± 0.02	96 ± 1

S. cerevisiae was grown in YEPD medium (300 µL) which was adjusted to the desired pH value using 1M HCl. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Growth rates (µ) and $MaxOD_{600}$ values are reported as a percentage of the OD_{600} obtained in control cultures at pH 7.0. $MaxOD_{600}$ values were taken as the highest OD_{600} at each pH value after 30 h, during stationary phase. Data are the means of five replicates and the standard deviations are shown.

S. cerevisiae grew to a constant $MaxOD_{600}$ of between 1.4 and 1.5 across the entire pH range tested. In general, with decreasing pH there was a reduction in growth rate and also an increase in lag phase (appendix 12.1.1, page 204). At pH 6.0 the growth rate was reduced to 82 % of that at pH 7.0, and further reduced to 56 % at pH 5.0. *S. cerevisiae* grew much more slowly, at a rate of approximately 56 %, in the cultures adjusted to pH values of 3.0, 4.0 and 5.0. Therefore, between a pH value of 3.0 – 5.0 there is minimal effect on the growth rate of *S. cerevisiae* grown in YEPD medium, with respect to these three cultures. In a similar way to *E. coli*, the biomass concentration reached a value

similar to control cultures across all pH values tested. A decrease in the pH increased the lag time of the cultures (appendix 12.1.1, page 204). In the control experiments, the cells grew at a rate of 0.444 h^{-1} and exhibited a lag time of just 1 hour. In pH 3.0 cultures, however, the growth rate was reduced to 0.251 h^{-1} and the lag time increased to approximately 6 hours. It should be noted that *S. cerevisiae* could grow at pH 3.0, whereas no growth was observed in *E. coli* cultures at pH 3 (appendix 12.1.1, page 204).

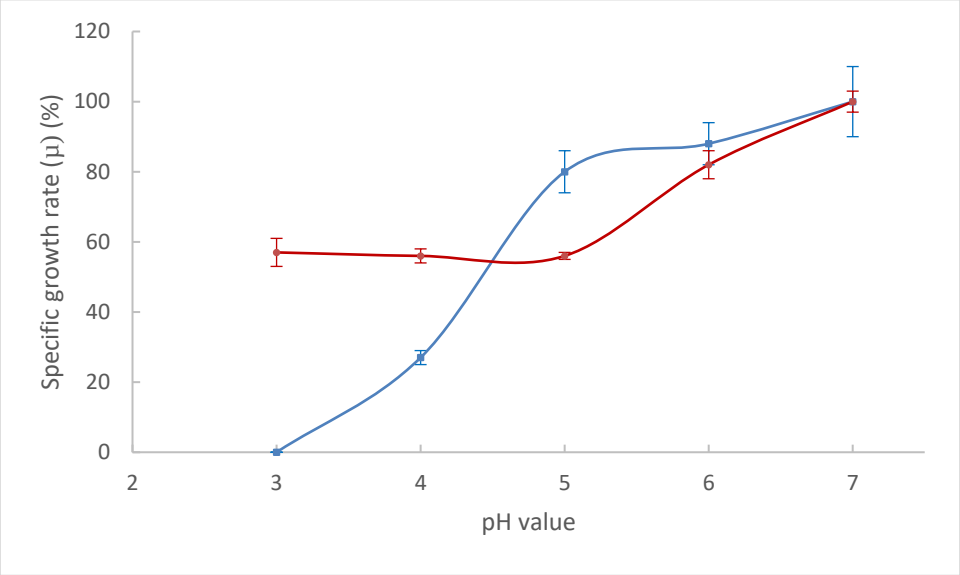


Figure 10. The effect of pH on growth rate of *E. coli* and *S. cerevisiae* in LB and YEPD medium, respectively

Stock solutions of LB and YEPD medium were adjusted to various pH values. *E. coli* MG1655 was incubated in LB medium (▪) and *S. cerevisiae* DSM 70449 was incubated in YEPD medium (●). Growth rates are expressed as a percentage of the control. Means of five replicates are shown and error bars represent standard deviation.

In conclusion, *S. cerevisiae* was the more tolerant organism of acidic conditions, showing a growth rate of 56 % and a final OD_{600} of 98 % at pH 3.0, compared to control cultures at pH 7.0. In comparison, *E. coli* showed no growth at pH 3.0, and a significantly slower growth rate of 27 % at pH 4.0. Between the pH values of 4.0 – 7.0, both organisms grew to final biomass concentrations similar to control cultures. The growth rates of *S. cerevisiae* demonstrated a gradual decline when the pH value was reduced from 7.0 to

5.0, however growth rates were extremely similar to one another between the pH ranges of 3.0 – 5.0 (Figure 10). A gradual reduction in growth rate was observed with decreasing pH value for *E. coli* cultures, demonstrating a significant drop below pH 5.0 to just 27 %.

Overall, the data suggest that *E. coli* could be a potential host biocatalyst for this process if the pH of the external medium was maintained at pH 4.0. However, below this there would be issues with cell inhibition due to acid shock. There was an indication that *S. cerevisiae* may be able to tolerate increased concentrations of acid at pH 3.0, allowing for a more economically sustainable process. However, this is dependent upon the ability of the organism to tolerate MAA itself at pH 3.0.

5.3 Effect of MAA on growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM70449 at pH 7

As both potential host organisms showed optimal growth at pH 7.0, the toxicity of MAA was determined at this pH value. At pH 7.0, MAA would be fully dissociated (H^+ and MAA^-) in aqueous solution. For the bioproduction of MAA to be considered as economically viable, the biocatalyst would be required to produce and tolerate a high concentration of MAA, which may inhibit cell growth.[6,7] Therefore, the effects of MAA on the growth of *E. coli* and *S. cerevisiae* were investigated in order to determine the concentration at which MAA would become toxic to the biocatalysts during its production at pH 7.0. The effect of MAA concentration was determined by growing *E. coli* MG1655 in LB medium, and *S. cerevisiae* DSM70449 in YEPD medium, which had been buffered to pH 7.0, in the presence of various concentrations of MAA (appendix 12.1.2.2, page 205) (Table 4).

Table 4. The effect of MAA concentration on the growth of *E. coli* and *S. cerevisiae* at pH 7.0

	***E. coli* MG1655**		***S. cerevisiae* DSM70449**	
	LB		**YEPD**	
Concentration of MAA (mM)	**μ (%)**	**MaxOD$_{600}$ (%)**	**μ (%)**	**MaxOD$_{600}$ (%)**
0	100 ± 1	100 ± 0	100 ± 2	100 ± 0
5	95 ± 0	98 ± 0	83 ± 9	100 ± 0
10	77 ± 1	96 ± 1	83 ± 13	97 ± 0
15	37 ± 4	94 ± 1	104 ± 4	94 ± 1
20	16 ± 1	86 ± 1	56 ± 9	89 ± 1
25	4 ± 1	20 ± 1	21 ± 0	72 ± 0
30	-	-	-	14 ± 2

E. coli MG1655 and *S. cerevisiae* DSM70449 were grown in LB and YEPD medium, respectively, buffered at pH 7.0. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Each well contained an appropriate amount of 1M MAA (appendix 12.1.2.1, page 205) to reach the desired final concentration of MAA, with a total well volume of 300µL. Growth rates (µ) and $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures at pH 7.0. $MaxOD_{600}$ values were taken as the highest OD_{600} value at each pH value after 48 h, during stationary phase. Data are the means of three replicates and the standard deviations are shown.

At pH 7.0, the growth rate of *E. coli* decreased as the concentration of MAA increased (Table 4). When exposed to a concentration of 5mM MAA, the growth rate was reduced to 95 % of control cultures, containing no MAA. At 10 mM MAA, the growth rate was further reduced to 77 %. This trend continued with increasing MAA concentration, until no growth was observed in the culture containing 30 mM MAA. Despite the steady decline in growth rate in the presence of between 0-30 mM MAA, the OD_{600} values were affected to a lesser extent by the presence of up to 20 mM MAA. A gradual decline in OD_{600} was observed with increasing MAA concentration, similarly to growth rate. However, at 20 mM MAA, where the growth rate was reduced by 84 %, the final biomass concentration was only reduced by 14 %. This was indicative of a greater effect of MAA on growth rate than on final biomass concentration. The highest concentration of MAA in which growth of *E. coli* was observed was

at 25 mM, albeit to an OD_{600} of just 20 % and at a growth rate of 4 %. This confirmed that MAA has significant inhibitory effects on the growth of *E. coli* cells grown in LB medium at pH 7.0.

S. cerevisiae demonstrated a slightly higher tolerance than *E. coli* towards the dissociated MAA species. It should be noted that the standard deviations are quite large for the *S. cerevisiae* tests in the cultures containing 5-20 mM MAA due to the formation of a biofilm. Biofilm formation occurs when cells aggregate together on a surface in order to minimize their exposure to a toxin. Hence, large deviations occurred in OD_{600} values as cells were not evenly distributed throughout the culture. At 20mM MAA, there was a slight reduction in OD_{600} to 89 % of control cultures. The growth rate was also reduced to 56 %, indicating that the dissociated form of MAA had inhibitory effects on the growth of *S. cerevisiae* at a concentration of 20 mM. In the presence of 25 mM MAA, there appeared to be a more significant effect on the growth rates and OD_{600} values observed. The growth rate was reduced to just 21 % of control cultures and the OD_{600} was reduced to 72 %. *S. cerevisiae* was therefore still tolerant of this concentration but exhibited much slower growth. At 30mM MAA, the cells were completely inhibited, reaching a biomass concentration of just 14 %, however the growth rate was so slow that it could not be calculated. Therefore, the toxic concentration of dissociated MAA towards *S. cerevisiae* at pH 7.0 was determined as between 25 – 30 mM, indicating that, similarly to *E. coli*, MAA has significant inhibitory effects towards *S. cerevisiae* when grown in complex medium, at pH 7.0.

To improve the economic viability of an industrial process, it would be desirable for the required growth medium to contain only the minimal nutrients that are essential for growth. Complex medium is costly, derived from plant and food materials and contains inorganic salts as a carbon and energy source for microbial growth. Minimal medium is chemically much less complex and offers reduced process costs, easier product recovery and cleaner waste streams.[33,35,201] Minimal medium contains a single carbon source, such as glucose, and a few trace elements, making this as a much cheaper option

than complex medium. However, the inhibitory effects of MAA may vary towards an organism when grown in a minimal medium, compared to a complex medium, which is rich in nutrients. Therefore, the effect of MAA was tested by growing *E. coli* MG1655 in MSX medium buffered to pH 7.0, in the presence of various concentrations of MAA. The results were compared with a control culture grown in MSX medium containing no MAA (12.1.2.2, page 205) (Table 5).

Table 5. Effect of MAA on the growth of *E. coli* in MSX medium at pH 7.0.

Concentration of MAA (mM)	μ (%)	MaxOD$_{600}$ (%)
0	100 ± 7	100 ± 3
5	66 ± 1	111 ± 3
10	25 ± 4	63 ± 4
15	-	18 ± 3
20	-	-
25	-	-
30	-	-

E. coli was grown in MSX medium buffered at pH 7.0. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Each well contained an appropriate amount of 1M MAA (appendix 12.1.2.1, page 205) to reach the desired final concentration of MAA, with a total well volume of 300µL. Growth rates (µ) and $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures at pH 7.0. $MaxOD_{600}$ values were taken as the highest OD_{600} value at each pH value after 48 h, during stationary phase. Data are the means of three replicates and the standard deviations are shown.

There is an observable effect on the growth of *E. coli* in the presence of MAA when the growth medium was changed from LB to MSX (Table 5). There was a fairly rapid decrease in growth rate with increasing concentrations of between 0-10 mM MAA. At 10 mM MAA, the growth rate reduced to just 25 % of control cultures containing no MAA. At concentrations of ≥ 15 mM MAA, *E. coli* showed very little, to no growth when cultured in MSX medium. Again, the OD_{600} was affected to a lesser extent than the growth rate. At 5 mM MAA, the

final biomass concentration reached was similar to control cultures, and at 10 mM, the OD_{600} was only reduced by 37 %. However, a significant reduction in OD_{600} was observed at 15 mM MAA, to just 18 % of control cultures. Above this concentration, *E. coli* was unable to grow in MSX medium at pH 7.0. Therefore, the growth of *E. coli* in MSX medium was affected significantly by lower concentrations of MAA, compared to when cultured in complex medium.

A comparison of the effect of MAA concentration on the growth rates of *E. coli* and *S. cerevisiae* in both minimal and complex media buffered at pH 7.0, are displayed below (Figure 11). *S. cerevisiae* was not tested in a minimal medium, as the growth of yeast requires complex medium to grow efficiently and therefore the results were likely to be inconclusive based on the specific inhibitory effects of MAA.

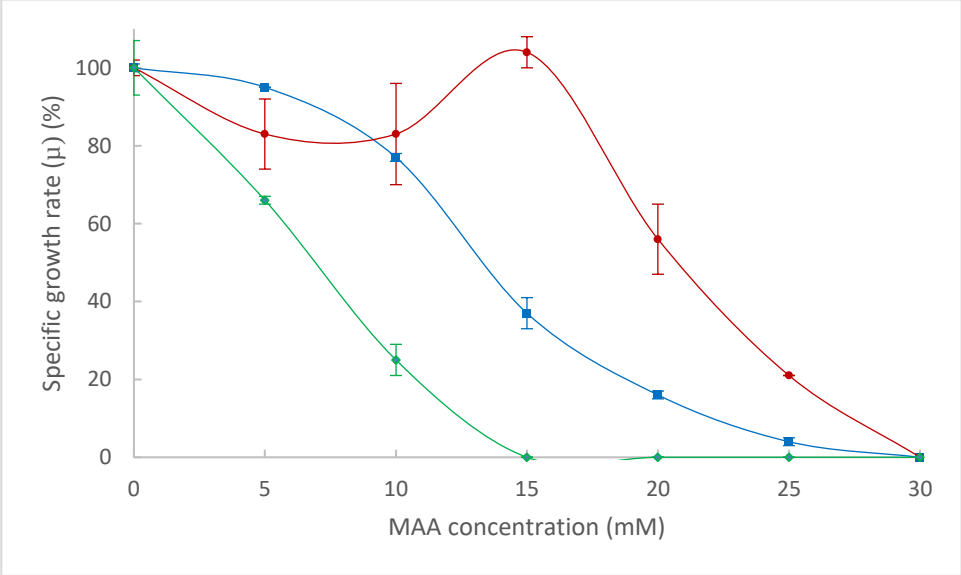


Figure 11. The effect of MAA concentration on the specific growth rates of *E. coli* and *S. cerevisiae* in complex and minimal medium buffered at pH 7.0

E. coli MG1655 was incubated in LB medium (▪), or MSX medium (♦). *S. cerevisiae* DSM 70449 was incubated in YEPD medium (●). Media contained various concentrations of MAA and was buffered at pH 7.0. Growth rates are expressed as a percentage of the control. Means of three replicates are shown and error bars represent standard deviation.

Based on the two organisms tested, *S. cerevisiae* tolerated the highest concentration of MAA, at pH 7.0 (Figure 11). The growth rate of *S. cerevisiae* in YEPD medium was only affected above a concentration of between 15 – 20 mM MAA, where the growth rate was reduced from 104 % to 56 %, respectively. *E. coli* demonstrated a lower tolerance towards MAA in both LB and MSX medium. In LB medium, the growth rate exhibited the most significant reduction between the concentrations of 10 – 15 mM MAA, where a reduction from 77 % to 37 % was observed. Additionally, when *E. coli* was cultured in MSX medium, the expressed tolerance of the organism towards MAA reduced even further. No growth was observed in the cultures containing 15 mM MAA and cultures containing 10mM MAA grew at a significantly reduced rate of just 25 % compared to control cultures, at pH 7.0.

5.4 Effect of MAA on growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM70449 at pH 4

As it was concluded that both *E. coli* and *S. cerevisiae* were able to grow in medium buffered at pH 4.0, it was also necessary to determine the toxicity of MAA at this pH value, to test the toxicity of the undissociated acid. At pH 4.0, only 22 % of the MAA would be dissociated, in contrast to the 100 % dissociation of MAA at pH 7.0. This increased percentage of MAA in its undissociated form could potentially improve the MAA recovery process, reducing downstream processing costs. Therefore, the effect of undissociated MAA was determined by growing *E. coli* MG1655 in LB medium and *S. cerevisiae* in YEPD medium that was pre-buffered to pH 4.0 (appendix 12.1.2.2, page 205) (Table 6).

Table 6. The effect of MAA concentration of the growth of *E. coli* and *S. cerevisiae* at pH 4.0

	***E. coli* MG1655**		***S. cerevisiae* DSM70449**	
	LB		**YEPD**	
Concentration of MAA (mM)	**μ (%)**	**MaxOD$_{600}$ (%)**	**μ (%)**	**MaxOD$_{600}$ (%)**
0	100 ± 8	100 ± 7	100 ± 25	100 ± 2
5	-	-	37 ± 2	74 ± 12
10	-	-	26 ± 4	63 ± 3
15	-	-	-	-
20	-	-	-	-
25	-	-	-	-
30	-	-	-	-

E. coli and *S. cerevisiae* were grown in LB and YEPD medium, respectively, adjusted to pH 4.0. The OD$_{600}$ was measured every 10 min using a Bioscreen C microplate reader. Each well contained an appropriate amount of 1M MAA (appendix 12.1.2.1, page 205) to reach the desired final concentration of MAA, with a total well volume of 300 µL. Growth rates (µ) and MaxOD$_{600}$ values are reported as a percentage of the values obtained in control cultures at pH 4.0. MaxOD$_{600}$ values were taken as the highest OD$_{600}$ at each pH value after 48 h, during stationary phase. Data are the means of three replicates and the standard deviations are shown.

At pH 4.0, *E. coli* was unable to grow in LB medium in the presence of MAA, showing no growth in the cultures that contained 5 mM MAA. Lower concentrations than 5mM were not tested, as this concentration equates to a production of 0.43 g/L, which would not make for a viable process. Therefore, the bioproduction of MAA at pH 4.0 would completely inhibit the growth of *E. coli*.

S. cerevisiae also showed a distinct lack of tolerance towards MAA when grown in YEPD medium at pH 4.0. At 5 mM MAA, a final biomass concentration of 74 % was achieved, albeit at a reduced growth rate of 37 % compared with control cultures at pH 4.0. In the cultures containing 10 mM MAA, there was a further reduction in both growth rate and MaxOD$_{600}$ to just 26 and 63 %, respectively. At this concentration there was also a significant increase in the lag phase, as the cells only began exponential growth 30 h after inoculation (appendix

12.1.2.2, page 205). Above 10 mM MAA, complete cell inhibition occurred and no growth was observed.

As MAA was found to be toxic towards *E. coli* at a concentration of <5 mM when cultured in complex medium at pH 4.0, the effects of MAA towards *E. coli* were not tested in MSX medium. The growth of *E. coli* at pH 7.0 had previously shown to be reduced when cultured in MSX medium compared to LB medium in the presence of MAA, and therefore it was anticipated a similar result would occur at pH 4.0.

Overall, the results confirmed that MAA toxicity would be a significant issue during its bioproduction. The toxicity of MAA towards *E. coli* and *S. cerevisiae* was significantly reduced at pH 7.0 and therefore it would be beneficial to carry out the process at pH 7.0 to produce the dissociated form of MAA. However, despite the improved tolerance of the MAA anion, toxicity would still be an issue at the concentrations required to create a viable process. Therefore, removal of the MAA from the aqueous phase would be necessary in order to reduce the concentration of MAA which would be in direct contact with the biocatalyst, thus preventing inhibition.

6 Toxicity of organic solvents towards growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM70449

As established in the previous chapter, MAA toxicity is a significant issue based on its accumulation in the aqueous phase during fermentation. Inhibition of the biocatalyst due to this accumulation will typically limit product titres, affect fermentation performance and operational procedures, and impact process economics.[6,7,9] Analysis of the literature indicates that two-phase extraction systems would be the best method for the recovery of MAA, reducing the concentration in the aqueous phase and therefore preventing inhibition of the biocatalyst.[9,16,19] Literature indicates that organic solvents are frequently used for this purpose, however are frequently toxic towards microorganisms.[9,16,202,203] Therefore, for this bioprocess to be considered sustainable, the toxicity of the solvent must be considered.

6.1 Effect of organic solvents on the growth of *E. coli* MG1655 and *S. cerevisiae* DSM70449 in complex medium

An assessment of the toxicity of various organic solvents towards *E. coli* MG1655 and *S. cerevisiae* DSM70449 was carried out. A range of organic solvents were selected, ensuring that each chosen solvent was hydrophobic, and therefore water immiscible, in order to produce the two-phase system. They were also selected based on their relative $\log P_{o/w}$ (logP) values, obtained from experimental values throughout the literature.[73,204,205] Solvents of logP values of between 2.1 and 6.8 and with a range of structures were selected.

The effect of organic solvents was tested by growing *E. coli* MG1655 and *S. cerevisiae* DSM70449 on LB and YEPD medium, respectively, in the presence of organic solvent (20 %v/v). Data for these tests was unable to be collected using the Bioscreen C MBR due to the solvents degrading the plastic well plates. Therefore, shake flasks were used, which had to be sampled manually. Growth of *E. coli* was monitored during the exponential phase by taking samples every 10 min in order to generate a growth curve and calculate the

growth rate (appendix 12.1.3, page 207). Growth rates were not calculated for *S. cerevisiae* because the duration of the lag phase was too unpredictable; the $MaxOD_{600}$ was recorded instead (Table 7).

Table 7. The effect of organic solvents on the growth of *E. coli* and *S. cerevisiae* in complex medium

		E. coli		***S. cerevisiae***
		LB		**YEPD**
Solvent	**$LogP_{o/w}$**	**μ (%)**	**$MaxOD_{600}$ (%)**	**$MaxOD_{600}$ (%)**
Control (No solvent)	-	100 ± 1	100 ± 1	100 ± 2
Benzene	2.1	-	-	-
Toluene	2.7	-	-	-
Tributyrin	3.0	90 ± 1	81 ± 0	79 ± 0
Cyclopentane	3.0	-	-	-
o-xylene	3.1	-	-	-
m-xylene	3.2	-	-	-
p-xylene	3.4	-	-	-
Cyclohexane	3.4	-	-	-
1,2,4-trimethylbenzene	3.6	-	-	-
isopropylbenzene	3.6	-	-	-
n-propylbenzene	3.7	-	-	-
Cycloheptane	4.0	-	-	-
Hexane	4.1	-	-	-
Heptane	4.3	92 ± 1	67 ± 1	-
Cyclooctane	4.5	91 ± 0	65 ± 0	-
Isooctane	4.6	99 ± 2	77 ± 0	-
1,4-diisopropylbenzene	5.0	56 ± 1	75 ± 1	-
Octane	5.2	93 ± 3	62 ± 0	-
Nonane	5.7	92 ± 0	93 ± 0	-

Decane	6.2	94 ± 2	96 ± 1	95 ± 0
Undecane	6.4	90 ± 2	82 ± 0	54 ± 1
Dodecane	6.8	91 ± 0	87 ± 1	82 ± 1

E. coli MG1655 was grown in LB medium and *S. cerevisiae* was grown in YEPD medium (7.9 mL) in the presence of organic solvent (2 mL, 20 %v/v). Tests were carried out using the shake-flask method, in 40 mL Teflon-sealed glass vials to prevent the loss of volatile solvents. Data was o The OD_{600} of the aqueous phase was measured after 72 h, diluting each sample by a factor of 10, using a UV/Vis spectrophotometer. Growth rates (μ) and $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures containing no organic solvent. Data are the means of three replicates and the standard deviations are shown.

Growth rates of *E. coli* were affected to a lesser extent than the $MaxOD_{600}$ values in the presence of the solvents. In the cultures containing solvents of a logP value greater than 4.1, the growth rates maintained a rate of between 90-99 % compared to control cultures. This observation is supported by various publications, which have reported that exponentially growing cells are more resistant to organic solvents, whereas the cell viability decreases significantly as soon as the cells reach stationary phase.[206,207] Solvents of a logP value of less than 4.3 caused complete inhibition of *E. coli*, with the exception of tributyrin. Tributyrin has a logP value of 3.0, however it allowed the growth of *E. coli* at a rate of 90 % compared to control cultures, similarly to solvents of a logP value of ≥ 4.3. The culture containing 1,4-diisopropylbenzene caused a significant reduction in growth rate to 56 % compared to control cultures containing no solvent. 1,4-diisopropylbenzene has a logP of 5, and therefore, when analysing the trend in toxicity based on this parameter, the toxicity cannot be attributed to the logP value of this solvent, as it is well above the toxic threshold value of 4.1. Therefore, the apparent inhibitory effects can be attributed to the chemical structure of this solvent itself. However, in general, when grown in complex medium in the presence of hydrocarbon solvents, *E. coli* cells grew at a rate similar to control cultures, despite the presence of the solvent.

The $MaxOD_{600}$ values showed more variation than the observed growth rates, with regards to toxic effects of the solvents. Final biomass concentrations were

observed to be affected by the presence of solvents, but to varying degrees, with little relationship to the logP value of the solvent. Generally, with decreasing logP value between the ranges of 6.8 to 4.3, the final biomass concentration decreased. Solvents with a logP value of between 4.3 and 5.7 caused a reduction in $MaxOD_{600}$ to between 65 – 77 % compared to control cultures. The solvents which exhibited the highest level of biocompatibility were the linear aliphatic solvents of C atom length C_9-C_{12}. These solvents are highly hydrophobic in nature and therefore will not partition in and disrupt the cellular membranes of *E. coli* as efficiently as the more polar solvents.[64,65]

S. cerevisiae had an extremely limited tolerance when exposed to the selection of organic solvents tested compared with *E. coli*, since only four solvents allowed growth (Table 7). All solvents with a logP value lower than that of decane (6.2) caused complete growth inhibition, with the exception of tributyrin (3.0), which allowed growth to a final biomass concentration of 79 % compared to control cultures. Therefore, the growth of *S. cerevisiae* was affected much more significantly by the presence of organic solvents compared to *E. coli*, when cultured in complex medium. Overall, solvents with a logP value of between 2.1 and 4.1 were toxic towards both organisms when cultured in complex medium. Solvents of a logP value of > 4.1 showed negligible toxicity towards *E. coli*, when grown in LB medium, however *S. cerevisiae* only tolerated solvents of a logP value of ≥ 6.2.

6.2 Effect of organic solvents on the growth of *E. coli* in minimal medium

As mentioned previously, MSX medium would be a more economically viable choice of growth medium for this process. However, when grown in a minimal medium, the cell membrane compositions, metabolic functions and therefore ability of the cells to deal with solvent stresses would be altered when compared to bacteria grown in complex medium. Therefore, the tolerance of *E. coli* towards organic solvents (20 % v/v) was tested in MSX medium. Growth rates could not be obtained easily because the lag phase was found to be

unpredictable. Additionally, the cultures in MSX medium formed emulsions with organic solvents and there was difficulty separating the cells from the solvents to measure OD during exponential growth. Therefore, a final $MaxOD_{600}$ value was measured after 72 h growth and separation of the phases. The results were compared with control cultures containing no organic solvent (Table 8).

Table 8. The effects of organic solvents on the growth of *E. coli* in MSX medium

Solvent	**$LogP_{o/w}$**	**$MaxOD_{600}$ (%)**
Control (No solvent)	-	100 ± 1
Benzene	2.1	-
Toluene	2.7	-
Tributyrin	3	117 ± 1
Cyclopentane	3	-
o-xylene	3.1	-
m-xylene	3.2	-
p-xylene	3.4	-
Cyclohexane	3.4	-
1,2,4-trimethylbenzene	3.6	-
isopropylbenzene	3.6	74 ± 5
n-propylbenzene	3.7	67 ± 3
Cycloheptane	4.0	116 ± 0
Hexane	4.1	87 ± 0
Heptane	4.3	132 ± 2
Cyclooctane	4.5	132 ± 4
Isooctane	4.6	131 ± 1
1,4-diisopropylbenzene	5	136 ± 0
Octane	5.2	127 ± 0
Nonane	5.7	122 ± 0

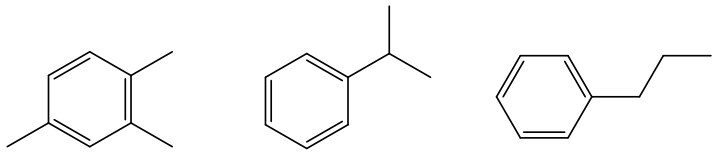
Decane	6.2	119 ± 0
Undecane	6.4	112 ± 1
Dodecane	6.8	104 ± 1

E. coli was grown in MSX medium at 37 °C, 280 rpm. Each test vial contained MSX inoculated with *E. coli* (8 mL) and organic solvent (2 mL), giving a total test volume of 10 mL, equating to 20 %v/v solvent/aqueous phase. The $MaxOD_{600}$ of the aqueous phase was measured after 72 h, diluting each sample by a factor of 10, using a UV/Vis spectrophotometer. $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures containing no organic solvent. Data are the means of three replicates and the standard deviations are shown.

In general, solvents with logP values of less than 3.6 caused inhibitory effects towards *E. coli* grown in MSX medium, whereas solvents with a logP value above 3.6 were biocompatible, due to the increased hydrophobicity of the solvents (Table 8). The aliphatic solvents containing both straight and branched chains were non-toxic, with only hexane causing a slightly reduced final biomass concentration of 87 % compared to control cultures. As before, tributyrin was also biocompatible with *E. coli* when grown in MSX medium regardless of its low logP value of 3.0.

A key observation was that in the presence of solvents with a logP value of greater than 4.0, *E. coli* cells grew to apparent final biomass concentrations of up to 36 % higher than control cultures. There was a distinct rise and fall in final biomass concentration for the solvents with a logP value of between 4.3 - 6.8, with *E. coli* cultures in the presence of 1,4-diisopropylbenzene (logP 5.0) demonstrating the highest OD_{600} reached of 136 % compared to control cultures containing no organic solvent. The cultures were observed post-solvent exposure under a microscope and emulsions were seen to have formed in the aqueous phase. Therefore, the excess growth may have been due to an increased amount of light scattering caused by the emulsions. Further tests would be required to explain the apparent increased OD of *E. coli* in MSX when exposed to these solvents, however this work was considered as outside the scope of this project and therefore was not investigated further in this case.

For solvents with logP values of between 3.6 and 4.1, an effect due to chemical composition was apparent. The toxic effects which were associated with 1,2,4-trimethylbenzene, isopropylbenzene and *n*-propylbenzene, indicated differences based on the structural variation of the molecules (Figure 12).



1,2,4-trimethylbenzene	isopropylbenzene	*n*-propylbenzene
LogP 3.6	LogP 3.6	LogP 3.7
0%	74%	67%

Figure 12. Chemical structures of 1,2,4-trimethylbenzene, isopropylbenzene and n-propylbenzene.

The relative logP values and respective final $MaxOD_{600}$ values of *E. coli*, expressed as a percentage of the control culture, are shown for each solvent.

The cultures containing 1,2,4-trimethylbenzene (logP 3.6) showed no growth, however, in the presence of isopropylbenzene (logP 3.6), *E. coli* cells to grew to 74 %, while the culture containing *n*-propylbenzene (logP 3.7) grew to 67 %, compared to control cultures. This suggested that branched alkyl-substituted cyclic, conjugated aromatic solvents, such as isopropylbenzene, have a reduced ability to accumulate in and disrupt bacterial cell membranes when compared with a methyl-substituted cyclic aromatic solvent. This observation is most likely due to steric factors which are introduced within molecules containing branched substituents. This was particularly interesting as many studies have generally attributed solvent toxicity to logP value, whereas this result shows a distinct structural effect.[63,65,69]

Another notable result was the difference in toxicity between cycloheptane (4.0) and hexane (4.1). Cultures grown in the presence of cycloheptane grew to a final biomass concentration of 116 %, whereas cultures containing hexane were slightly inhibited and grew to a final biomass concentration of 87 %

compared to control cultures. Cycloheptane adopts a twist chair conformation to prevent eclipsing hydrogens.[208] Thus, it is essentially more sterically hindered than a hexane molecule, which is a flexible linear chain. This could potentially allow hexane molecules to enter the membranes more easily than cycloheptane molecules based on their conformations. However, this is speculative and further tests investigating the interactions of the solvents with cell membranes at a molecular level would be required to explain this result. Additionally, aliphatic and heterocyclic hydrocarbons have been suggested to have different inhibitory effects on cellular activities once they have entered the cells.[65] It has been reported that when exposed to hexane, the inner and outer membranes of *E. coli* cells were found to be detached from one another and the periplasmic space was expanded, suggesting damage of the inner membrane.[209] This could also explain the observed difference in toxicity of both of hexane and cycloheptane, which may not be attributed to logP value.

7 Extraction of MAA into biocompatible organic solvents

Several biocompatible organic solvents were identified in the previous chapter. These solvents could potentially be used in two-phase systems for the *in situ* extraction of MAA, during its biocatalytic production. However, the viability of the process is dependent upon the extraction efficiency of each solvent, and whether MAA will partition preferentially in the organic phase as opposed to the polar aqueous phase. The solvents which were found to be inhibitory towards the growth of *E. coli* MG1655 and *S. cerevisiae* DSM70449 were not tested for their ability to extract MAA, as the presence of these solvents in the two-phase system would be detrimental to the organisms and therefore they were deemed unsuitable as potential extractants.

In order to determine the extraction efficiencies, the partition coefficient (K_D) can be calculated for MAA in each solvent system.

$$K_D = \frac{[\mathrm{MAA_{org}}]}{[\mathrm{MAA_{aq}}]} = D = \frac{[\mathrm{MAA_{org}}]\mathrm{total}}{[\mathrm{MAA_{aq}}]\mathrm{total}}$$

Equation 1.

K_D is a measure of how well extracted a species is in an immiscible two-phase system. A large K_D value indicates that extraction into the organic solvent is favourable. It is calculated by dividing the concentration of MAA in the organic phase by the concentration of MAA in the aqueous phase, in a system which is at dynamic equilibrium, as shown by Equation 1, where [MAA] represents the concentration of MAA in either the organic or aqueous phase, as denoted by MAA_{org} or MAA_{aq}, respectively.

To evaluate the efficiency of an extraction, the total concentration of MAA in each phase must be considered. This is known as the distribution ratio, *D*. However, in the case of this particular two-phase system, as the only factor affecting the extraction efficiency is the partitioning of MAA between the two

phases, K_D and D will have the same value and can therefore be referred to interchangeably (Equation 1).

$$E(\%) = \frac{[\mathrm{MAA_{org}}]}{[\mathrm{MAA_{total}}]} \text{ x } 100$$

Equation 2.

The extraction efficiency (E) can be calculated, as shown by Equation 2, where [MAA_{total}] represents the total concentration of MAA in the system and [MAA_{org}] remains as previously defined. E represents the percentage transfer of MAA from the MSX phase into the organic solvent, and therefore quantifies the efficiency of the extraction process.

In these particular two-phase extraction systems, MSX medium was used as the aqueous phase and therefore, as MSX is buffered at pH 7.0, the data obtained are based on the presence of the anionic species of MAA in the aqueous phase, as this was deemed the less toxic of the two species in Chapter 5. The concentration of MAA in the aqueous phase was determined using HPLC-UV (appendix 12.2.1, page 217). It should be noted that the results were obtained based on sampling of the MSX phase only. MAA is a non-volatile compound and therefore vaporization of the acid would be unlikely to occur under the conditions used for these tests. Therefore, the concentrations obtained using this method would be sufficient in order to calculate the concentration of MAA in the solvent phase, by subtracting the final concentration in the aqueous phase from the starting concentration. Each method differed slightly based on the variable which was being investigated i.e. temperature, concentration of MAA and the phase ratio.

It was important to establish the kinetics of the extraction process. Initial tests monitored the extraction of MAA by taking samples at specific time intervals (Table 9). The aim of this was to establish the quantity of MAA that could be extracted *in situ* during continuous production, as it would be in an industrial process, at various time intervals.

Table 9. Efficiency of MAA extraction into heptane at various time intervals

	Extraction Efficiency (*E*) (%)	
Time (h)	**30 °C**	**37 °C**
1	24 ± 1	26 ± 2
2	25 ± 2	27 ± 2
3	24 ± 2	25 ± 1
24	26 ± 2	24 ± 2
21 days	24 ± 2	27 ± 2

Extractions were carried out in 40mL Teflon sealed glass vials. Biphasic mixtures of 500 mM MAA in MSX medium (8 mL) and organic solvent (2 mL, 20 %v/v) were agitated using a magnetic flea over a period of 24 h, at 30 and 37 °C, 280 rpm. There was significant mixing of the phases as a large vortex was visible during the mixing period. A 10 min phase separation period was allowed prior to the sampling of the MSX phase (100 µL). The sample was then diluted with MSX (900 µL), before injecting into the HPLC. The samples were left to phase separate for 21 days, without agitation, to achieve dynamic equilibrium. The concentration of MAA in each phase was then calculated. *E* (%) was calculated using Equation 2. Data are the means of three replicates and the standard deviations are shown. No visible emulsion formation occurred once mixing was stopped.

The removal of MAA from the aqueous phase by each solvent reached its maximum extraction efficiency before 1h (Table 9). The increase in MAA extraction efficiency into heptane after 1h and 21 days was not statistically significant. However, despite the maximum extraction by the solvent seeming to occur immediately, to ensure that accurate values of *D* were calculated, the two-phase systems containing MAA were left to equilibrate for 21 days before sampling. Thus, all data reported in this chapter is based upon the results obtained after vigorous agitation, followed by a 21-day period to allow for phase separation to ensure equilibria had been established.

7.1 Effect of temperature

Initial tests focused on obtaining the distribution ratio (*D*) of MAA and the extraction efficiency (*E*) of each solvent at two different temperatures, focusing on the optimal growth temperatures of 30 ℃ and 37 °C for *S. cerevisiae* and *E. coli,* respectively. This would mimic the conditions in which the continuous extraction process would be carried out during the

bioproduction of MAA. The aim of this work was to determine the effects of temperature on the partitioning of MAA between the two phases, and the consequent extraction efficiency of each solvent. The effect of temperature on the extraction efficiency of solvents was tested by agitating two-phase systems of MSX containing a known concentration of MAA and organic solvent at different temperatures and analysing the concentration of MAA in each phase after extraction (Table 10).

Table 10. The effect of temperature on the extraction of MAA into organic solvents

		Distribution Ratio (*D*)		Extraction Efficiency (*E*) (%)	
Solvent	**logP**	**30°C**	**37°C**	**30°C**	**37°C**
Tributyrin*	3.0	0.89 ± 0.09	0.98 ± 0.07	47 ± 2	50 ± 2
Isopropylbenzene	3.6	0.45 ± 0.15	0.48 ± 0.01	30 ± 8	33 ± 1
n-propylbenzene	3.7	0.41 ± 0.02	0.47 ± 0.01	29 ± 2	32 ± 1
Cycloheptane	4.0	0.38 ± 0.01	0.46 ± 0.04	27 ± 1	31 ± 2
Hexane	4.1	0.30 ± 0.02	0.47 ± 0.02	23 ± 1	32 ± 1
Heptane	4.3	0.31 ± 0.03	0.38 ± 0.03	24 ± 2	27 ± 2
Cyclooctane	4.5	0.30 ± 0.02	0.37 ± 0.02	23 ± 1	27 ± 3
Isooctane	4.6	0.35 ± 0.03	0.37 ± 0.05	26 ± 2	27 ± 2
1,4-diisopropylbenzene	5.0	0.37 ± 0.02	0.50 ± 0.06	27 ± 1	33 ± 3
Octane	5.2	0.32 ± 0.13	0.41 ± 0.07	23 ±7	29 ± 3
Nonane	5.7	0.29 ± 0.05	0.41 ± 0.05	20 ± 3	29 ± 3
Decane	6.2	0.21 ± 0.06	0.27 ± 0.01	17 ± 4	19 ± 1
Undecane	6.4	0.09 ± 0.01	0.16 ± 0.02	9 ± 2	14 ± 2
Dodecane	6.8	0.10 ± 0.01	0.09 ± 0.02	9 ± 1	9 ± 1

Two-phase solvent extractions were carried out in 40 mL Teflon sealed glass vials containing organic solvent (2 mL) and a buffered stock solution of 500 mM MAA in MSX (8 mL). Each solvent system was incubated at either 30 or 37°C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. There was significant mixing of the phases as a large vortex was visible during the mixing period. No visible emulsion formation occurred once mixing was stopped unless indicated*. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each solvent system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown. *Tributyrin formed an emulsion at the phase boundary, however full separation of the phases occurred after 3 h of no agitation.

As mentioned previously, a high distribution ratio is indicative of a greater concentration of MAA in the organic phase. Thus, the higher the value of *D*, the greater the extraction efficiency of that particular solvent. The solvent which showed the highest extraction efficiencies was tributyrin, showing an efficiency of 47 and 50 % at 30 and 37 °C, respectively. The least efficient

solvent was dodecane, demonstrating an efficiency of just 9 % at both temperatures. There is a clear relationship between the logP value of the solvent, and the resulting values of *D* and *E*. As the logP value increases, *D* and *E* decreased, confirming that the more efficient solvents for the extraction of MAA were solvents with a lower logP values. This corresponds with the phenomenon that like dissolves like, with respect to the polarity of a solvent and solute.

A particularly notable result was the slight increase in *E* for systems containing 1,4-diisopropylbenzene, at 37 °C. MAA has a carbonyl group conjugated to the C=C double bond. This conjugation within the MAA molecule may give rise to π-π interactions between itself the aromatic ring in 1,4-diisopropylbenzene, which could explain the similar extraction efficiencies of isopropylbenzene, *n*-propylbenzene and 1,4-diisopropylbenzene, despite the differences in logP value.

D and *E* were slightly higher for all solvents at 37 °C when compared to 30 °C. By considering the process as a macroscopic thermodynamic extraction process, the distribution ratio can be fitted into Equation 3 in order to determine the change in enthalpy (ΔH) in relation to the change in temperature.

$$\ln D = \mathrm{C} + (-\Delta H)/RT$$

Equation 3.

Equation 3 shows the relationship between change in enthalpy and the distribution ratio, where *D* is the distribution ratio, C is constant, ΔH is the change in enthalpy of the extraction process, *R* is the molar gas constant (8.314 J mol^{-1} K^{-1}), and *T* is the absolute temperature (K). By inputting the values of *D* at each temperature and solving the equation using linear regression, the change in enthalpy of the extraction process (ΔH) could be calculated for each solvent system. The extraction process was endothermic, with all ΔH values for each solvent equating to a positive value (appendix 12.2.2, page 218). This

indicated that an increase in temperature improved the efficiency of extraction by means of an increase in energy within the system, however the effects of temperature variation were not hugely significant.

As only four of the most hydrophobic solvents tested were biocompatible with *S. cerevisiae*, if it was to be chosen as the host organism, the resulting MAA recovery would be extremely poor. Decane, undecane and dodecane demonstrated the lowest efficiencies of between 9 – 19 %, and therefore their use as extractants would not make for a viable process. The increase in temperature which would come with choosing *E. coli* as the host organism resulted in an increase in *E* of between 1-6 %, which suggests temperature to be fairly insignificant during the extraction process. The highest performing solvent, tributyrin, showed biocompatibility with both *S. cerevisiae* and *E. coli*, suggesting this solvent may have the potential to be used as the extractant in the process. However, efficiencies of 47 % and 50 % are still fairly low and would be unlikely to produce an industrially viable process.

7.2 Effect of phase ratio

In order to create a viable process, it would be beneficial for the organic extractant layer to be of minimal volume to decrease costs. A solvent which has a high capacity for MAA, and therefore large distribution ratio, would be ideal, particularly as a smaller phase ratio of solvent/aqueous phase could then be utilized. Therefore, the aim of this work was to investigate the effects of minimizing the volume of the organic phase on the recovery of MAA in a heptane/MSX system.

As all the solvents with a logP value of between 3.6 - 5.7 had similar extraction efficiencies of between 29 and 33 %, it was anticipated that each solvent would be affected in a similar way with regards to varying the phase ratio. Therefore, heptane was chosen as the representative solvent to demonstrate the effects of changing these variables. Heptane has a logP value of 4.3, which is in the middle range of all the solvents tested. It is also frequently used by Lucite International for the extraction of MAA during its current chemical production.

The effect of phase ratio (%) on the extraction of MAA into organic solvents was tested by agitating two-phase systems of MSX containing a constant concentration of MAA with various volumes of heptane. The concentration of MAA in each phase was analysed after extraction (Table 11).

Table 11. The effect of phase ratio on the extraction of MAA into heptane

	Distribution Ratio (*D*)		**Extraction Efficiency (*E*) (%)**	
Phase ratio (% v/v)	**30 °C**	**37 °C**	**30 °C**	**37 °C**
5	0.02 ± 0.02	0.10 ± 0.03	2 ± 2	9 ± 3
10	0.11 ± 0.03	0.17 ± 0.01	10 ± 3	14 ± 1
20	0.31 ± 0.03	0.38 ± 0.03	24 ± 2	27 ± 2

Solvent extractions were carried out in 40 mL Teflon sealed glass vials containing a buffered stock solution of 500 mM MAA in MSX and phase ratios equalling 5, 10 and 20 % v/v of heptane, resulting in a total volume of 10mL. Each solvent system was incubated at either 30 or 37 °C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. There was significant mixing of the phases as a large vortex was visible during the mixing period. No visible emulsion formation occurred once agitation was stopped. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each solvent system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown.

As the phase ratio increased, there was an increase in *D* and *E*. At a phase ratio of 5 %, at 30 °C, the extraction efficiency of heptane was only 2 %, indicating that basically no extraction is occurring at 5 % v/v heptane. With an increase in temperature to 37 °C, E only increased to 9 % which was still extremely poor. When the phase ratio was increased in 10 %, *E* increased, however only by a negligible amount of 5 – 8 %. At 20 % v/v heptane/MSX medium, the highest efficiency was obtained (24 and 27 % at 30 and 37 °C, respectively). This suggested that for an increased concentration of MAA to be recovered using organic solvents, a large volume of solvent would be required. It would be anticipated that increasing the phase ratio further would also increase *D* and

E, however this would increase the cost of the process, making it uneconomical.

7.3 Effect of MAA concentration

The current production titres of MAA by *E. coli* are low, at around 170 µM, without an additional extraction phase.[6] Therefore, it was of interest to investigate the effects of MAA concentration on the extraction efficiencies of each solvent system. If *E* decreases with decreasing starting concentration of MAA in the MSX phase, this could affect the viability of the process as a whole. As was previously shown, a starting concentration of 500 mM MAA resulted in poor extraction efficiencies of between 9 – 50 %. Therefore, decreasing this concentration further could indicate that there would be significant issues with using organic solvents as extractants in this particular process.

The effect of MAA concentration on the extraction efficiency of organic solvents was tested by agitating two-phase systems of MSX containing different concentrations of MAA, and organic solvent and analysing the concentration of MAA in each phase after extraction (Table 12).

Table 12. The effect of MAA concentration on the extraction of MAA into heptane

	Distribution Ratio (*D*)		Extraction Efficiency (*E*) (%)	
Concentration of MAA (mM)	**30 °C**	**37 °C**	**30 °C**	**37 °C**
6.25	-	-	-	-
12.5	-	-	-	-
25	-	-	-	-
50	0.05 ± 0.01	0.09 ± 0.01	5 ± 1	9 ± 1
100	0.08 ± 0	0.17 ± 0.03	7 ± 0	15 ± 3
200	0.18 ± 0.02	0.20 ± 0.05	15 ± 2	17 ± 4
300	0.24 ± 0.1	0.26 ± 0.01	19 ± 7	21 ± 1
400	0.25 ± 0.08	0.33 ± 0.02	20 ± 5	25 ± 1
500	0.31 ± 0.03	0.38 ± 0.03	24 ± 2	27 ± 2

Solvent extractions were carried out in 40mL Teflon sealed glass vials. A stock solution of 500 mM MAA in MSX was diluted to the desired concentration for each test, using fresh MSX medium. Each vial contained a specific concentration of MAA in MSX (8 mL) and heptane (2 mL, 20 %v/v). The solvent systems were incubated at either 30 or 37 °C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. There was significant mixing of the phases as a large vortex was visible during the mixing period. No visible emulsion formation occurred once agitation was stopped. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each solvent system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown.

Between the concentrations of 6.25 and 25 mM MAA no detectable extraction occurred, therefore the production of a concentration of ≤ 25 mM MAA when using organic solvents as extraction medium would not create a viable process. At a concentration of between 50 – 500 mM MAA, *D* and *E* increased linearly with increasing concentration of MAA at both 30 and 37 °C. As the concentration of MAA doubled, the extraction efficiency increased by between 2 – 8 % (Figure 13).

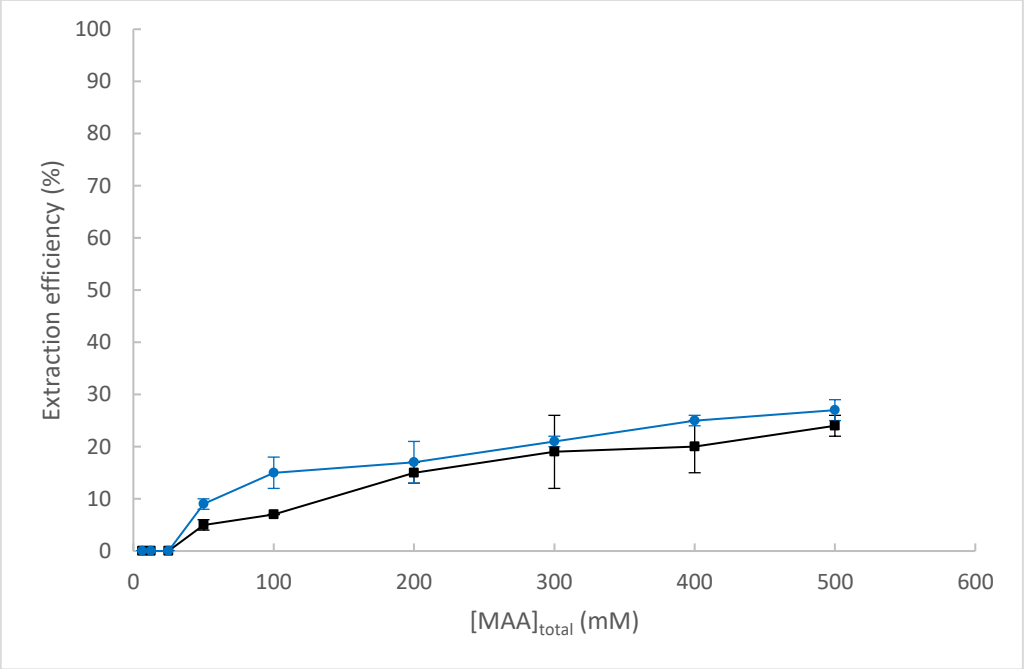


Figure 13. The effect of MAA concentration on the extraction efficiency of heptane

Various concentrations of MAA in MSX medium were agitated in the presence of heptane (20 %v/v) for 24 h, 280 rpm. Extraction efficiencies were calculated using Equation 2 after equilibrium was reached at 30°C (▪) and 37 °C (●). Means of three replicates are shown and error bars represent standard deviation.

Increasing the temperature of the system showed a slight improvement in extraction efficiency, similarly to the previous results obtained at a constant concentration. However, an increase in temperature had a greater influence on the systems containing lower MAA concentrations. As the temperature increased from 30 to 37 °C, there was still no detectable extraction in the tests containing ≤ 25 mM MAA. However, *E* almost doubled in value in the tests containing 50 and 100 mM MAA (Figure 13). Whereas, in the more concentrated samples of greater than 200 mM MAA, an increase in temperature had a smaller effect, increasing *E* by around 2-5 %. This could be linked to the solubility of MAA in heptane and the thermal effects on mass transfer from the initially more concentrated MSX phase. However, overall, to recover a high enough concentration of MAA to create an economically sustainable process using organic solvents, a higher concentration of MAA would need to be produced by the biocatalysts.

Ultimately, the use of the select few biocompatible organic solvents for the extraction of MAA would not create a viable process. The increased logP values which result in a reduction in toxicity towards the biocatalyst, unfortunately reduces the affinity of MAA towards the solvent, resulting in poor extraction efficiencies. Therefore, using organic solvents as extraction medium would not create a viable process and therefore alternative extraction solvents must be investigated for the *in situ* recovery of MAA.

8 ILs as alternative solvents for the liquid-liquid extraction of MAA

Although organic solvents are low cost, recyclable and have low viscosities, using them in liquid-liquid extraction processes involving microorganisms can create issues with toxicity.[18,19,153] As was demonstrated in Chapters 6 and 7, organic solvents are limited with regards to their biocompatibility when extracting polar compounds, such as MAA. The polar solvents, which would offer the highest extraction efficiencies, were unfortunately detrimental to *E. coli* and *S. cerevisiae*. Additional drawbacks when using organic solvents in industrial bioprocesses include high volatility and flammability, implying additional environmental hazards, and the formation of stable emulsions in fermentation systems, hindering product recovery.[74,142,210]

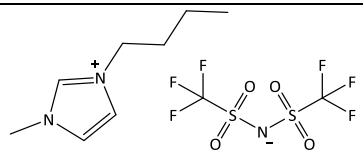
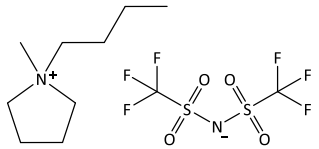
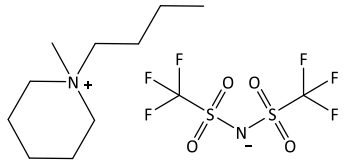
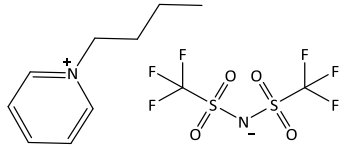
Ionic liquids (ILs) have received increasing attention with regards to their potential use as solvents in two-liquid-phase fermentation processes.[111,137,153,155] The structure of ILs can be tailored to lower their specific toxicities towards the biocatalysts.[176,211] Additionally, they offer favourable, tunable solvating properties for a number of polar compounds, including organic acids.[119,153,155] Therefore, the use of ILs as alternative solvents for the ISPR of MAA has been investigated.

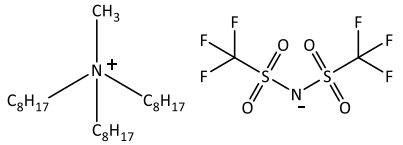
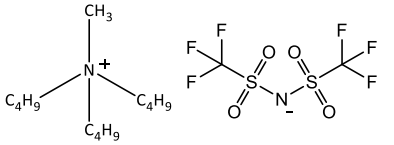
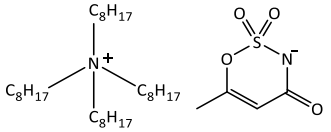
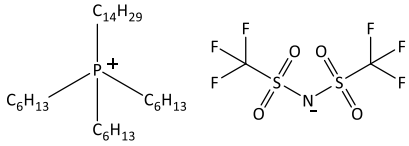
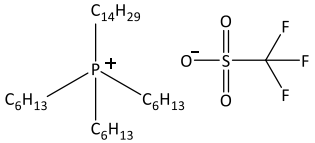
8.1 The selection and synthesis of ILs

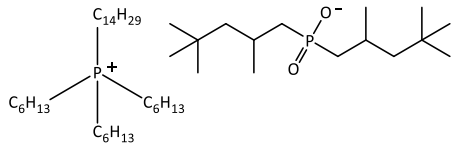
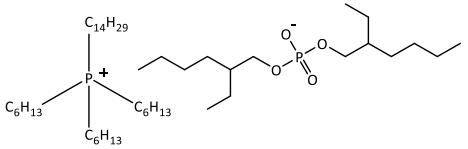
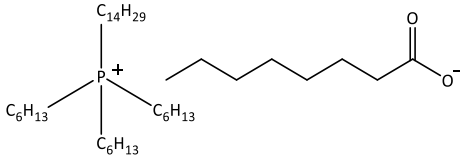
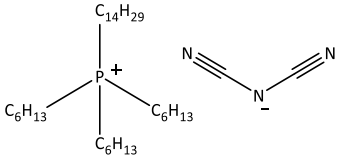
The selection and synthesis of ILs focused on a logical and systematic approach. IL solvent properties are mainly determined by the ability of the sale to act as a hydrogen-bond donor and/or acceptor, and the degree of localization of the charges on the anions.[212] Therefore, considering this, along with toxicity trends reported in the literature, a selection of common cations and anions were chosen. Additionally, room-temperature ionic liquids (RTILs) were also selected based upon their hydrophobicity, ensuring the formation of a two-phase system necessary for the liquid-liquid extraction process. The density of each IL was also an important factor. At both lab and plant scale, if

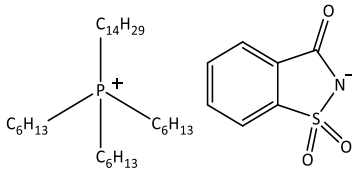
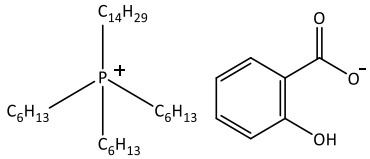
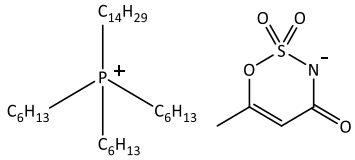
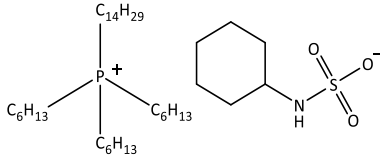
the density of the IL is very close to that of the aqueous solution then separation of the phases will be slow, which needed to be taken into account during extraction experiments. A total of twenty-two ILs were synthesised, using adapted synthetic procedures reported throughout the literature. The purity of each IL was confirmed by nuclear magnetic resonance (NME) spectroscopy, mass spectrometry (MS) and ion chromatography (IC) prior to their use in any further experiments. The structures of the combined cation and anion moieties forming each IL, along with their abbreviated nomenclature, by which they will be referred to throughout this *thesis* are displayed below (Table 13).

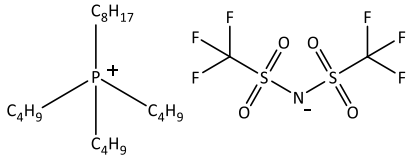
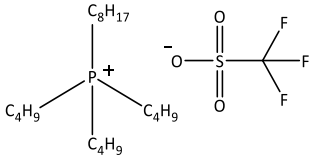
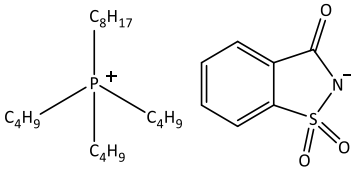
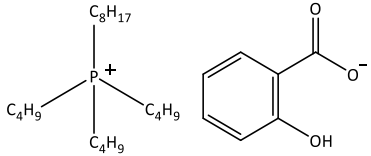
Table 13. Synthesized IL structures and their common abbreviations

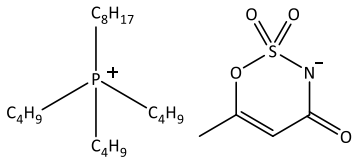
Name	Abbreviation	ρ (30˚C) (g/mL)	η (30˚C) (mPa·s)	Structure	Reference
1-n-Butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide	[BMIm][NTf_2]	1.43	69.0		134
1-n-Butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide	[BMPyrr][NTf_2]	1.41	85.0		184
1-n-Butyl-1-methylpiperidinium bis(trifluoromethane)sulfonimide	[BMPip][NTf_2]	1.46	58.4		186
1-n-Butylpyridinium bis(trifluoromethane)sulfonimide	[BPyr][NTf_2]	1.45	56.8		187

Trioctyl(methyl)ammonium bis(trifluoromethane)sulfonimide	$[N_{8881}][NTf_2]$	1.09	218.0		188
Tributyl(methyl)ammonium bis(trifluoromethane)sulfonimide	$[N_{4441}][NTf_2]$	1.32	241.5		133
Tetraoctylammonium Acesulfame	$[N_{8888}][Ace]$	0.94	472.7		Novel compound
Trihexyl(tetradecyl)phosphonium bis(trifluoromethane)sulfonimide	$[P_{66614}][NTf_2]$	1.07	224.9		177
Trihexyl(tetradecyl)phosphonium trifluoromethanesulfonate	$[P_{66614}][OTf]$	0.99	336.5		189

Trihexyl(tetradecyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate	$[P_{66614}][(^{i}C_8)_2PO_2]$	0.95	98.3		191
Trihexyl(tetradecyl)phosphonium bis(2-ethylhexyl)phosphate	$[P_{66614}][\ (^{i}C_8O)_2PO_2]$	0.96	124.3		191
Trihexyl(tetradecyl)phosphonium octanoate	$[P_{66614}][C_7COO]$	0.92	66.4		190
Trihexyl(tetradecyl)phosphonium dicyanamide	$[P_{66614}][DCA]$	0.93	125.2		192

Trihexyl(tetradecyl)phosphonium saccharinate	$[P_{66614}][Sacch]$	1.03	349.5		Novel compound
Trihexyl(tetradecyl)phosphonium salicylate	$[P_{66614}][Sal]$	0.98	141.6		193
Trihexyl(tetradecyl)phosphonium acesulfame	$[P_{66614}][Ace]$	1.01	241.1		194
Trihexyl(tetradecyl)phosphonium cyclamate	$[P_{66614}][Cyc]$	0.99	148.7		Novel compound

Tributyl(octyl)phosphonium bis(trifluoromethane)sulfonimide	$[P_{4448}][NTf_2]$	1.02	186.9		195
Tributyl(octyl)phosphonium trifluoromethanesulfonate	$[P_{4448}][OTf]$	1.00	224.1		195
Tributyl(octyl)phosphonium saccharinate	$[P_{4448}][Sacch]$	1.12	140.5		Novel compound
Tributyl(octyl)phosphonium salicylate	$[P_{4448}][Sal]$	1.02	56.9		Novel compound

Tributyl(octyl)phosphonium acesulfame	$[P_{4448}][Ace]$	1.09	160.4		Novel compound

In order to evaluate the use of ILs as solvents in this process, the cations selected were commonly used heterocyclic aromatic and non-aromatic structures, and also ammonium and phosphonium-based structures. With regards to the heterocyclic cations such as *N*–alkyl-*N*-methylimidazolium and *N*-alkyl-*N*-methylpyrrolidinium ILs, there have been many publications claiming alkyl chain length to be a key factor in increasing or decreasing the biocompatibility of these particular ILs.[158,213] It is widely accepted that toxicity, based on the cation structure, increases significantly with increasing alkyl chain length, particularly over eight carbon atoms in length.[158,162,168] Therefore, the side chains of the imidazolium, pyrrolidinium, piperidinium and pyridinium ILs were limited to 4 carbon atoms in length. The aim of this was to prevent the exposed hydrophobic alkyl chain from permeating the cell membrane of the biocatalyst.[214]

The choice of anion generally governs the physical properties of ILs.[114] Therefore, in order for the heterocyclic cations to be both water immiscible and in a liquid state at room temperature it was essential for them to be coordinated with hydrophobic, fluorinated anions such as $[NTf_2]^-$. As these ILs were being tested with the aim of eventually being scaled up to create an industrial process, cost was highly important. The salt, $[LiNTf_2]$, is an extremely expensive starting material, costing around £3500/kg (Sigma-Aldrich). Therefore, alternative starting materials with significantly lower cost, a fraction of the price, have been used to produce ILs. Furthermore, anions such as saccharinate $[Sacch]^-$, salicylate $[Sal]^-$, cyclamate $[Cyc]^-$ and acesulfame $[Ace]^-$ are generally recognised as safe (GRAS). The sodium salts of these anions are significantly cheaper than the more commonly used starting materials, costing around £80/kg (Sigma-Aldrich). Additionally, they also show promise of being non-toxic, due to their GRAS status, and offer higher polarities and increased hydrogen bonding abilities, which could potentially increase the efficiency of extraction of MAA. However, due to their hydrophilic nature, these anions could only be coordinated with phosphonium cations, which are highly hydrophobic, to give a water immiscible IL.

With respect to phosphonium-based ILs, it has been shown that longer chains, such as trihexyl(tetradecyl)phosphonium $[P_{66614}]^+$ ILs, have significantly reduced toxicities as opposed to their shorter chain equivalents.[113] This is believed to be due to the high charge density phosphonium core being shielded by the long lipophilic chains.[189] This potentially reduces the attraction of the positively charged atom to the negatively charged phospholipid cell membrane within microorganisms. The $[P_{66614}]^+$ cation was also paired with other hydrophilic anions including $[DCA]^-$, $[C_7COO]^-$, $[(^iC_8O)_2PO_2]^-$, $[(^iC_8)_2PO_2]^-$, and $[TfO]^-$.

The shorter chained phosphonium cation $[P_{4448}]^+$ was also investigated in order to determine if a decrease in alkyl chain length would improve physical properties that may affect the extraction process, such as water solubility and viscosity. However, the choice of anion was limited with this cation due to its reduced hydrophobicity when compared to $[P_{66614}]^+$. When paired with a hydrophilic anion, the resulting IL became water soluble and was therefore unsuitable for use in this process. Three tetraalkylammonium ILs were also investigated, however combinations of cation and anion were again limited when producing a water immiscible IL. After synthesis of a number of these ILs, many of the combinations produced either water soluble ILs, or salts with melting points above room temperature and therefore could not be used in this project.

8.2 Toxicity of ILs towards growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM 70449

It was essential to determine the toxicity of each IL towards *E. coli* and *S. cerevisiae*, in order to evaluate the possibility of using them for the ISPR of MAA. In order to create a viable process, the IL must demonstrate biocompatibility with the biocatalyst to ensure that productivities will not be affected during MAA production.

8.2.1 Effect of ILs on the growth of *S. cerevisiae* DSM70449

The toxicity of each IL was tested by growing *S. cerevisiae* DSM70449 in YEPD medium in the presence of IL (20 %v/v) (appendix 12.1.4, page 209) (Table 14).

Table 14. Effect of ILs on the growth of *S. cerevisiae* DSM70449 in complex medium

IL	μ (%)	MaxOD$_{600}$ (%)
[P_{66614}][($^{i}C_8O$)$_2PO_2$]	108 ± 3	98 ± 3
[P_{66614}][DCA]	101 ± 5	92 ± 11
[P_{66614}][NTf_2]	92 ± 4	103 ± 1
[P_{66614}][Ace]	86 ± 3	97 ± 2
[P_{66614}][Sacch]	86 ± 4	91 ± 5
[P_{66614}][Sal]	71 ± 0	94 ± 2
[P_{66614}][OTf]	64 ± 5	97 ± 3
[P_{66614}][($^{i}C_8$)$_2PO_2$]	56 ± 4	87 ± 4
[P_{66614}][C_7COO]	70 ± 8	42 ± 2
[P_{66614}][Cyc]	68 ± 11	38 ± 4
[P_{4448}][NTf_2]	68 ± 9	102 ± 3
[P_{4448}][Sacch]	22 ± 2	46 ± 8
[P_{4448}][Ace]	36 ± 8	26 ± 5
[P_{4448}][Sal]	17 ± 4	19 ± 2
[P_{4448}][OTf]	-	-
[N_{4441}][NTf_2]	74 ± 12	100 ± 1
[N_{8888}][Ace]	14 ± 4	25 ± 8
[N_{8881}][NTf_2]	-	-
[BMIm][NTf_2]	-	-
[BMPyrr][NTf_2]	-	-
[BMPip][NTf_2]	-	-
[BPyr][NTf_2]	-	-

S. cerevisiae was grown in YEPD medium (240 µL) in the presence of ILs (60 µL, 20 %v/v). A negative control containing no IL was also run, along with an uninoculated IL control to ensure that the OD_{600} readings were not affected by light scattering due to the formation of emulsions. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Growth rates (µ) and MaxOD$_{600}$ values are reported as a percentage of the values obtained in control cultures containing no IL. MaxOD$_{600}$ values were taken as the highest OD_{600} value after 72 h, during stationary phase. Data are the means of three replicates and the standard deviations are shown. ILs have been grouped together based on their cation type for ease of discussion.

The four heterocyclic nitrogen containing ILs, [BMIm][NTf_2], [BMPyrr][NTf_2], [BMPip][NTf_2] and [BPyr][NTf_2] caused significant inhibition of growth. These ILs also inhibit the growth of fungi.[215] Generally, the cationic components of ILs are known for their ability to interact with the cell membrane of microorganisms, causing expansion *via* insertion of the alkyl chain into the phospholipid bilayer, ultimately resulting in cell death.[214] However, a recent publication speculated that the mechanism of toxicity of imidazolium ILs towards *S. cerevisiae* may also be through interactions with the mitochondria.[216]

The tetraalkylammonium IL, [N_{8881}][NTf_2], also inhibited the growth of *S. cerevisiae* entirely, and [N_{8888}][Ace] caused a reduction in growth rate to just 14 % of control cultures. This confirmed that long chained quaternary ammonium cations have detrimental effects on growing cells of *S. cerevisiae*. In contrast, the shorter chained quaternary ammonium, [N_{4441}][NTf_2], was one of the more biocompatible ILs. In its presence, cells reached a final biomass similar to control cultures. Therefore, a decrease in cation alkyl chain length reduced the toxicity of quaternary ammonium ILs, which is generally agreed with throughout the literature.[171,174]

Comparison of the toxicities of ILs containing a phosphonium cationic core was slightly more complex. Specific toxic effects due to the individual cationic and anionic components were observed. The majority of $[P_{66614}]^+$ ILs were biocompatible with *S. cerevisiae*, with cultures reaching OD_{600} values of ≥ 87 % compared to control cultures. Two exceptions included the coordination of $[P_{66614}]^+$ with cyclamate and octanoate anions. The cultures containing [P_{66614}][Cyc] and [P_{66614}][C_7COO] grew at rates of 68 and 70 %, with respect to the control culture, however only reached final biomass concentrations of 38 and 42 %, respectively. This indicated that *S. cerevisiae* was sensitive to the cyclamate and octanoate anions, specifically, rather than the cationic component. However, when paired with the $[P_{66614}]^+$ cation, all other anions created an IL that was biocompatible with *S. cerevisiae*. Many reports have claimed that the anionic components of ILs have little to no effect on

antimicrobial activities.[159,169] However, a small number of publications on the contribution of anion specific effects towards the toxicity of ILs have more recently been reported, although the mechanism in which they act upon cells is much more poorly understood than cationic effects.[67,177,217]

Of the shorter chain phosphonium ILs, $[P_{4448}][NTf_2]$ was the only IL which demonstrated complete biocompatibility with *S. cerevisiae*. This culture grew to an OD_{600} similar to the control, albeit at a slightly reduced rate of 68%. $[P_{4448}]$[Sacch], $[P_{4448}]$[Sal], $[P_{4448}]$[Ace] and $[P_{4448}]$[OTf], all inhibited growth significantly, the latter of which inhibited growth completely. Again, this indicated a specific anion effect, with the toxicity increasing in the order of $[OTf]^- > [Sal]^- > [Ace]^- > [Sacch]^-$ when coordinated with the $[P_{4448}]^+$ cation. This observed trend could be attributed to an increased solubility of the IL in the aqueous phase, allowing increased interactions of both that cation and anion with cell surfaces. This is further supported by the observation that $[P_{4448}][NTf_2]$ demonstrated complete biocompatibility with *S. cerevisiae* cells. 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.[114] These anions are highly water soluble, and can therefore interrupt the hydrogen bonding network between water molecules. Therefore, a toxic effect due to the chaotropicity of the anionic moiety was indicated. Furthermore, upon coordination of salicylate and saccharinate with the more highly hydrophobic cation $[P_{66614}]^+$, cell growth was no longer inhibited by the presence of the IL. This indicated that the toxicity had been reduced significantly on extension of the alkyl chains within the cation. This suggested that it is the combination of the $[P_{4448}]^+$ cation with the chaotropic anions which creates the inhibitory effects, and supports the statement that an IL is only as toxic as its most toxic component.[162] However, there was a strong indication that chaotropicity of the anion and hydrophilicity of both the cation and anion played a large role in the resulting IL toxicity.

The majority of the $[P_{66614}]^+$ ILs were biocompatible with *S. cerevisiae*. An increase in alkyl chain length decreased the resulting toxicity. This effect

opposed those observed for the ILs containing an ammonium cationic core, where an increase in toxicity was observed on extension of the alkyl chain. This trend was clearly demonstrated when comparing the toxicities of the $[NTf_2]^-$ ILs that did not completely inhibit the growth of *S. cerevisiae.* $[P_{66614}][NTf_2]$, $[P_{4448}][NTf_2]$ and $[N_{4441}][NTf_2]$ were the most biocompatible with regards to cultures reaching the highest biomass concentrations in their presence. Growth rates of *S. cerevisiae* in the presence of ILs of various cation type followed the trend $[P_{66614}]^+ > [P_{4448}]^+ > [N_{4441}]^+ > [N_{8881}]^+$. Therefore, long chain phosphonium ILs were the least toxic, and long chain quaternary ammonium ILs were the most toxic, when paired with an $[NTf_2]^-$ anion. These results, with regards to the trends in toxicity of the phosphonium ILs, contest previous reports that have stated that there is an increase in toxicity of ILs with increasing alkyl chain length.[158,168,169] Moreover, it is speculated that the increased water solubility of short chain ILs may have a reduced interaction with enzymes and hence more rapid excretion from cells, resulting in a reduced toxic effect.[218] However, in the case of phosphonium based ILs, these results suggested that these trends are reversed.

As there appears to be both completely inhibitory and entirely biocompatible ILs containing the $[NTf_2]^-$ anion, this demonstrated the effect of the cation type with regards to altering the toxicity of an IL. The combination of both cation and anion had significant effects on the toxicities of $[NTf_2]^-$ ILs. $[P_{66614}][NTf_2]$, $[P_{4448}][NTf_2]$ and $[N_{4441}][NTf_2]$ all demonstrated complete biocompatibility with *S. cerevisiae*, whereas $[BMIm][NTf_2]$, $[BPyrr][NTf_2]$, $[BPip][NTf_2]$, $[BPyr][NTf_2]$ and $[N_{8881}][NTf_2]$, completely inhibited growth. This highlighted a cationic influence on toxicity. ILs containing the acesulfame anion also demonstrated a strong cationic influence on toxicity. When comparing ILs containing the acesulfame anion, $[P_{66614}][Ace]$ did not significantly affect the growth of *S. cerevisiae*, whereas $[N_{8888}][Ace]$ reduced the growth rate to just 14 % of control cultures, and only reached a final biomass concentration of 25 %. This further supported the statement that long chain tetraalkylammonium ILs are significantly more toxic than long chain tetraalkylphosphonium based

ILs. However, similarly to [N_{8888}][Ace], [P_{4448}][Ace] also indicated a significant effect on the growth of *S. cerevisiae*, reducing the biomass concentration to 26 % in its presence. This suggested that it was either solely a toxic effect caused by the cation moiety, or alternatively, it further supported the theory that when the chaotropic anion, acesulfame, is paired with a more hydrophilic cation, a greater concentration of the IL would be in the aqueous phase. Therefore, inhibition occurred due to an increased concentration of IL able to interact with the *S. cerevisiae* cells.

8.2.2 Effect of ILs on the growth of *E. coli* MG1655

The toxicity of each IL was tested by growing *E. coli* MG1655 in LB medium in the presence of IL (20 %v/v) (appendix 12.1.4, page 209) (Table 15).

Table 15. Effect of ILs on the growth of *E. coli* MG1655 in complex medium

IL	μ (%)	MaxOD$_{600}$ (%)
[P_{66614}][DCA]	99 ± 9	86 ± 8
[P_{66614}][Ace]	89 ± 1	70 ± 2
[P_{66614}][NTf_2]	86 ± 1	78 ± 5
[P_{66614}][($^iC_8$)$_2PO_2$]	86 ± 1	78 ± 1
[P_{66614}][Sacch]	84 ± 2	63 ± 7
[P_{66614}][Cyc]	84 ± 3	53 ± 8
[P_{66614}][C_7COO]	82 ± 5	66 ± 2
[P_{66614}][OTf]	76 ± 3	74 ± 4
[P_{66614}][(^iC_8O)$_2PO_2$]	74 ± 6	83 ± 1
[P_{66614}][Sal]	31 ± 2	19 ± 1
[P_{4448}][Ace]	200 ± 10	46 ± 1
[P_{4448}][Sacch]	129 ± 30	45 ± 2
[P_{4448}][Sal]	102 ± 5	68 ± 3
[P_{4448}][NTf_2]	78 ± 5	86 ± 3
[P_{4448}][OTf]	39 ± 8	70 ± 2
[N_{8888}][Ace]	88 ± 2	66 ± 7
[N_{4441}][NTf_2]	66 ± 10	43 ± 1
[N_{8881}][NTf_2]	55 ± 3	49 ± 1
[BMIm][NTf_2]	-	-
[BMPyrr][NTf_2]	-	-
[BMPip][NTf_2]	-	-
[BPyr][NTf_2]	-	-

E. coli MG1655 was grown in LB medium (240 µL) in the presence of ILs (60 µL, 20 %v/v). A negative control containing no IL was also run, along with an uninoculated IL control to ensure that the OD_{600} readings were not affected by light scattering due to the formation of emulsions. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Growth rates (µ) and MaxOD$_{600}$ values are reported as a percentage of the values obtained in control cultures containing no IL. MaxOD$_{600}$ values were taken as the highest OD_{600} value after 72 h, during the stationary phase. Data are the means of three replicates and the standard deviations are shown. ILs have been grouped together based on their cation type for ease of discussion.

When examining the effects of ILs on the growth of *E. coli* in complex media, it was clear that the toxicity of each IL was organism specific, due to the differing cell morphologies (Table 15). In the presence of ILs, the growth rates of *E. coli* were affected to a greater extent than final biomass concentrations. As was observed for growing cultures of *S. cerevisiae*, [BMIm][NTf_2], [BMPyrr][NTf_2], [BMPip][NTf_2] and [BPyr][NTf_2] ILs were toxic to *E. coli*, inhibiting growth completely. Of the $[NTf_2]^-$ based ILs that allowed growth, the growth rate decreased in the order of $[P_{66614}]^+ > [P_{4448}]^+ > [N_{4441}]^+ > [N_{8881}]^+$, which was consistent with the trends observed for *S. cerevisiae*. Therefore, both *E. coli* and *S. cerevisiae* cells cultured in complex media followed the same trends in toxicity with regards to the effects of cationic species and alkyl chain length, when associated with the same anion. This suggested a similar mechanism of toxicity with respect to the interactions of the cationic components of these particular ILs with both *E. coli* and *S. cerevisiae* cellular envelopes.

E. coli grew at a rate of between 70-100 % compared to control cultures, in the presence of the majority of ILs, and such ILs were considered as biocompatible with *E. coli*. The four exceptions, which caused significantly reduced growth rates, were [P_{66614}][Sal], [P_{4448}][OTf], [N_{8881}][NTf_2] and [N_{4441}][NTf_2]. Although [P_{4448}][OTf] appeared to cause a reduction in growth rate to 38%, the culture reached final biomass concentration of 70 % and was therefore still considered as biocompatible, whereas, [P_{4448}][OTf] completely inhibited the growth of *S. cerevisiae*. The observed tolerance of *E. coli* towards [P_{4448}][OTf] could be attributed to the additional outer cell membrane, which may improve tolerance towards hydrophilic ILs. Further tests involving molecular dynamic or spectroscopic studies at a cellular level would be necessary to gain a greater understanding of how various IL structures interact with the cell walls/membranes of different microorganisms.[219,220] However, these tests were considered to be outside the scope of this project.

Many of the ILs tested had an inhibitory effect on the final biomass concentration reached by *E. coli* cultures, however three ILs in particular

should be discussed. $[P_{4448}][Sal]$, $[P_{4448}][Ace]$ and $[P_{4448}][Sacch]$ caused a reduction in OD_{600} to between 45 – 60 % of control cultures, yet the cells grew at a significantly faster rate in the presence of the ILs. It was speculated that the cells were using these ILs as a carbon and energy source, resulting in increased growth rates. However, after the tests were repeated in the absence of glucose, this was found not to be the case. There is the possibility of co-metabolism of both glucose and also the IL. However, further tests would be required and therefore the reason for the increased growth rates can only be speculated from this data.

The toxicity of the tetraalkylammonium based ILs towards *E. coli* increased in the order $[N_{4441}][NTf_2]$ > $[N_{8881}][NTf_2]$ > $[N_{8888}][Ace]$. *E. coli* demonstrated a much higher tolerance of $[N_{8888}][Ace]$ and $[N_{8881}][NTf_2]$ than *S. cerevisiae*, the latter of which caused complete inhibition of *S. cerevisiae*. However, $[N_{4441}][NTf_2]$ was entirely biocompatible with *S. cerevisiae*, whereas *E. coli* cultures only reached a final biomass concentration of 43 % in its presence. Conclusively, *E. coli* was more tolerant of long chain quaternary ammonium ILs than *S. cerevisiae*, potentially due to the additional outer cell membrane in *E. coli*.

$[P_{66614}][Sal]$ was the only $[P_{66614}]^+$ containing IL which showed significant toxicity towards growing cultures of *E. coli* in complex medium. This suggested that the toxicity of this IL was due to the salicylate anion. However, $[P_{4448}][Sal]$ was fairly non-toxic, its presence having no effect on growth rate and only reducing the final biomass concentration by 30 %. Thus, there was a specific toxic effect due to the combination of the $[P_{66614}]^+$ cation and $[Sal]^-$ anion and the toxicity cannot be attributed solely to one individual component. The salicylate anion did not show any toxic effects towards *S. cerevisiae*, suggesting a mechanism of toxicity specifically towards *E. coli* cells. It could be that the interactions between the salicylate anion and phosphonium cation are weaker when coordinated with the $[P_{66614}]^+$ cation, due to shielding of the positive charge at the phosphonium core by the extended alkyl chains. As a result of this, the anion may be more readily available to interact with cell membranes,

causing detrimental effects on the growth of *E. coli*. This theory could also be supported by the observation that *S. cerevisiae* can tolerate the salt forms of acidic molecules much more effectively than *E. coli*, as was shown in Chapter 5. Therefore, *S. cerevisiae* may be able to tolerate and grow in the presence of the more loosely associated salicylate anion, whereas *E. coli* cannot.

The effect of ILs on the growth of *E. coli* MG1655 in MSX medium was also investigated. The aim of this was to establish whether the effects of ILs on the growth of *E. coli* was also affected by the available nutrients in the medium (appendix 12.1.4, page 209) (Table 16).

Table 16. Effect of ILs on the growth of *E. coli* MG1655 in minimal medium

IL	μ (%)	MaxOD$_{600}$ (%)
[P_{66614}][Ace]	89 ± 12	67 ± 1
[P_{66614}][($^{i}C_8$)$_2$$PO_2$]	69 ± 3	128 ± 2
[P_{66614}][NTf_2]	60 ± 2	66 ± 9
[P_{66614}][OTf]	55 ± 8	85 ± 10
[P_{66614}][Sacch]	55 ± 3	68 ± 2
[P_{66614}][DCA]	49 ± 6	96 ± 2
[P_{66614}][C_7COO]	37 ± 1	123 ± 2
[P_{66614}][($^{i}C_8O$)$_2$$PO_2$]	29 ± 10	64 ± 2
[P_{66614}][Cyc]	21 ± 2	74 ± 1
[P_{66614}][Sal]	-	-
[P_{4448}][Ace]	118 ± 28	131 ± 7
[P_{4448}][Sal]	110 ± 9	99 ± 2
[P_{4448}][Sacch]	99 ± 20	129 ± 31
[P_{4448}][NTf_2]	51 ± 6	87 ± 2
[P_{4448}][OTf]	31 ± 15	80 ± 11
[N_{8881}][NTf_2]	51 ± 2	59 ± 1
[N_{8888}][Ace]	33 ± 1	81 ± 1
[N_{4441}][NTf_2]	24 ± 3	59 ± 10
[BMIm][NTf_2]	-	-
[BMPyrr][NTf_2]	-	-
[BMPip][NTf_2]	-	-
[BPyr][NTf_2]	-	-

E. coli MG1655 was grown in MSX medium (240 µL) in the presence of ILs (60 µL, 20 %v/v). A negative control containing no IL was also run, along with an uninoculated IL control to ensure that the OD_{600} readings were not affected by light scattering due to the formation of emulsions. The OD_{600} was measured every 10 min using a Bioscreen C microplate reader. Growth rates (µ) and MaxOD$_{600}$ values are reported as a percentage of the values obtained in control cultures containing no IL. MaxOD$_{600}$ values were taken as the highest OD_{600} value after 72 h, during stationary phase. Data are the means of three replicates and the standard deviations are shown. ILs have been grouped together based on their cation type for ease of discussion.

The growth rates of *E. coli* were slower when cultured in MSX compared to LB medium, when in the presence of a particular IL. This indicated that *E. coli* cells grown in MSX medium were more sensitive towards the majority of the ILs. Most of the previously observed trends with regards to cation family and alkyl chain length were consistent with those observed for *E. coli* cells when cultured in complex medium. Thus, the toxicity of quaternary ammonium salts increased with decreasing alkyl chain length of the cation and, in general, tetraalkylammonium ILs were more inhibitory towards cell growth than tetraalkylphosphonium ILs.

The most interesting observations concerned the $[P_{4448}]^+$ ILs. The toxicities of $[P_{4448}]$[Ace], $[P_{4448}]$[Sacch] and $[P_{4448}]$[Sal] decreased significantly towards *E. coli* cells, when the growth medium was changed from LB to MSX medium. These cultures all grew at similar rates to control cultures. However, $[P_{4448}]$[Ace], $[P_{4448}]$[Sacch] and $[P_{4448}]$[Sal] had much greater inhibitory effects on the final biomass concentrations in cultures grown in LB than MSX, generally reducing them to approximately 50 % of that of the control. Therefore, these ILs were much more toxic towards *E. coli* when grown in LB medium compared to when grown in MSX medium, confirming that growth medium has an effect on the tolerance of organisms to ILs.

$[P_{4448}]$[OTf] was completely inhibitory towards *S. cerevisiae*, however for *E. coli* cells cultured in both complex and minimal medium, $[P_{4448}]$[OTf] appeared to be reasonably biocompatible and allowed fairly consistent growth alongside the control. This further supports the varied susceptibility of different organisms towards ILs.[67] Interestingly, upon comparison of the $[P_{66614}]^+$ and $[P_{4448}]^+$ ILs consisting of the same anionic counterpart, the $[P_{66614}]^+$ ILs were less toxic to *E. coli* cultures in complex media. One discrepancy was observed when comparing the toxicities of $[P_{4448}][NTf_2]$ and $[P_{66614}][NTf_2]$, for which $[P_{4448}][NTf_2]$ was the more biocompatible of the two. However, upon cultivation of *E. coli* in minimal medium, the opposite result was observed in that the shorter chained phosphonium IL was the least toxic.

Concerning the two ILs containing bulky phosphonium anions, $[P_{66614}][(^{i}C_8O)_2PO_2]$ and $[P_{66614}][(^{i}C_8)_2PO_2]$, similarities in biocompatibility were seen for both organisms when grown in complex medium. Both ILs were biocompatible with *S. cerevisiae* and *E. coli*, with $[P_{66614}][(^{i}C_8O)_2PO_2]$ showing the highest degree of biocompatibility with *S. cerevisiae* when grown in complex medium. Upon exposure of *E. coli* to $[P_{66614}][(^{i}C_8)_2PO_2]$ in MSX medium, there was no observable effect on growth, whereas $[P_{66614}][(^{i}C_8O)_2PO_2]$ caused a significant reduction in both the growth rate and final biomass concentration. This may be indicative of a more highly susceptible cell membrane to the $[(^{i}C_8O)_2PO_2]^-$ anion specifically, however only in *E. coli* grown in a minimal medium.

The growth rates of *E. coli* in MSX medium were significantly reduced to less than 50%, upon exposure to $[P_{66614}][C_7COO]$ and $[P_{66614}][DCA]$. However, the cultures did reach a biomass concentration similar to control cultures after 72 h. Growth of *E. coli* in complex medium, however, was reasonably unaffected by exposure to $[P_{66614}][DCA]$ and $[P_{66614}][C_7COO]$. This indicated that when grown in LB medium, *E. coli* had a higher tolerance towards the dicyanamide and octanoate anions, compared to when cultured in MSX medium.

A detailed explanation for the observable differences in the toxicity of ILs towards organisms when grown in various types of growth media has yet to be published in the literature. MSX contains many different salts, buffers and various other components (4.5.5, page 73). As there are increasing reports on the ability of ILs to extract solutes from water, it could be suggested that they are in fact able to extract components out of the growth medium. Removal of these essential nutrients could hinder cell growth. Therefore, this could be considered as a reasonable suggestion for the enhanced inhibition of growth seen upon exposure of *E. coli* when grown in MSX medium in the presence of ILs. This theory was suggested towards the final stages of this project and therefore limited information to help prove this was obtained. However, upon analysing both the IL and aqueous phases of the system using ion chromatography (IC), initial tests indicated that traces of halide salts were

present in the IL layer. This could therefore begin to explain the apparent growth inhibition of *E. coli* in the presence of some ILs when grown in MSX medium, due to essential nutritive salts potentially being extracted from the aqueous phase. However, detailed tests would be required to confirm this relationship by using IC to investigate the salt components within the aqueous and IL phases after mixing.

8.2.3 Conclusions

A number of water-immiscible ILs have been identified as being biocompatible with *E. coli* MG1655 and *S. cerevisiae* DSM70449. It has been shown that the toxicity of ILs is mainly dependant on the chemical structure of the IL, i.e. the alkyl chain length, cation family and anionic moiety, and also the morphology of the organism. The growth of *E. coli* in the presence of ILs was also affected by the type of growth medium, due to the resulting differing cell membrane compositions, components and also tolerance mechanisms in place in order for the cells to respond effectively to salt and solvent stresses.

Cation family and alkyl chain length had a significant effect on IL toxicity. ILs containing heterocyclic, nitrogen containing cations were the most toxic, completely inhibiting the growth of both organisms. The majority of quaternary ammonium ILs, particularly ones containing long alkyl chains, inhibited the growth of both organisms. Phosphonium ILs that were coordinated with a specific anion showed a decrease in toxicity corresponding with an increase in the alkyl chain of the cation for both organisms when cultured in complex medium. However, the opposite trend was observed for *E. coli* cells when cultured in MSX media. An increase in alkyl chain produced a more toxic IL towards *E. coli* grown in MSX media, compared to the shorter chained counterpart.

Generally, $[P_{66614}]^+$ ILs, irrespective of the anion counterpart, demonstrated biocompatibility with both organisms. The exceptions included $[P_{66614}][Sal]$, which inhibited the growth of *E. coli* cultured in LB and MSX medium, and $[P_{66614}][C_7COO]$ and $[P_{66614}][Cyc]$, which significantly inhibited the growth of *S.*

cerevisiae. This indicated a specific inhibitory effect based solely upon the toxicity of the anionic moiety. In case of less toxic cations, the coordinated anions significantly influenced the overall toxicity of the resulting IL. The chaotropicity of the anion had a large influence on the toxicity of ILs. However, this was generally only when paired with short-chain phosphonium ILs, likely due to the production of a more hydrophilic IL. Ultimately, it can be said that the anion type contributed to the toxicity of the ILs, particularly for shorter alkyl chained ILs, hence the varied toxicities of the $[P_{4448}]^+$ ILs.

Overall, *S. cerevisiae* was more tolerant of the $[P_{4448}]^+$ based ILs, than *E. coli*. However, *E. coli* cells grown in complex media, were more tolerant of a wider range of ILs. Complete growth inhibition only occurred in the presence of four of the twenty-two ILs tested, whereas six of the ILs were toxic towards *S. cerevisiae*. The ILs which were considered as biocompatible towards both organisms, based mainly on final biomass concentrations, included $[P_{66614}][DCA]$, $[P_{4448}][NTf_2]$, $[P_{66614}][OTf]$, $[P_{66614}][NTf_2]$, $[P_{4448}][Sal]$, $[P_{66614}][Sacch]$, $[P_{66614}][Ace]$. However, if an IL were to be used in this particular process as a water-immiscible extraction phase, the effects of the IL used would be entirely organism specific based on its apparent inhibitory effects to the host biocatalyst. Therefore, all ILs which showed a degree of biocompatibility with either organism were taken forward for further testing in the next stages of the project. Further investigation in the potential use of $[BMIm][NTf_2]$, $[BMPyrr][NTf_2]$, $[BMPip][NTf_2]$, $[BPyr][NTf_2]$ and $[P_{66614}][Sal]$ was not carried out, based on their inhibitory effects towards both *E. coli* MG1655 and *S. cerevisiae* DSM70449.

8.3 Physical properties of ILs

It is of great importance to consider the physical properties of ILs that may affect their use as solvents in extraction processes. The appealing designer aspect, in that their physicochemical properties can be tuned by altering the cation/anion combinations, has drawn attention to their potential use in bioprocessing applications.[121,142] In particular, properties such as viscosity, interfacial surface tension and polarity can affect extraction performance *via* mass transfer limitations and solute recovery, essentially determining the viability of their use as extraction solvents.[111,120] Additionally, these properties may also be linked to the observed biocompatibilities with various microorganisms, discussed in the previous section. Therefore, both the viscosity and polarity, with regards to water solubility, of ILs have been investigated. The effect of temperature on water uptake and viscosity of ILs was evaluated in order to determine which microorganism would be a more favourable host biocatalyst, based on the increased or decreased system temperature required for the optimal growth of *E. coli* and *S. cerevisiae*, respectively.

8.3.1 Water content

The key characteristics of a liquid that is to function as a solvent are those that determine how it will interact with potential solutes. For traditional molecular solvents, this is most commonly recorded as the polarity of the pure liquid, as expressed through its dielectric constant. However, it is unfortunately impossible to determine the polarities of ILs using this scale.[114] Assessing the polarity of an IL is a more complex process, involving the determination of Kamlet-Taft solvatochromic parameters.[221] This model breaks the solvent strength down into three component parts; the hydrogen bond acidity (α), hydrogen bond basicity (β) and dipolarity/polarizability effects (π^*).[221] To date, solvatochromic studies have indicated that ILs have a polarity similar to those of short chain alcohols and other polar solvents, which suggests they should interact strongly with other polar solutes.[134] It is evident from reports in the

literature that determination of the polarity of ILs is difficult and correlation between IL structure and polarity is not as simple as for organic solvents.[108] Therefore, a rapid and simple method for determining the polarity of an IL could be to determine the hydrophilicity/hydrophobicity of ILs by investigating their maximum water uptake. If water uptake could act as a reasonable approximation for polarity, based upon the phenomenon that "like dissolves like", then it could also act as an indication of the potential level of interaction of the ILs with other polar molecules, such a MAA.

The presence of water in ILs can affect properties such as their polarity and viscosity.[126] This occurs due to a decrease in the intermolecular interaction between the anionic and cationic components of an IL, as a result of water uptake. Studies found that the addition of water to IL solutions affected the density, viscosity, surface tension, specific conductivity and excess volume values.[222] They attributed this to the change in the molecular organization of the solution. It is important to determine the solubility of water in ILs for this particular process as the ILs will be used in an aqueous/IL two-phase system for the ISPR of MAA. Therefore, their physicochemical properties will be affected by water during the process so it is important to determine the properties in the presence of water.

The main goal of this work was to investigate the relationship between the ionic structures of hydrophobic ILs and their ability to solubilize water. Determination of the saturated water content within an IL could potentially allow for the correlation of water uptake with viscosity measurements, toxicity and interactions of the ILs with polar molecules such as MAA. The water content of an IL can easily be determined using Karl Fischer titration. This technique can detect parts per million (ppm) traces of water in the samples, and can serve as an indication of the hydrophilic/hydrophobic nature of the IL. This coulometric technique involves the following reaction scheme

$$ROH + SO_2 + RN \rightarrow (RNH)SO_3R$$

Equation 4

$$(RNH)SO_3R + 2RN + I_2 + H_2O \rightarrow (RNH)SO_4R + 2(RNH)I$$

Equation 5

Where ROH represents an alcohol such as methanol, and RN represents a base. The determination of water content involves the electrochemical generation of iodine (I_2) from iodide (I^-). Upon contact of I_2 with water in the IL sample, water is titrated according to the above Equations 4 and 5. The amount of water in the IL is then calculated to a high accuracy according to Equation 6, by measuring the current required during the generation of I_2 from I^-.

$$2I^- \rightarrow I_2 + 2e^-$$

Equation 6

The saturated water content of each IL was tested by agitating each IL (20%v/v) with MSX medium at various temperatures. A 21-day period was then allowed in order for the system to reach dynamic equilibrium. The IL phase was then sampled in triplicate and the water content determined in ppm (Table 17).

Table 17. The saturated water content of ILs

	Water content (ppm)	
Ionic liquid	**30 °C**	**37 °C**
$[P_{66614}][(^{i}C_8)_2PO_2]$	157600	173400
$[P_{4448}][Sal]$	153900	153700
$[P_{66614}][C_7COO]$	149400	120300
$[P_{4448}][Sacch]$	82350	89270
$[P_{66614}][(^{i}C_8O)_2PO_2]$	68150	82350
$[P_{4448}][Ace]$	79560	80410
$[P_{66614}][Cyc]$	67010	76340
$[P_{66614}][Sacch]$	25920	41730
$[P_{66614}][Ace]$	23070	33190
$[N_{8888}][Ace]$	31300	32160
$[P_{66614}][DCA]$	31050	28720
$[P_{4448}][OTf]$	17310	25720
$[P_{66614}][OTf]$	17060	21800
$[N_{4441}][NTf_2]$	5755	7410
$[N_{8881}][NTf_2]$	3881	5131
$[P_{4448}][NTf_2]$	3767	4748
$[P_{66614}][NTf_2]$	3097	4037

Tests were carried out in 40 mL Teflon sealed glass vials. A mixture of MSX medium (8 mL) and IL (2 mL, 20 %v/v) was agitated at 30 and 37 °C for 24 h. The biphasic mixtures were left for 21 days with no agitation to achieve full phase separation. The IL phase was then sampled and water content was determined using KF titration. Water content is expressed in ppm and are an average of triplicate experiments.

The water content of ILs was strongly influenced by the nature of the cation and anion (Table 17). When paired with the same anion, the ILs consisting of shorter chained $[P_{4448}]^+$ cations had a higher capacity for water sorption, than ILs composed of the longer chained $[P_{66614}]^+$ cations. Therefore, short chain phosphonium ILs are more hydrophilic than their long chain equivalents. The cation alkyl chain length is known to influence the polarity of an IL and

ultimately its water solubility.[223,224] In general, the hydrophobic nature of an IL increases with increasing alkyl chain length, which is consistent with the observed water uptake (Table 17).[223] For example, $[P_{4448}]$[Sacch] and $[P_{66614}]$[Sacch] were shown to contain significantly different amounts of water. $[P_{4448}]$[Sacch] contained almost four times the amount of water than $[P_{66614}]$[Sacch] at 30°C, and double the amount at 37°C. This same decrease in water content was seen when changing the cationic moiety from $[P_{4448}]^+$ to $[P_{66614}]^+$, when paired with the same anion. This indicates that the cation also has an influence on the hydrophilicity of the IL and can be altered to further tune this property. This observation supports the trends in the literature which claim that ILs with short chained tetraalkylphosphonium and tetraalkylammonium cations have increased water sorption than their longer chain equivalents.[225] This is a well-established trend, by which an increase in the size of the aliphatic moieties attached to the cation core leads to an increase in the IL hydrophobicity, and therefore to a decrease in their mutual miscibility with water.[114] Therefore, it can be speculated that the shorter chained ILs could potentially recover increased concentrations of MAA from aqueous systems, if hydrophilicity is a key factor in its extraction.

In ILs, water molecules are assumed to be bound to either the cation or the anion, depending on their kosmotropicity/chaotropicity, H-bond acidity/basicity and nucleophilicity.[226] However, usually the dominant interaction between ILs and water is through hydrogen bonding. The role of water is complex and depends on the molecular structure of the IL.[227] There was a clear dominating influence of the anion species on water sorption (Table 17). In general, when comparing ILs of the same cationic moiety, the mutual water solubilities due to anion variation decreased in the order of $[(^iC_8)_2PO_2]^-$ > $[Sal]^-$ > $[C_7COO]^-$ > $[(^iC_8O)_2PO_2]^-$ > $[Cyc]^-$ > $[Sacch]^-$ > $[Ace]^-$ > $[DCA]^-$ > $[OTf]^-$ > $[NTf_2]^-$. This can be used as an indication of the relative hydrophobicity of each IL and how the anion can change the polarity of the IL as a solvent. Therefore, these results could help indicate which ILs may be more successful at extracting MAA, based on the hydrophilicity of the anionic moiety. The anions

bis(2,4,4-trimethylpentyl)phosphinate, salicylate and octanoate demonstrated the highest hydrophilic character of all the anions tested. This was likely due to the presence of multiple oxygen-rich functional groups which have the ability to hydrogen bonding with water. Additionally, salicylate and octanoate contain a carboxylate group which may further enhance the polarity of these ILs, consequently improving water uptake.

The lowest water content was observed in the ILs containing $[NTf_2]^-$ and $[OTf]^-$ anions. These anions contain hydrophobic, electronegative fluorine atoms which results in a delocalization of charge across the molecule. ILs containing the $[NTf_2]^-$ anion demonstrated the lowest degree of miscibility with water. $[NTf_2]^-$ is a particularly large anion and the negative charge is delocalized amongst the nitrogen and sulfur atoms, with the oxygen atoms and CF_3 groups shielding its charge from other charges.[224] This results in a decrease in the ability of the anion to interact with water molecules. For the ILs containing the $[NTf_2]^-$ anion, their water sorption capacity followed the order of $[N_{4441}][NTf_2]$ > $[N_{8881}][NTf_2]$ > $[P_{4448}][NTf_2]$ > $[P_{66614}][NTf_2]$, suggesting ILs containing ammonium cores to be more hydrophilic than phosphonium-based ILs.

The highest concentration of water was observed in $[P_{66614}][(^iC_8)_2PO_2]$, which contains a large phosphinate anion. It was speculated that the bulky alkyl chains within the anion may essentially trap water molecules in the IL layer by forming aggregates. Alternatively, as a result of the size of the ionic components, the cation and anion may be more loosely coordinated to one another, allowing increased interactions between the anion and water molecules. However, $[P_{66614}][(^iC_8O)_2PO_2]$ was observed to contain half the quantity of water compared with $[P_{66614}][(^iC_8)_2PO_2]$. This was unexpected, as the $[(^iC_8O)_2PO_2]^-$ anion contains two additional oxygen atoms, which would be anticipated to create a more polar IL and therefore have increased levels of water uptake. However, this was found not to be the case, the reasons for which are not fully understood. It could be that in $[P_{66614}][(^iC_8O)_2PO_2]$ the charge density is pulled away from the O^- atom by the neighbouring O atoms, whereas in $[P_{66614}][(^iC_8)_2PO_2]$ charge density is pushed onto the O^- atom by the

aliphatic chains. Another significant difference between the two anions is the higher degree of branching in the $[(^{i}C_8)_2PO_2]^-$ anion, which may somehow contribute to the increased levels of water sorption of the IL.

The water content of ILs increased with increasing temperature of the system. This was likely due to a decrease in the viscosity of the ILs, which is known to occur with increasing temperature.[228] Interestingly, $[P_{4448}][Sal]$ and $[P_{66614}][C_7COO]$ showed a decrease in water uptake when the system temperature was increased to 37 °C. These ILs both demonstrated extremely high water uptake capacities, due to the anions ability to partake in extensive hydrogen bonding with water molecules *via* the polar carboxylate groups. However, the reason for the decrease in water sorption with increasing temperature was unexpected and cannot be explained from this data alone.

Overall, it was concluded that the mutual solubilities between water and ILs are most strongly influenced by the chaotropicity, H-bond acidity/basicity and nucelophilicity of the anion. The cationic influence on water uptake was directly related to the increase in the hydrophobic character of the cation, derived from the increase of the alkyl chains. However, water uptake was also governed by other factors, such as the charge distribution of the central atom and consequent charge distribution at the aliphatic moieties, and the accessibility of water to the charged part of the cation. Ultimately, the determination of water content within ILs appeared to offer a simple method for determining its relative polarity. Ideally, comparison of this data with Kamlet-Taft parameters reported in the literature would indicate whether there is a correlation between the hydrogen bond acidity and basicity of the IL components and water uptake. Unfortunately, the majority of Kamlet-Taft data available are for imidazolium and a small number of tetraalkylammonium ILs, whereas, data for phosphonium-based ILs appears to be extremely limited.[229,230] Therefore, without personally determining the Kamlet-Taft parameters of the ILs used in this study, a comparison cannot be made.

8.3.2 Viscosity

The viscosity of fluids in liquid-liquid extraction processes is an important factor to consider when selecting a solvent. Viscosity has a major influence on stirring, mixing and transport properties, such as diffusion.[231] Therefore, it is of fundamental importance to understand the various physicochemical processes of a particular system under a specific set of conditions. During an extraction process, viscosity can affect the mass transfer of the solute into the solvent phase, the recyclability of the solvent and also the mixing and downstream processing costs.[232]

The viscosity of a fluid can be affected by the strength of the interactions between the molecules within the liquid, and ambient conditions such as temperature and pressure.[228] The viscosities of ILs are typically much higher than those of traditional organic solvents, ranging from 10-1000 mPa·s.[114] The cation and anion structure have a large influence on the subsequent viscosity of the liquid, for example, longer cation chain lengths will cause an increase in viscosity, while bulky anion groups, such as $[NTf_2]^-$ reduce the viscosity of imidazolium based ILs.[233] The increase in viscosity with alkyl chain length can be explained through the stronger van der Waals interactions in the hydrophobic domains of the liquid.[124] The viscosity of ILs can also be influenced by temperature, and the addition of co-solvents, such as water.[234]

During an *in situ* extraction process using ILs as solvents, the IL would be fully saturated with water from the aqueous phase, affecting its viscosity. Therefore, it was important to investigate the viscosity of ILs after exposure to water in a biphasic system. The viscosity of ILs was measured using an Anton-Paar Microviscometer (AMVn), a falling ball viscometer, which calculates the viscosity using known values of tube diameter and a specific size, weight and density of ball. The method relates the drag force experienced by a falling ball to the balls constant velocity in a liquid of known viscosity. When the terminal velocity, size and density of the ball, and density of the IL are known, the absolute viscosity can be calculated using Stokes Law

$$\eta = (\frac{2}{9}) \frac{(\rho_s - \rho) g R^2}{\upsilon}$$

Where η is the viscosity, ρ_s is the density of the ball, ρ is the density of the liquid, g is the gravitational constant (980 cms^{-2}), R is the radius of the ball, and υ is the velocity of the ball. From this, the viscosity of each IL at a particular temperature, in the presence of water, can be calculated. The aim was to determine the ILs that had the lowest viscosities in order to prevent the problems associated with high viscosity solvents in large scale extraction processes. The viscosity of ILs was tested by agitating each IL (20 %v/v) with MSX medium at various temperatures. A 21-day period was then allowed in order for the system to reach dynamic equilibrium. The IL phase was then sampled in triplicate and the viscosity determined (Table 18).

Table 18. The dynamic viscosities of water saturated ILs

	Dynamic viscosity (η) (mPa·s)	
Ionic liquid	**30 °C**	**37 °C**
$[N_{8888}][Ace]$	472.7 ± 8.0	302.0 ± 4.9
$[P_{66614}][Sacch]$	349.5 ± 6.6	258.1 ± 5.3
$[P_{66614}][OTf]$	336.5 ± 6.7	252.8 ± 5.1
$[P_{66614}][Ace]$	241.1 ± 4.8	181.9 ± 2.4
$[N_{8881}][NTf_2]$	218.0 ± 2.8	179.0 ± 1.9
$[N_{4441}][NTf_2]$	241.5 ± 3.2	174.6 ± 3.0
$[P_{66614}][NTf_2]$	224.9 ± 2.9	173.8 ± 2.8
$[P_{4448}][OTf]$	224.1 ± 3.0	170.7 ± 2.9
$[P_{4448}][NTf_2]$	186.9 ± 2.2	133.7 ± 2.1
$[P_{4448}][Ace]$	160.4 ± 2.3	114.8 ± 1.9
$[P_{66614}][Cyc]$	148.7 ± 2.2	114.4 ± 1.8
$[P_{4448}][Sacch]$	140.5 ± 1.2	91.57 ± 0.71
$[P_{66614}][DCA]$	125.2 ± 1.3	89.09 ± 1.12
$[P_{66614}][(^{i}C_8O)_2PO_2]$	124.3 ± 1.7	82.49 ± 1.41
$[P_{66614}][(^{i}C_8)_2PO_2]$	98.32 ± 1.18	74.32 ± 0.92
$[P_{66614}][C_7COO]$	66.37 ± 0.39	48.15 ± 0.29
$[P_{4448}][Sal]$	56.98 ± 0.44	40.07 ± 0.33

Tests were carried out in 40 mL Teflon sealed glass vials. A mixture of MSX medium (8 mL) and IL (2 mL, 20 %v/v) was agitated at 30 and 37 °C for 24 h. The biphasic mixtures were left for 21 days with no agitation to achieve full phase separation. The IL phase was then sampled and the viscosity determined at an 80° angle using an Anton-Parr AVMn falling ball Microviscometer. Dynamic viscosities are expressed in mPa·s and are an average of six experiments.

The viscosities of all of the ILs tested were higher than those of water (1.002 mPa·s) or any other conventional solvent (Table 18). For example, at room temperature toluene maintains a viscosity of 0.590 mPa·s, whereas the lowest viscosity presented in this work was that of $[P_{4448}Sal]$ at 37 °C, which equated to 40.07 mPa·s. At the opposite end of the scale, the highest viscosity

demonstrated was that of $[N_{8888}][Ace]$, which exhibited a viscosity of 472.7 mPa·s at 30 °C.

There is a clear indication of the influence of temperature on the viscosity of ILs. For every IL tested, there was a significant reduction in the viscosity of the liquid when increasing the system temperature from 30 to 37 °C. This has been reported previously and it is known that the general trend, irrespective of structural composition, is that an increased temperature results in a decrease in the viscosity of a liquid.[235] This occurs as the kinetic motion at higher temperatures promotes the breaking of intramolecular bonds, allowing less hindered movement of the particles. The energy is therefore transferred more quickly as the particles in the fluid move faster, resulting in a reduced resistance to flow and a subsequently lower viscosity.[122] Therefore, it can be said that with regards to process design, as *E. coli* requires a slightly elevated temperature of 37 °C for optimum growth compared to *S. cerevisiae*, which requires a temperature of 30 °C, *E. coli* would be more favourable due to the significant decrease in viscosity of the ILs at this temperature.

With regards to the effect of the cationic structure on viscosity, a number of observations have been made. When comparing ILs containing a phosphonium cationic core, upon extension of the alkyl chains within the cation was correlated with an increase in viscosity. For example, $[P_{4448}][NTf_2]$ exhibited a viscosity of 133.7 mPa·s at 37 °C, whereas the long chained congener, $[P_{66614}][NTf_2]$ demonstrated an increased viscosity of 173.8 mPa·s, at the same temperature. This increase in viscosity was observed for the $[P_{66614}]^+$ ILs sharing the same anion species as its shorter chain $[P_{4448}]^+$ congener. Similarly, when evaluating the effect of alkyl chain length on the viscosity of ILs containing ammonium cationic cores, the same trend was observed at 37 °C. For example, $[N_{4441}][NTf_2]$ presented a viscosity of 174.6 mPa·s, whereas the longer chain equivalent, $[N_{8881}][NTf_2]$ demonstrated an increased viscosity of 179.0 mPa·s. The reasons for the viscosity increase with alkyl chain length can be explained through the stronger van der Waals interactions in the hydrophobic domains of the IL, increasing the resistance of the fluid to flow.[124]

The significant difference in the magnitude of increase in viscosity, due to increasing alkyl chain length in phosphonium and ammonium ILs, should be noted. The viscosities of $[NTf_2]^-$ phosphonium-based ILs were seen to increase by 40mPa·s upon extension of the alkyl chain length within the cation, whereas the ammonium equivalents only increased by 5 mPa·s. Furthermore, at 30 °C the viscosity of $[N_{8881}][NTf_2]$ was observably lower than that of $[N_{4441}][NTf_2]$, contradicting the trends observed at 37 °C. One explanation for the apparent reduced effect of temperature on the viscosity of ammonium ILs at lower temperatures could be due to the interactions between the anion and the central ammonium atom. The strength of anion−cation interactions is known to play a pivotal role in the viscosity of ILs.[129] The strength or disruption of intramolecular forces determines whether the molecules stick together or move freely, which in turn effects the viscosity of the fluid.[129] Therefore, the greater the interactions between the cation and anion, the higher the viscosity. It could be that as the nitrogen core maintains a high charge density, there is a much stronger interaction between the cation and anion, compared to the interaction between a phosphonium-based IL and the equivalent anion. Therefore, the increase in temperature, which brings about the breaking of intramolecular bonds and as a result increases the fluidity of the liquid, may not be as effective due to the increased strength of the columbic interactions between the IL components. This also may help to explain the reason for the slightly increased viscosities observed for ammonium-based ILs at the same temperature, particularly $[N_{8888}][Ace]$. Generally, the trends in viscosity due to the cationic species are seen to increase in the order of $[N_{8888}]^+ > [N_{8881}]^+ > [N_{4441}]^+ > [P_{66614}]^+ > [P_{4448}]^+$. However, the viscosities of $[N_{8881}][NTf_2]$, $[N_{4441}][NTf_2]$ and $[P_{66614}][NTf_2]$ were all observed to be fairly similar at 37 °C, and there appear to be many exceptions to this rule. This statement particularly holds true when involving $[P_{66614}]^+$ ILs paired with hydrophilic anions, indicating that the anion may have a greater influence on the resulting viscosity than the cationic moiety.

When examining the effects of viscosity due to the structure of the anion, the viscosity of anions coordinated to $[P_{66614}]^+$ based ILs are seen to increase in the order $[Sacch]^-$ > $[OTf]^-$ > $[Ace]^-$ > $[NTf_2]^-$ > $[Cyc]^-$ > $[DCA]^-$ > $[(^iC_8O_2)PO_2]^-$ > $[(^iC_8)_2PO_2]^-$ > $[C_7COO]^-$. The large differences in ILs viscosity as a function of the anion type confirm that the strength of anion−cation interactions are a fundamental component in the resulting viscosities (Table 18). Interestingly, the anions coordinated to $[P_{4448}]^+$ cations are seen to increase in viscosity following a different order than was seen for the $[P_{66614}]^+$ congeners (Table 18). The viscosities of $[P_{4448}]^+$ ILs, due to the anionic component, followed the order of $[OTf]^-$ > $[NTf_2]^-$ > $[Ace]^-$ > $[Sacch]^-$ > $[Sal]^-$. This suggested an influence on the viscosity of ILs based upon the combination of cation and anion and that there are not just simply effects due to each individual moiety. This is likely due to the specific interactions between the cation and anion and also between individual IL molecules composing the liquid. The stronger the interactions between the cation and anion, the higher the viscosity. The more common intramolecular forces involved in ILs that will have an effect on the apparent viscosities are coulombic interactions, van der Waals forces and hydrogen bonding.[130,131] Viscosity trends have shown to be influenced by the hydrogen bond donor (HBD) ability, non-polar (NP) regions and hydrogen bond acceptor (HBA) sites of the individual anions.[235]

Overall, the results indicate that if an IL of low viscosity is to be designed, the molecular interactions between the ionic components and the molecule as a whole must be considered. Cations of shorter alkyl chain length reduced the van der Waals interactions within the hydrophobic domains, and ultimately produced a lower viscosity IL. Additionally, ensuring the central atom of the cation had a lower charge density, reduced the coulombic interactions between the ionic counterparts, resulting in a decreased viscosity. Finally, utilizing chaotropic, hydrophilic anions in the presence of water, increased water uptake through hydrogen bonding, reducing the viscosity of the IL.

8.3.3 Conclusions

The water content and subsequent viscosities of each biocompatible IL have been established. Clear trends based on cation and anion structures and combinations were observed, along with correlations between the two physicochemical properties, which are fundamental when designing an extraction solvent. The most promising ILs with regards to both a low viscosity and increased water sorption were $[P_{4448}][Sal]$, $[P_{66614}][C_7COO]$ and $[P_{66614}][(^iC_8)_2PO_2]$. However, ILs containing hydrophilic counterparts, such as $[P_{66614}][(^iC_8O)_2PO_2]$ and $[P_{66614}DCA]$, also seemed promising, indicating reasonably low viscosities. Additionally, phosphonium-based ILs offered more favourable physiochemical properties than the ammonium-based ILs, as they generally demonstrated increased water content and decreased viscosities, due to the previously discussed interactions within the ILs.

The cation alkyl chain length influenced the solubility of water within ILs, possibly due to the hydrophobic nature of the longer alkyl chains. This resulted in a reduced ability of water molecules to disrupt the domain structure of the ILs, ultimately increasing the viscosity. The greater van der Waals interactions between the longer chains may explain the weakening effect water uptake has on the coulombic interactions between the cation and the anion, and therefore the reduced viscosities observed with increased water content.

With increasing temperature, the water content increased and the viscosity of ILs decreased significantly. Therefore, it could be said that with respect to the design of this process, it would be more favourable to use *E. coli* as the host biocatalyst as its optimal growth temperature is higher than that required for the cultivation of *S. cerevisiae*. Consequently, the viscosity of the ILs would be reduced and the mixing, separation and processing costs would be significantly reduced, improving the economic viability of this process and supporting the use of ILs as alternative solvents.

8.4 Extraction of MAA using biocompatible ILs

Organic acids, such as MAA, have a strong affinity for water. Therefore, when using organic solvents as extractants, the efficiency of the process can be fairly low, as was observed in Chapter 7. The solvents which demonstrated high levels of biocompatibility with growing cultures of *E. coli* and *S. cerevisiae*, were unfortunately the non-polar, highly hydrophobic solvents. Therefore, their interactions with polar MAA molecules were limited. This resulted in fairly low extraction efficiencies of around 25 %, which would not make for a viable process. ILs can be tailored to contain polar domains, whilst remaining hydrophobic in nature, due to the possibility of different cation and anion combinations.[114] Therefore, the MAA extraction capacities of biocompatible ILs was investigated, anticipating stronger interactions between the MAA molecules and the ILs than was seen for conventional solvents.

All ILs that had shown a degree of biocompatibility with either *E. coli* or *S. cerevisiae* were tested, in order to determine their potential as an extractant in the bioproduction of MAA. Therefore, the ILs which were not tested included [BMIm][NTf_2], [BMPyrr][NTf_2], [BMPip][NTf_2], [BPyr][NTf_2], [N_{8881}][NTf_2] and [P_{66614}][Sal]. These ILs showed to have significant detrimental effects on the growth and viability of both *E. coli* and *S. cerevisiae*, and therefore could not be used as solvents in this process. As the remaining seventeen ILs were biocompatible with the potential host organisms, in order to be considered a viable option, they must also demonstrate high distribution ratios and ultimately, a high capacity for MAA.

The ILs which were investigated had been carefully designed in order for them to be water immiscible, thus distribution ratios (*D*) and extraction efficiencies (*E*) could be calculated for each IL. These results could then be compared to the values obtained for the biocompatible organic solvents in order to assess the viability of using ILs as alternative solvents in this process. The effect of the nature of the anion was evaluated along with temperature, MAA

concentration and phase ratio, as were investigated for organic solvents, using the same methods.

8.4.1 Cation and anion effects

The individual and combined effects of each cation and anion were investigated with regards to their extraction efficiencies. The affinity of MAA towards the IL phase was measured by the distribution coefficient, at equilibrium, between the aqueous and organic phases. It was desirable for the organic phase to possess a high distribution coefficient to minimise the volume of the IL required. This has economic benefit with regards to cost, recovery and recycling within the bioprocess. Reducing the affinity of the acid towards water is of highest importance in order to concentrate the target molecule within the extractant layer. This would be aided by strong interactions, such as coulombic, hydrogen bonding and van der Waals interactions, of the acid with the IL.

The aim of this work was to determine the effects of cation and anion species on the partitioning of MAA between the two phases, and the subsequent extraction efficiency of each IL. The effect of IL structure on the extraction efficiency was tested by agitating two-phase systems of MSX containing a known concentration of MAA and IL at different temperatures and analysing the concentration of MAA in each phase after extraction (Table 19).

Table 19. The effect of cation and anion species on the extraction of MAA into ILs

	Distribution Ratio (*D*)		Extraction Efficiency (*E*) (%)	
Ionic Liquid	**30 °C**	**37 °C**	**30 °C**	**37 °C**
$[P_{66614}][C_7COO]$	11.00 ± 2.95	12.83 ± 0.96	91 ± 2	93 ± 1
$[P_{66614}][Cyc]$	6.04 ± 0.06	8.09 ± 0.12	86 ± 0	89 ± 0
$[P_{4448}][Sal]$	6.78 ± 0.84	5.64 ± 0.62	87 ± 1	86 ± 1
$[P_{4448}][Ace]$	6.58 ± 0.23	5.23 ± 0	87 ± 1	86 ± 0
$[P_{4448}][Sacch]$	5.71 ± 0.08	5.07 ± 0.09	84 ± 1	84 ± 0
$[N_{8888}][Ace]$	4.58 ± 1.27	5.30 ± 0.28	81 ± 4	84 ± 1
$[P_{66614}][Ace]$	4.47 ± 0.29	4.92 ± 0.75	82 ± 1	83 ± 2
$[P_{66614}][DCA]$	3.80 ± 0.18	5.08 ± 0.71	79 ± 1	83 ± 2
$[P_{66614}][(^iC_8O)_2PO_2]$*	4.05 ± 0.92	4.34 ± 0.09	79 ± 4	81 ± 0
$[P_{4448}][OTf]$	4.22 ± 0.43	4.31 ± 0.39	80 ± 2	81 ± 1
$[P_{66614}][Sacch]$	4.40 ± 0.20	3.95 ± 0.15	81 ± 1	80 ± 1
$[P_{66614}][(^iC_8)_2PO_2]$*	3.57 ± 0.11	3.68 ± 0.42	78 ± 0	78 ± 2
$[P_{66614}][OTf]$	1.53 ± 0.02	1.62 ± 0.08	60 ± 0	62 ± 1
$[N_{4441}][NTf_2]$	0.59 ± 0.03	0.62 ± 0.05	35 ± 0	36 ± 0
$[P_{4448}][NTf_2]$	0.50 ± 0.02	0.53 ± 0.02	33 ± 1	34 ± 1
$[N_{8881}][NTf_2]$	0.49 ± 0.02	0.52 ± 0.01	33 ± 1	34 ± 1
$[P_{66614}][NTf_2]$	0.44 ± 0.02	0.47 ± 0.02	31 ± 1	32 ± 1

Extractions were carried out in 40 mL Teflon sealed glass vials a stock solution of 500 mM MAA in MSX (8 mL) and IL (2mL, 20 %v/v). Each IL system was incubated at either 30 or 37°C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each IL system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown. *Emulsification occurred during mixing, however after 21 days the IL and aqueous phase had visibly separated into two distinct phases.

The majority of ILs extracted 80-90 % of the MAA from the aqueous phase, indicating the huge potential of these ILs to be used as solvents during the

bioproduction of MAA (Table 19). The ILs paired with the more polar, hydrophilic anions, such as $[Sal]^-$, $[Cyc]^-$ and $[Ace]^-$, extracted greater quantities of MAA than those paired with highly hydrophobic anions, such as $[NTf_2]^-$ and $[OTf]^-$. The high extraction efficiencies indicated by these ILs are likely due to the enhanced polarities of the anion. This suggested a relationship between the polarity of the anion and the resulting extraction efficiencies, which has been observed in similar studies throughout the literature.[155] For example, $[P_{66614}][(^iC_8)_2PO_2]$ and $[P_{66614}][C_7COO]$ can participate in hydrogen bonding with MAA, due to the presence of oxygen atoms containing lone pairs of electrons.[155]

MAA extraction efficiency due to the anionic species followed the order $[C_7COO]^- > [Cyc]^- > [Sal]^- > [Ace]^- > [DCA]^- > [(^iC_8O)_2PO_2]^- > [Sacch]^- > [(^iC_8)_2PO_2]^- > [OTf]^- > [NTf_2]^-$. The large distribution ratios of ILs containing $[Cyc]^-$, $[Sal]^-$, $[Ace]^-$, $[(^iC_8O)_2PO_2]^-$, $[Sacch]^-$ and $[(^iC_8)_2PO_2]^-$ anions could be attributed to their HBA abilities. Additionally, the aromatic $[Sal]^-$ and $[Sacch]^-$ anions also offered the potential for π-π interactions with the unsaturated double bond of the MAA molecules, which may also enhance the extraction abilities of these ILs. However, the majority of the ILs tested were appropriate as solvents for MAA extraction from aqueous medium.

$[P_{66614}][C_7COO]$ presented the highest extraction efficiencies of 91 and 93 % at 30 and 37°C, respectively. The carboxylate group within the anionic structure would interact strongly with the MAA anion, as they both contain the same functionality. However, the extraction process would likely involve other interactions, such as coulombic and van der Waals. It should be noted that in the presence of water, carboxylic acids do not dimerise, they instead form H bonds with water molecules (Figure 14). It has been reported that long chain carboxylic acids, such as octanoic acid, can disrupt the hydrogen bonding interactions between acids and water, which may hold true for MAA and water.[236] This may explain the high extraction efficiencies observed for $[P_{66614}][C_7COO]$.

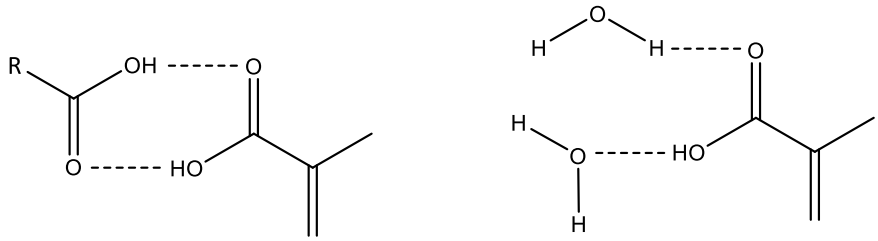


Figure 14. The potential dimerization *via* hydrogen bonds between a carboxylic acid and MAA in non-aqueous conditions (left) and aqueous conditions (right).

In contrast, the ILs which had the lowest capacity for MAA extraction were the $[NTf_2]^-$ containing ILs. When coordinated with a $[NTf_2]^-$ anion, both ammonium and phosphonium cation families, with either long or short alkyl chain lengths, exhibited similar extraction efficiencies of between 31-34 %. These ILs included $[P_{4448}][NTf_2]$, $[N_{4441}][NTf_2]$, $[N_{8881}][NTf_2]$ and $[P_{66614}][NTf_2]$. It was speculated that as $[NTf_2]^-$ and $[OTf]^-$ ILs have such high hydrophobicities they do not mix efficiently with aqueous medium. Therefore, they extracted reduced amounts of MAA due to a reduced contact with the IL phase. This was supported by the statement that like dissolves like. Therefore, a greater concentration of MAA would be extracted by polar ILs which, in this case, is governed to a greater extent by the anion. Similar results have been reported, which attributed low extraction efficiencies of acids to the hydrophobicity of the anion. During the extraction of gallic acid using ILs, it was concluded that the increased hydrophobicity of these ILs made them less able to solubilise the negatively charged form of gallic acid, resulting in the reduced extraction efficiencies.[237] Therefore, in theory, the extraction of the negatively charged species of MAA would be poorer in the case of hydrophobic ILs, if hydrophobicity was the sole factor which determines extraction of an acid by ILs.

Extraction efficiencies were affected to a lesser extent by the cation variation, compared to the influence of the anion. For example, the ILs containing the acesulfame anion, $[N_{8888}][Ace]$, $[P_{4448}][Ace]$ and $[P_{66614}][Ace]$, showed very little difference in extraction efficiencies. $[P_{4448}][Ace]$ demonstrated slightly

improved efficiencies compared to the longer chained congeners containing both phosphonium and ammonium cores, whereas these two longer chained ones demonstrated almost identical efficiencies of 83 and 84 %. This indicated a slightly improved interaction of MAA with ILs composed of cations containing shorter alkyl chains. This could be linked to an increased polarity which occurs when the alkyl chain lengths are reduced. Alternatively, it could also be linked to a more exposed positively charged cationic core, which could potentially partake in coulombic interactions with the negatively charged MAA anion. Whereas, the longer alkyl chains may shield these interactions, resulting in a decrease in *E*. An improvement in *E* can be seen when comparing all shorter chained phosphonium cations paired with the same anions as the longer chained equivalent phosphonium ILs.

8.4.2 Effect of temperature

Similar effects due to temperature variation were observed for the extraction of MAA by ILs, as were seen for extraction using organic solvents. An increase in the temperature of the system from 30 to 37 °C showed an improvement in the extraction efficiencies of each IL by around 2 – 3 %. This indicated the extraction process to be endothermic. Therefore, an increase in temperature of the system was favourable for the extraction of MAA by the IL. The increase in extraction efficiency could be attributed to a decrease in the viscosity of the ILs with increasing temperature, as demonstrated in section 8.3.2. A decrease in viscosity would result in a decrease in mass transfer limitations and therefore may improve diffusion of the MAA from the aqueous phase to the IL phase. However, an increase of 2 – 3 % is a fairly insignificant improvement in extraction efficiency and therefore it can be concluded that temperature only plays a minimal role with regards to its influence on the viability of the process. Therefore, the effects on MAA recovery due to temperature were considered insignificant.

8.4.3 Effect of MAA concentration

The current production titres of MAA by *E. coli* are low, at around 170 µM, without the *in situ* removal of the acid.[6] The ILs have so far demonstrated extremely high extraction efficiencies of 80-90 %, however, that is only from an aqueous phase containing 500 mM MAA. If this concentration was to reduce, the extraction efficiency must remain high in order to maintain the viability of the extraction process. Therefore, the effects of reducing the concentration of MAA, mimicking lower production titres, was investigated.

As all of the ILs demonstrated similar extraction efficiencies of > 80 %, with the exceptions of $[NTf_2]^-$ based ILs, it was anticipated that all of the ILs would be affected in a similar way with regards to varying MAA concentrations and phase ratios. Therefore, $[P_{66614}]$[DCA] was chosen as the representative IL to demonstrate the effects of changing these variables. The effect of MAA concentration on the extraction efficiency of ILs was tested by agitating two-phase systems of MSX, containing different concentrations of MAA, and $[P_{66614}]$[DCA] and analysing the concentration of MAA in each phase after extraction (Table 20).

Table 20. The effect of MAA concentration on the extraction efficiency of $P_{66614}DCA$

	Distribution Ratio (*D*)		Extraction Efficiency (*E*) (%)	
Concentration of MAA (mM)	**30 °C**	**37 °C**	**30 °C**	**37 °C**
6.25	0.11 ± 0	0.12 ± 0	10 ± 1	10 ± 0
12.5	0.12 ± 0.01	0.12 ± 0.01	10 ± 1	10 ± 1
25	0.28 ± 0.01	0.26 ± 0	22 ± 0	21 ± 0
50	1.08 ± 0	1.04 ± 0.04	52 ± 0	51 ± 1
100	2.22 ± 0.09	2.63 ± 0.07	69 ± 1	73 ± 1
200	3.94 ± 0.06	4.09 ± 0.33	80 ± 0	80 ± 1
300	4.72 ± 0.06	4.83 ± 0.06	82 ± 0	83 ± 0
400	5.03 ± 0.23	5.11 ± 0.14	83 ± 1	84 ± 0
500	3.80 ± 0.18	5.08 ± 0.71	79 ± 1	83 ± 2

Extractions were carried out in 40 mL Teflon sealed glass vials. A buffered stock solution of 500mM MAA in MSX was diluted to the desired MAA concentration for each test. Each vial contained a specific concentration of MAA in MSX (8 mL) and $P_{66614}DCA$ (2 mL, 20 % v/v). The IL systems were incubated at either 30 or 37 °C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each IL system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown.

As the concentration of MAA decreased from 500 mM to 12.5 mM, the extraction efficiency decreased. At MAA concentrations of 6.25 and 12.5 mM, *E* remained constant at a value of 10 %, indicating this to be the maximum extraction efficiency at concentrations of < 12.5 mM MAA. As the concentration increased to 25mM and 50mM, *E* increased linearly (Figure 15). At 100 mM MAA, the relationship between MAA concentration and *E* was no longer linear. Once the concentration of MAA in the system reached 200 mM, a plateau in *E* was observed. *E* remained consistent at between 80 – 84 % at concentrations above this concentration. This indicated that the maximum extraction efficiency possible by $[P_{66614}][DCA]$, at a phase ratio of 20 %v/v, was 84 % at a concentration of ≥ 200 mM MAA.

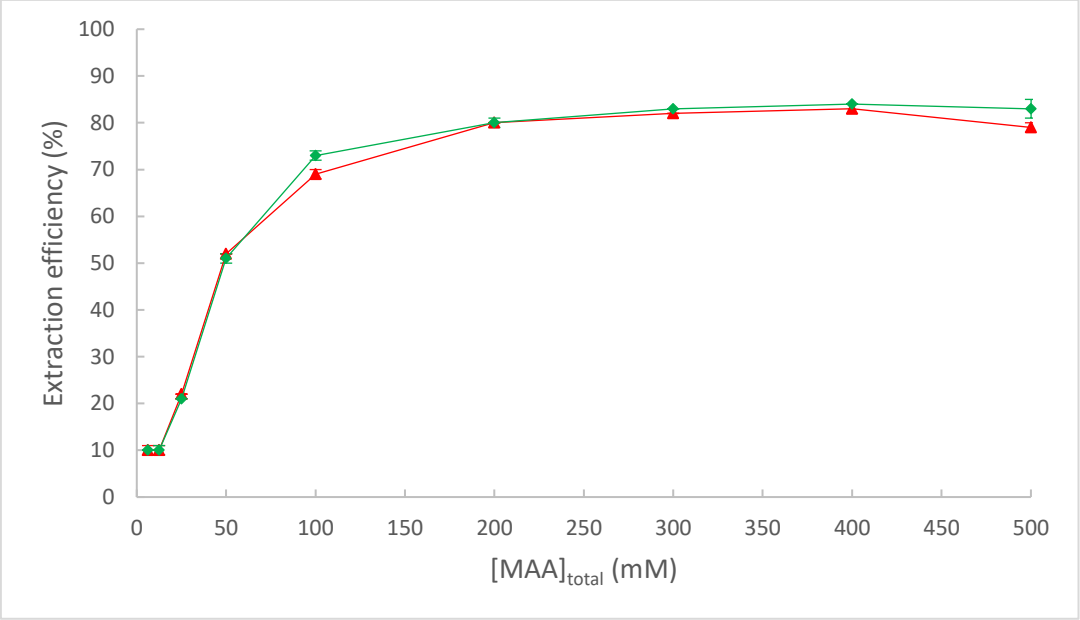


Figure 15. The effect of MAA concentration on the extraction efficiency of $[P_{66614}][DCA]$

Various concentrations of MAA in MSX medium were agitated in the presence of $[P_{66614}][DCA]$ (20 %v/v). Extraction efficiencies were calculated using Equation 2 after the equilibria was reached at 30 °C (▲) and 37 °C (♦). Means of three replicates are shown and error bars represent standard deviation.

The extraction efficiency increased initially with increasing MAA concentration, however at concentrations of below 12.5 mM and above 200 mM, the efficiency remained constant. In general, however, there was an increase in *E* with increasing MAA concentration. This relationship contests literature which reported that during the extraction of caffeic and ferrulic acid using ILs, an increase in concentration of acid in the system resulted in a decrease in the extraction efficiency.[137] They attributed this phenomenon to the reduced distance of molecules to one another, therefore enhancing the interaction between the acid molecules and reducing the interaction of the acid with the IL. This opposing trend could potentially be due to the use of ILs of different structure, as Yan-Ying *et al.* investigated imidazolium ILs rather than phosphonium based ILs. Therefore, ILs of differing cation families may demonstrate different trends in the relationship between extraction efficiency and solute concentration.

Increasing the temperature of the system showed minimal improvement in extraction efficiency, similarly to the previous results obtained at a constant concentration. This was surprising as it was anticipated that the resulting decrease in viscosity upon increase in the temperature of the system would improve the mass transfer and diffusion of MAA into the IL. However, as this hypothesis was found to be incorrect, it was concluded that the viscosity of the IL had an insignificant effect on the extraction efficiencies of ILs. Ultimately, to recover a high concentration of MAA using ILs as extractants, a higher production, and therefore concentration of MAA in the system, would be necessary.

8.4.4 Effect of phase ratio

To reduce the costs of the extraction process, a smaller volume of IL should be used. However, the viability of this is dependent upon whether the extraction efficiency remains high when the phase ratio of IL/aqueous phase is reduced. Therefore, the effect of reducing the volume of the IL phase was investigated. The effect of phase ratio on the extraction efficiency of ILs was tested by agitating two-phase systems of MSX, containing different phase ratios of IL/MSX. The concentration of MAA in each phase was then analysed (Table 21).

Table 21. The effect of phase ratio on the extraction efficiency of $P_{66614}DCA$

	Distribution Ratio (*D*)		Extraction Efficiency (*E*) (%)	
Phase ratio (% v/v)	**30 °C**	**37 °C**	**30 °C**	**37 °C**
5	1.10 ± 0.05	1.36 ± 0.01	52 ± 1	58 ± 2
10	2.49 ± 0.12	3.43 ± 0.16	71 ± 1	77 ± 1
20	3.80 ± 0.18	5.08 ± 0.71	79 ± 1	83 ± 2

IL extractions were carried out in 40 mL Teflon sealed glass vials containing a stock solution of 500 mM MAA in MSX, and phase ratios equalling 5, 10 and 20 % v/v of $P_{66614}DCA$, resulting in a total volume of 10 mL. Each IL system was incubated at either 30 or 37 °C for 24 h with constant agitation (280 rpm), then allowing a 21-day equilibration period with no agitation. Concentrations of MAA in the MSX phase were detected using HPLC-UV (210 nm). The peak area output was then fitted to a calibration curve to obtain the final concentration of MAA in the MSX phase for each IL system. *D* and *E* (%) were calculated using Equations 1 and 2. Data are the means of three replicates and the standard deviations are shown.

As the phase ratio increased from 5 % to 20 % v/v, the extraction efficiency increased (Table 21). In the system containing 5 % $[P_{66614}][DCA]$, the distribution ratios indicated that over half of the MAA had been extracted, despite the extremely small volume of IL in the system. However, a reduction in volume of extractant did negatively affect the efficiency of MAA recovery from the aqueous phase. The efficiency was greatly improved in the systems containing a phase ratio of 10 and 20 %v/v, indicating a 77 and 83 % transfer of MAA into $[P_{66614}][DCA]$, at 37 °C. It is likely that the increase in efficiency with increasing volume of the IL phase occurred due to the increase in solvent molecules which can interact with the MAA, therefore extracting a higher concentration out of the aqueous phase. At 5 %v/v, the solvent molecules available to extract the MAA would be in sparse quantities. Therefore, extraction would be hindered due to the strong interactions of MAA with the polar water molecules. It is anticipated that increasing the phase ratio to above 20 %v/v could further improve extraction efficiencies, however additional tests would be required to confirm this. There may instead be a plateau in extraction above a particular phase ratio when the maximum MAA capacity of

the IL is reached, as was seen when the concentration of MAA was increased to above 200 mM.

8.5 Conclusions and discussion

The potential of using ILs as alternative solvents for the ISPR of MAA was evaluated. Since organic solvents had shown such high toxicities and low extraction efficiencies (Chapters 6 and 7) it was important to investigate alternative extraction solvents, which may improve the viability of the process. Twenty-two ILs were selected based on their hydrophobic nature and previously established toxicity trends reported in the literature.[67,162,218] The toxicity of each IL was determined towards growing cultures of the two potential host biocatalysts, *E. coli* MG1655 and *S. cerevisiae* DSM70449. Seventeen biocompatible ILs were identified and their physicochemical properties investigated with regards to their viscosities and water solubilities. Finally, the MAA extraction efficiencies of each IL/aqueous system was determined. The effects of IL structure, temperature of the system, MAA concentration and IL/aqueous phase ratio on extraction efficiency were evaluated in order to establish the optimal conditions required to create a viable process for the ISPR of MAA, using ILs as *in situ* extractants.

It was of interest to explore the relationships between physical properties, toxicity and extraction in relation to the use of ILs. IL toxicity has been suggested as being directly linked to the lipophilicity of the IL.[168,238,239] Therefore, it could be suggested that as the hydrophobicity of an IL increases, the toxicity would also increase. Hence, ILs that take up reduced amounts of water in aqueous two-phase systems, due to an increased hydrophobic character, would likely inhibit the biocatalysts. Thus, by determining the water content of an IL, the toxicity could be predicted. During the determination of the water content of ILs, it was found that water uptake was dependent on both the cation and anion structures. ILs consisting of more highly polar, chaotropic anions had the highest water contents, whereas ILs containing bulky cations with long alkyl chains, paired with more hydrophobic anions, had

the lowest water contents. For example, ILs consisting of the $[NTf_2]^-$ anion had very low water contents of between 3000-7500 ppm, irrespective of the cation structure, indicating these ILs to be the most hydrophobic. Therefore, if toxicity was able to be predicted based on water uptake, the $[NTf_2]^-$ containing ILs would be expected to exhibit the highest toxicities. However, $[P_{66614}][NTf_2]$ and $[P_{4448}][NTf_2]$ were in fact two of the most biocompatible ILs, with both *E. coli* and *S. cerevisiae*, suggesting the correlation between water content and toxicity to be poor. However, the tetraalkylammonium ILs containing $[NTf_2]^-$ anions, particularly the ones containing cations with shorter alkyl chains, demonstrated greater inhibitory effects than the phosphonium $[NTf_2]^-$ containing ILs. Therefore, the cation clearly had a strong influence on the resulting IL toxicity, whereas the anion had a greater influence on the water content and resulting hydrophobicity. Further supporting this, the water content of the $[P_{4448}]^+$ ILs was significantly higher than the $[P_{66614}]^+$ ILs, when paired with the same anion. This indicated the $[P_{4448}]^+$ ILs to be more hydrophilic, which would suggest these ILs to be less toxic. However, this was found not to be the case and in fact the $[P_{4448}]^+$ ILs had greater inhibitory effects on the biocatalysts. In conclusion, hydrophobicity, and therefore water content, cannot be used as a method for predicating the toxicity of an IL. Too many structural, chemical and physical properties influence the toxicity of ILs, which are also organism and strain specific. Therefore, toxicity must be determined for each specific IL towards each different organism and strain to assess the biocompatibility of an IL for use in a particular bioprocess.

By contrast, a correlation between the water uptake of ILs and their MAA extraction efficiencies was observed. In a similar study investigating the potential of ILs for the extraction of gallic acid (pKa = 4.41) at pH 7, it was reported that the extraction efficiency was governed by the hydrophobicity of the IL, which was mainly influenced by the anion type, as previously discussed.[237] If this holds true for the extraction of MAA at pH 7, the extraction efficiencies of each IL would correlate with the observed trend in water content. Water uptake increased based on the anion type in the order

$[(^{i}C_8)_2PO_2]^-$ > $[Sal]^-$ > $[C_7COO]^-$ > $[(^{i}C_8O)_2PO_2]^-$ > $[Cyc]^-$ > $[Sacch]^-$ > $[Ace]^-$ > $[DCA]^-$ > $[OTf]^-$ > $[NTf_2]^-$. When comparing the water content of ILs with the extraction efficiencies, at 37 °C, a correlation between the two was observed (Figure 16).

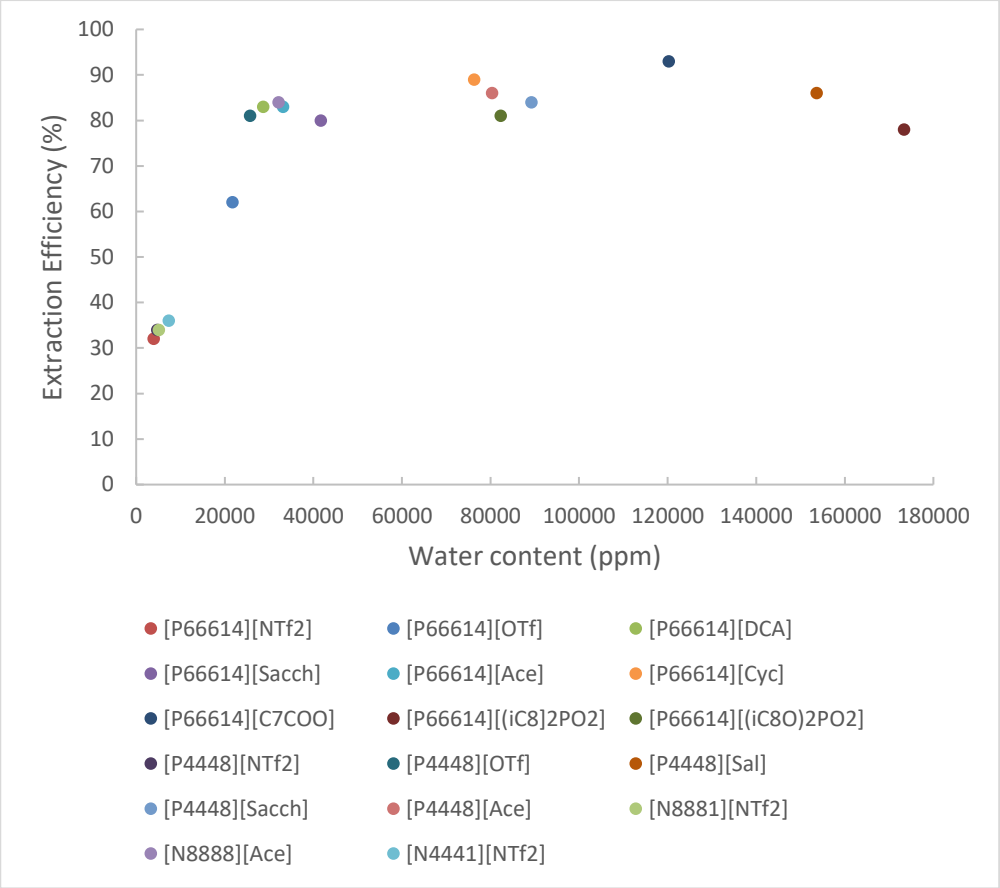


Figure 16. The relationship between water content and MAA extraction efficiencies of ILs at 37 °C

The ILs situated in the lower left region of the plot represented the ILs containing $[NTf_2]^-$ and $[OTf]^-$ anions, indicating their reduced water content and resulting poor extraction efficiencies (Figure 16). Generally, as water sorption of the IL increased, there was an initial increase in extraction efficiency, however it reached a limit. Therefore, these data support the theory that the recovery of MAA by ILs can be improved by increasing the hydrophilicity of the anion. It should be noted, however, that the ILs which contained hydrophilic anions all had relatively similar extraction efficiencies of 80-90 % despite the seemingly different water contents. Therefore, the anion

structure and functionalities must affect the water content but as long as the anion is hydrophilic in nature, extraction efficiency will always be high. This suggests that lower costing ILs can be utilized without reducing the efficiency of the process. Despite the poor efficiencies of the $[NTf_2]^-$ and $[OTf]^-$ containing ILs, it should be noted that they still had an improved performance over the biocompatible traditional solvents, with the exception of tributyrin. Therefore, this further supports the statement that ILs are superior at extracting MAA, as even the most hydrophobic ILs indicated higher distribution ratios than the tested organic solvents. Ultimately, to achieve high extraction efficiencies the most important property is the hydrophilicity of the IL. Water-immiscible yet hydrophilic ILs are needed, and these properties are generally governed by the structure of the anion. Therefore, we can design ILs which are hydrophobic based on their cationic component, but significantly improve the extraction of polar compounds by coordinating these cations with hydrophilic anions.

As viscosity is generally considered to be a key physical property in determining the selection of a solvent for a biphasic extraction process, it was considered as important to reduce the viscosity of the ILs as much as possible. In theory, if viscosity plays a key role in the extraction efficiency of ILs, decreasing the viscosity of the IL should decrease any mass transfer limitations and therefore extraction of MAA into the IL would increase. As the viscosities were determined post water uptake, the correlation between water sorption and viscosity could be evaluated. The viscosity of an IL was shown to be highly influenced by water content. The higher the capacity for water uptake by the IL, the lower the viscosity. As the water concentration increases in an IL, the ionic polar network gradually breaks down with the formation of water aggregates in the IL.[135] Eventually, water hydrogen-bonded networks are formed at high water concentrations.[240] Water molecules ultimately weaken the cation-anion interactions and disrupt the nanostructural organisation of the IL, hence decreasing the viscosity of the ILs in general.[135] The three ILs which had lowest viscosities were $[P_{4448}][Sal]$, $[P_{66614}][C_7COO]$ and $[P_{66614}][(^iC_8)_2PO_2]$. This correlates with the ILs which contained the highest

water content, indicating a clear relationship of an increased water content, and therefore hydrophilicity, with a reduced viscosity. Furthermore, ILs with longer alkyl chains demonstrated higher viscosities, which may offer an explanation for the observed decreased water sorption due to reduced mass transfer rates, therefore making it more difficult for water to be absorbed into the bulk of the ILs.

The viscosity of an IL is also highly affected by temperature, as discussed previously. Therefore, an increase in extraction efficiency was expected with increasing temperature, due to the huge reduction in viscosity of the IL with a small increase in temperature of just 7 °C. Surprisingly, this was found not to be the case and extraction efficiency was only increased by around 2-3 % when the temperature of the system was increased. This suggested that temperature, and therefore viscosity, does not significantly affect the ability of ILs to extract MAA from aqueous systems. This finding was interesting as the viscosity of ILs has long been thought to be one of the main issues with their use in industrial extraction processes. Although mixing costs would still be increased with increasing extractant viscosity, these data indicated that the extraction efficiencies remain extremely high despite mass transfer limitations which come with high viscosities at reduced temperatures. It is anticipated that the interfacial surface tension may be more influential than viscosity on extraction capabilities of ILs, and there is scope for investigation into this.[111]

Overall, the work carried out in this chapter indicated a number of ILs that were promising solvents for the ISPR of MAA, and also ones which were not appropriate. ILs consisting of $[NTf_2]^-$ and $[OTf]^-$ anions were the poorest performers as extractants in the process. These ILs exhibited high viscosities of between 170 – 240 mPa·s and low water contents of between 3000 – 7500 ppm, which correlated with the poorest extraction efficiencies of between 30-35 %. Additionally, these ILs are synthesised from the most expensive starting materials and therefore their use in this process would not be economically viable. Their use would increase process costs, due to mixing to account for high viscosities, increased costs for production of the IL itself and also the

recovery of low concentrations of MAA. Fortunately, all other ILs, containing polar anions, performed exceptionally. They exhibited reduced viscosities of as low as 40 mPa·s, increased water contents of up to 17000 ppm, reduced synthetic costs and improved MAA extraction efficiencies of up to 93 %. Overall, ILs have shown to perform exceptionally well as alternative solvents for the ISPR of MAA. When comparing the extraction of MAA into [P_{66614}][DCA] and heptane, there was a 60 % difference in extraction efficiencies (Figure 17).

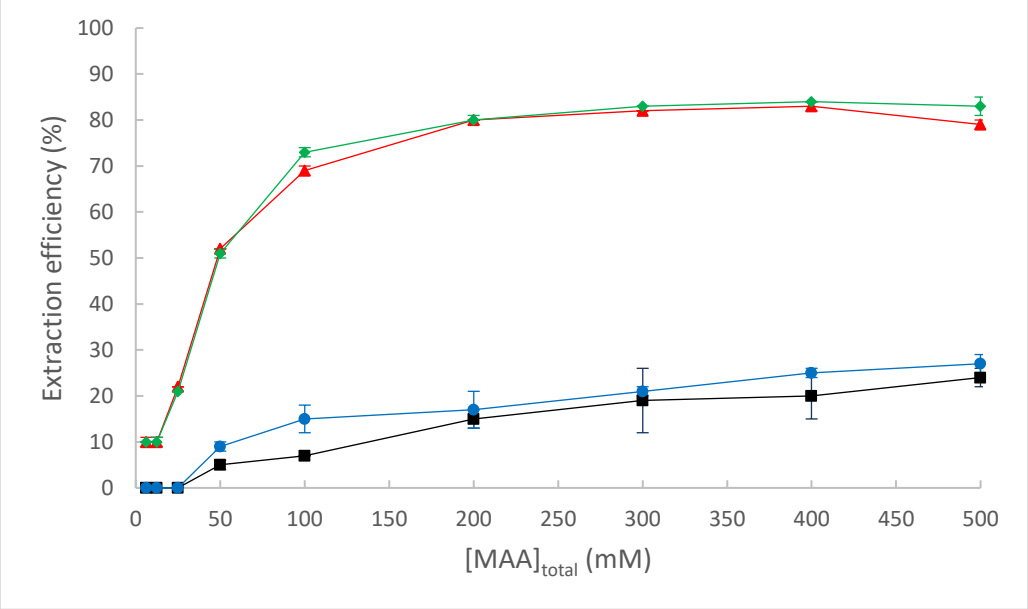


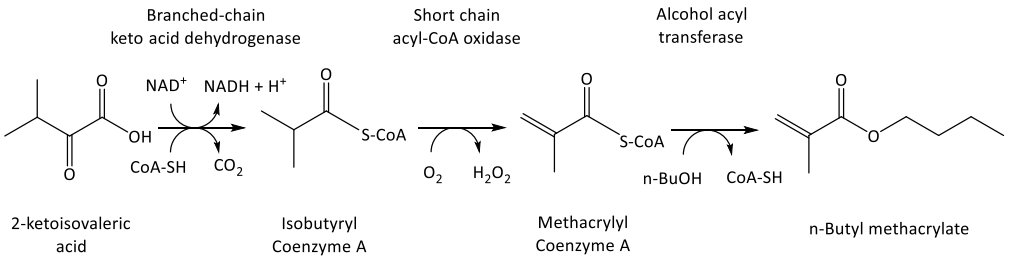
Figure 17. The effect of MAA concentration on the extraction efficiency of P_{66614}DCA at 30 °C (▲) and 37 °C (♦) and heptane at 30 °C (▪) and 37 °C (●)

However, a detailed techno-economic analysis of their implementation into the process at a large scale would be necessary, as many factors play a part in whether this process would be sustainable or not. At a concentration of 50 mM MAA, [P_{66614}][DCA] recovered ~50 % of the MAA from the aqueous phase. For an economical and sustainable process to be achieved using ILs as extraction solvents, a concentration of ≥ 200 mM MAA would need to be produced by the biocatalyst. This would achieve maximum efficiency of the process (Figure 17). At this production concentration, 32 mM MAA would remain in the aqueous phase in contact with the biocatalyst. As discussed in

chapter 5, MAA was toxic towards *E. coli* at 20 mM in LB medium and 10 mM in MSX medium. *S. cerevisiae* was also inhibited by MAA at concentrations of 25 mM. Therefore, despite the extremely efficient extraction of MAA by ILs when compared to traditional organic solvents, the concentration of MAA would still be at a toxic level in the aqueous phase. Therefore, growth and productivities of the biocatalyst would likely be hindered during this process, even during the *in situ* removal of MAA. Hence, an alternative route towards the bioproduction of MMA must be investigated, with focus on the production of alkyl methacrylates.

9 Towards the bioproduction of alkyl methacrylates

The potential for the direct production of alkyl methacrylate esters has been investigated by Lucite International and Ingenza. Production of methacrylate esters allows for simple product recovery through phase separation of the methacrylate, above a critical concentration. A novel, unpublished pathway for the direct production of butyl methacrylate (BMA) has recently been developed, involving a 3-step transformation of 2-ketoisovaleric acid to produce n-BMA, using *E. coli* as the host microorganism (Scheme 6).



Scheme 8. Pathway for the bioproduction of n-BMA in *E. coli*

However, for this process. cerevisises to be considered as economically viable, a production titre of 100 g/L would be required (Graham Eastham, Lucite International, personal communication). Hence, the same issue remains that the ester may present inhibitory effects towards the biocatalyst, as such high production concentrations are required. Therefore, a range of esters were tested to determine which was the least toxic towards the potential host biocatalysts, *Escherichia coli* MG1655 and *Saccharomyces cerevisiae* DSM70449, and hence was the best target molecule.

9.1 Effect of alkyl methacrylates towards growing cultures of *E. coli* MG1655 and *S. cerevisiae* DSM 70449

The effects of methyl methacrylate (MMA), ethyl methacrylate (EMA), isopropyl methacrylate (iPMA) and n-butyl methacrylate (BMA) were tested by growing *E. coli* MG1655 and *S. cerevisiae* DSM70449 in LB and YEPD medium, respectively, in the presence of various concentrations of the alkyl methacrylates. As *E. coli* and *S. cerevisiae* require different optimum growth temperatures, the required volumes of each liquid component were calculated based on the solubility limit of each ester at both 30 and 37 °C (Table 22).

Table 22. The relative solubility limits of the alkyl methacrylate esters at 30 and 37 °C

	Solubility Limit (g/L)	
Ester	**30°C**	**37°C**
MMA	14.50	21.25
EMA	5.50	7.00
iPMA	3.30	5.90
BMA	0.22	0.37

Final biomass concentrations were recorded after 72 h and were reported as a percentage of control cultures (appendix 12.1.5, page 215). Growth rates were not calculated because the duration of the lag phase was too unpredictable; the $MaxOD_{600}$ was recorded instead. Final biomass concentrations were used to calculate the inhibitory concentration (IC_{50}) for each ester. This is the concentration of ester that halves the maximum optical density ($MaxOD_{600}$) compared to growth in the absence of the ester (Table 23).

Table 23. The calculated IC_{50} values of alkyl methacrylates towards *E. coli* and *S. cerevisiae* grown in complex medium

		IC_{50} range (g/L)	
Methacrylate ester	**$logP_{o/w}$**	***S. cerevisiae***	***E. coli***
MMA	0.95	0.73 – 1.45	3.19 – 4.25
EMA	1.49	1.10 – 1.65	0.70 – 1.05
iPMA	1.81	0.66 – 0.99	1.18 – 1.77
BMA	2.57	0.04 – 0.07	0.07 – 0.11

E. coli MG1655 was grown in LB medium and *S. cerevisiae* DSM70449 was grown in YEPD, medium in the presence of alkyl methacrylates, at 37 and 30 °C, 280 rpm. Tests were carried out in 40 mL Teflon-sealed glass vials to prevent the loss of volatile solvents. Component volumes can be found in Table 32, page 214. Ester concentrations were calculated based on the relative solubility limits at the optimum growth temperature for each organism (Table 22). The OD_{600} of the aqueous phase was measured after 72 h using a UV/Vis spectrophotometer. The toxicity of each ester is represented by an IC_{50} range where the final biomass concentration was reduced to 50 % of control cultures. The pH was tested at the end of each experiment to confirm there was no change in pH due to hydrolysis of the esters. Data are the means of three replicates and the standard deviations are shown. Experimental $logP_{o/w}$ values were taken from a report published by Fujisawa and Masuhara.[241]

All esters were highly toxic towards both *E. coli* and *S. cerevisiae* (Table 23). As the concentration of MMA, EMA, iPMA and BMA increased in the aqueous phase, there was a subsequent decrease in the final biomass concentration reached by *E. coli* and *S. cerevisiae* (Appendix 12.1.5). In general, as the logP value of the ester increased, the toxicity also increased. The toxicity of EMA towards *E. coli* was the only exception to this rule, showing a lower IC_{50} range than expected, suggesting *E. coli* to have a reduced tolerance towards EMA, specifically. MMA, iPMA and BMA were more toxic towards *S. cerevisiae* than *E. coli*, halving the final biomass concentration of *S. cerevisiae* cultures at concentrations of between 0.04 and 1.45 g/L. EMA, however, was more toxic towards *E. coli* than *S. cerevisiae*. BMA was the most toxic methacrylate ester, reducing the OD_{600} value by 50% of control cultures at concentrations of between 0.07 – 0.11 g/L for *E. coli* and 0.04 – 0.07 g/L for *S. cerevisiae*. MMA was the least toxic methacrylate ester, particularly towards *E. coli*, demonstrating an IC_{50} value between a concentration of 3.19 – 4.25 g/L.

Although BMA was the most toxic alkyl methacrylate, it has a significantly lower water solubility of 0.22 and 0.37 g/L at 30 and 37°C, respectively. Thus, phase separation would occur at this reduced concentration. MMA, however, would phase separate at a significantly higher concentration of 14.5 and 21.25 g/L, at the respective temperatures. Toxicity tests of the esters at a concentration above their critical water solubility (20 %v/v) were also carried out (Table 24).

Table 24. The effect of alkyl methacrylates (20 %v/v) on the growth of *E. coli* and *S. cerevisiae* in complex medium

	OD_{600} (%)	
Methacrylate ester	***E. coli***	***S. cerevisiae***
MMA	17 ± 1	9 ± 0
EMA	10 ± 1	9 ± 1
iPMA	5 ± 0	8 ± 0
BMA	16 ± 0	9 ± 0

E. coli MG1655 was grown in LB medium and *S. cerevisiae* was grown in YEPD medium (8 mL) in the presence of alkyl methacrylates (2 mL, 20 %v/v). Tests were carried out in 40 mL Teflon-sealed glass vials to prevent the loss of volatile solvents. The OD_{600} of the aqueous phase was measured after 72 h, diluting each sample by a factor of 10, using a UV/Vis spectrophotometer. The pH was tested at the end of each experiment to confirm there was no change in pH due to hydrolysis of the esters. MaxOD_{600} values are reported as a percentage of the values obtained in control cultures containing no methacrylate ester. Data are the means of three replicates and the standard deviations are shown.

Both organisms were unable to grow in the presence of 20 %v/v of all four esters (Table 24). Therefore, the biocatalysts would be inhibited by MMA, EMA, iPMA and BMA before the concentration reached would be high enough for phase separation to occur. Consequently, both *E. coli* and *S. cerevisiae* grown in complex media would not be appropriate host biocatalysts for the production of alkyl methacrylates at an industrially viable production concentration.

The toxicities of MMA, EMA, iPMA and BMA were also determined towards *E. coli* grown in MSX medium. The aim was to assess whether or not changing the growth medium would affect the tolerance of *E. coli* cells towards the esters. Final biomass concentrations were used to calculate the inhibitory concentration (IC_{50}) for each ester (Table 25).

Table 25. The calculated IC_{50} values of alkyl methacrylates towards *E. coli* grown in MSX medium

Methacrylate ester	$logP_{o/w}$	IC_{50} range (g/L)
MMA	0.95	3.19 – 4.25
EMA	1.49	1.40 – 2.10
iPMA	1.81	1.18 – 1.77
BMA	2.57	0.07 – 0.11

E. coli MG1655 was grown in MSX medium in the presence of alkyl methacrylates, at 37 °C, 280 rpm. Tests were carried out in 40 mL Teflon-sealed glass vials to prevent the loss of volatile solvents. Component volumes can be found in Table 32, page 214. Ester concentrations were calculated based on the relative solubility limits at the optimum growth temperature for each organism (Table 22). The OD_{600} of the aqueous phase was measured after 72 h using a UV/Vis spectrophotometer. The toxicity of each ester is represented by an IC_{50} range where the final biomass concentration was reduced to 50 % of control cultures. The pH was tested at the end of each experiment to confirm there was no change in pH due to hydrolysis of the esters, Data are the means of three replicates and the standard deviations are shown. Experimental $logP_{o/w}$ values were taken from a report published by Fujisawa and Masuhara.[241]

The IC_{50} decreased with increasing logP value of the methacrylate esters. The IC_{50} ranges for MMA, iPMA and BMA were similar for *E. coli* cells whether grown in LB or MSX medium. This indicated that growth medium did not have an effect on the tolerance of *E. coli* towards these particular alkyl methacrylates. The IC_{50}, however, was higher (1.4 – 2.10 g/L) towards *E. coli* grown in MSX medium. This indicated that *E. coli* cells cultured in MSX medium are more tolerant of EMA than cells cultured in LB medium. *E. coli* grown in MSX medium tolerated double the concentration of EMA than *E. coli* grown in LB medium. *S. cerevisiae* cells indicated an improved tolerance to EMA compared to *E. coli* cells grown in LB, however *E. coli* cells grown in MSX

medium demonstrated the highest tolerance of all towards EMA. This observation suggests that the toxicity of EMA is specific to each organism and also influenced by the growth medium.

In general, when comparing methacrylate ester toxicity towards *E. coli* and *S. cerevisiae*, *E. coli* indicated a higher tolerance towards each ester, suggesting it to be a more appropriate choice of biocatalyst for the development of this process. Additionally, using a minimal growth medium did not affect ester tolerance and therefore offers the potential for reduced process costs. However, ultimately, the concentrations at which the esters inhibited the growth of both organisms were too low, and suggested that the process could not be sustainable. In fact, the esters were much more toxic than MAA (IC_{50} = 0.86 – 2.58 g/L). However, there is the potential for a process combining ester production and ISPR of the product. The partition coefficients of the esters would be much higher than that of MAA and therefore extraction should be more efficient. However, further tests would be required to confirm this concept.

Table 26. The effect of alkyl methacrylates (20%v/v) on the growth of *E. coli* in minimal medium

	OD_{600} (%)
Methacrylate ester	***E. coli***
MMA	8 ± 0
EMA	6 ± 0
iPMA	6 ± 0
BMA	79 ± 3

E. coli was grown in MSX medium at 37 °C, 280 rpm. Tests were carried out in 40 mL Teflon sealed glass vials. Each test vial contained MSX inoculated with *E. coli* (8 mL) and methacrylate ester (2 mL, 20 %v/v), giving a total test volume of 10mL. The $MaxOD_{600}$ of the aqueous phase was measured after 72 h, diluting each sample by a factor of 10, using a UV/Vis spectrophotometer. The pH was tested at the end of each experiment to confirm there was no change in pH due to hydrolysis of the esters. $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures containing no methacrylate ester. Data are the means of three replicates and the standard deviations are shown.

In the tests containing biphasic mixtures of *E. coli* in MSX and MMA, EMA and iPMA (20% v/v), the growth of *E. coli* was entirely inhibited (Table 26). However, an unexpected result was discovered during the toxicity tests involving BMA. BMA (20 %v/v) inhibited the growth of *E. coli* cells completely, when grown in LB medium. However, in the MSX grown cultures containing BMA (20 %v/v), after a 48 h lag period the culture grew to an OD_{600} of 79 % of that of the control (Table 26). This observation suggested that during the extended lag phase, the *E. coli* cells had either adapted or mutated in order to remove or tolerate the large concentrations of BMA.

9.2 Discovery and isolation of a BMA tolerant *E. coli* mutant

In order to confirm the discovery of a BMA tolerant *E. coli* mutant, it was necessary for the strain to be isolated and undergo further toxicity testing. From the culture, which had successfully grown over 72 h in MSX in the presence of 20 %v/v BMA, an aliquot was taken (100 µL) and added to fresh MSX (7.9 mL) containing BMA (2 mL, 20 %v/v), exactly as before. Alongside this test, two more tests were run. The growth of the original wild type *E. coli* MG1655 strain was re-tested in MSX medium in the presence of BMA (20% v/v). This was carried out in order to demonstrate that the wild type *E. coli* MG1655 strain was inhibited by BMA and the mutations and subsequent tolerance was not present in the parent strain. The second test vial was a control, containing wild type *E. coli* MG1655 in MSX in the absence of BMA, to ensure the strain was growing normally. The results showed that the wild type *E. coli* did not grow in the presence of BMA after 24 h. By contrast, the sample of the putative BMA-resistant mutant grew to an OD_{600} of 1.52, compared to the control culture which reached an OD_{600} of 1.85. There was no longer a significant lag phase for the putative mutant. Therefore, it was believed a tolerance to BMA had indeed been developed. Therefore, the mutant was isolated. A sample of this culture was streaked out on an LB plate, five different colonies were isolated and respective cryostocks were prepared. The five mutant strains were named colony 1-5. Each of the five mutants were then re-

tested for BMA tolerance in MSX medium and the growth rates monitored over 30 h. The calculated growth rates and $MaxOD_{600}$ values reached after 30 h are displayed in Table 27 and the raw data can be found in appendix 12.1.6, page 216.

Table 27. The growth of wild type *E. coli* MG1655 and the five mutant strains in MSX medium in the presence of BMA (20 %v/v)

Strain	**Specific growth rate (μ) (h^{-1})**	**μ (%)**	**$MaxOD_{600}$**	**$MaxOD_{600}$ (%)**
Control	0.571	100	2.03	100
Wild type *E. coli* MG1655	-	-	-	-
Colony 1	-	-	0.89	44
Colony 2	0.575	100	1.34	66
Colony 3	0.575	100	1.15	57
Colony 4	-	-	0.79	39
Colony 5	0.661	115	1.54	76

E. coli was grown in MSX medium at 37 °C, 280 rpm. Tests were carried out in 40 mL Teflon sealed glass vials. Each test vial contained MSX inoculated with *E. coli* (8 mL) and BMA (2 mL, 20 %v/v), giving a total test volume of 10 mL. Growth rates (μ) and $MaxOD_{600}$ values are reported as a percentage of the values obtained in control cultures containing no IL. $MaxOD_{600}$ values were taken as the highest OD_{600} value after 30h. Data are the means of three replicates and the standard deviations are shown. "-" data was unmeasurable

Wild type *E. coli* MG1655 strain did not grow in the presence of BMA and the control containing no BMA grew as normal (Table 27) (appendix 12.1.6, page 216). In the presence of BMA (20 %v/v), the colonies 2,3 and 5 matched the growth rate of the control. Colonies 2,3 and 5 showed a small increase in lag time of approximately 2 h. The OD_{600} values reached after 30 h varied between 57 – 76 %, with colony 5 exhibiting the greatest cell growth in the presence of BMA. Whereas, colonies 1 and 4 grew so slowly that the rate could not be calculated. Colonies 1 and 4 had an increased lag phase of 8 h, however, when sampled after 30 h the cultures had grown to 44 and 39 %, respectively, compared to control cultures. This indicated that colonies 1 and 4 had a

reduced tolerance towards BMA, compared to colonies 2, 3 and 5. The cultures were incubated for a further 24 h and there was no observable change in OD_{600} value, indicating that the maximum growth was reached after 24 h for each mutant strain.

In order to establish whether the BMA tolerance was specific to *E. coli* cells cultured in MSX medium, the tests were repeated in LB medium. None of the mutants or the wild type *E. coli* could grow in the presence of BMA (20 %v/v) when cultured in LB medium. This lack of growth and tolerance in complex medium suggested that in order for the cells to exhibit tolerance towards BMA, it is essential they be cultured in MSX medium. The cultures were incubated for a further 5 days, during which time no growth was seen in any of the LB cultures, confirming the lack of tolerance of the strains towards BMA when grown in LB.

As the previous tests had shown that the mutant strains could grow in LB medium in the absence of BMA, instead of growing the inocula in LB medium, the inocula were initially grown in MSX medium in the absence of BMA. LB medium was then inoculated using these cultures, and grown in the presence of BMA (20 %v/v) (Table 28) (appendix 12.1.6, page 216). This would indicate whether the strains were utilizing a component of the MSX medium during early stage replication, resulting in specific cell membrane components and metabolic processes which create a tolerance towards BMA.

Table 28. The growth of wild type *E. coli* MG1655 and the five mutant strains in LB medium containing BMA (20 % v/v) inoculated with an overnight culture of each strain grown in MSX medium

Name	Specific growth rate (μ)(h^{-1})	μ (%)	$MaxOD_{600}$	$MaxOD_{600}$ (%)
Control (Wild type)	0.374	100	2.1	100
Wild type + BMA	-	-	-	-
Colony 1	-	-	0.92	44
Colony 2	0.326	87	1.01	48
Colony 3	0.260	70	1.01	48
Colony 4	-	-	0.79	38
Colony 5	0.314	84	1.14	54

Wild type *E. coli* and the five *E. coli* mutant strains were grown in MSX medium (25 mL). An aliquot of each culture containing a different strain was then taken and used to inoculate LB medium (8 mL). BMA (2 mL, 20 %v/v) was then added to each test and the cultures were incubated at 37 °C, 280 rpm for 30 h. Growth rates (μ) and $MaxOD_{600}$ values are reported as a percentage of the control cultures containing no BMA. $MaxOD_{600}$ values were taken as the highest OD_{600} value at each pH value after 30h. Data are the means of three replicates. "-" data was unmeasurable.

All five mutant strains grew in LB medium, in the presence of BMA (20 %v/v), that had been inoculated with cultures grown in MSX medium. The $MaxOD_{600}$ values reached for all mutant strains were significantly lower than if the cultures were inoculated with an overnight culture of the strains grown in MSX medium. Therefore, growth of the inoculum and/or the experimental culture in MSX medium was essential for the tolerance to be fully expressed. Consistent with the previous results obtained for the growth of the strains in MSX medium in the presence of BMA (Table 27), colonies 2, 3 and 5 demonstrated the highest OD_{600} values reached along with growth rates of over 70 % of control cultures. Much longer lag phases were observed for colonies 1 and 4, however colony 1 reached similar OD_{600} values to the other mutant strains, whereas colony 4 showed little growth after 30 h, indicating a significantly reduced tolerance of this strain to BMA.

The tests were repeated, however this time inoculating MSX medium containing BMA (20 %v/v) with overnight cultures which were grown in LB medium. It was anticipated that the cultures would not grow in LB medium in the presence of BMA as seen previously, however this was not the case (Table 29).

Table 29. The growth of wild type *E. coli* MG1655 and the five mutant strains in MSX medium containing BMA (20 % v/v) inoculated with an overnight culture of each strain grown in LB medium

Name	$MaxOD_{600}$	Max OD_{600} (%)
Control (Wild type)	4.44	100
Wild type + BMA	-	-
Colony 1	0.68	15
Colony 2	0.52	12
Colony 3	0.46	10
Colony 4	0.59	13
Colony 5	0.48	11

Wild type *E. coli* and the five *E. coli* mutant strains were grown in LB medium (25 mL). An aliquot of each culture containing a different strain was then taken and used to inoculate MSX medium (8 mL). BMA (2 mL, 20 % v/v) was then added to each test and the cultures were incubated at 37 °C, 280 rpm for 30 h. $MaxOD_{600}$ values are reported as a percentage of the control cultures containing no BMA. $MaxOD_{600}$ values were taken as the highest OD_{600} value at each pH value after 30 h. Data are the means of three replicates.

Having inoculated with cultures previously grown in MSX, all mutants were now somewhat able to grow in LB and BMA, albeit extremely poorly, reaching final biomass concentrations of between 10-15 % compared to control cultures (Table 29).The significantly reduced growth of the strains in LB suggests that when originally cultured in MSX, essential structural compositions and metabolic pathways are established, which are responsible for the improved tolerance to BMA. The data suggests that the strains cannot develop and express the necessary tolerance mechanisms when cultured in LB medium. Ultimately, the inocula must be cultured in MSX medium, or the cultures must be grown in MSX medium during exposure to BMA, in order for the mutants to express full tolerance towards the product. Therefore, it can

be concluded that MSX is the ideal medium for growth of the mutants in the presence of BMA and its presence seems vital for the expression of BMA tolerance.

As these growth tests have indicated a difference in levels of tolerance between the five mutant strains, colonies 2, 3 and 5 showing a strong tolerance towards BMA and colonies 1 and 4 indicating a much lower tolerance, important information could be gained by sequencing the genomic DNA of the mutants. In order to determine the mutations responsible for the tolerance of the mutant strains towards BMA, genomic DNA was extracted from wild type *E. coli* MG1655 as a reference parent strain, and from the five *E. coli* mutant strains (4.6.1, page 77). The DNA samples were then sent for sequencing at the Centre for Genetics and Genomics, located at the University of Nottingham. Genomic sequencing was carried out by Sunir Malla (Deep Seq). The sequencing and bioinformatics data was analysed by Luca Rossoni and therefore the raw data is not presented in this *thesis*. However, the analysis and results are discussed below.

9.3 Interpretation of DNA sequencing results

By comparing the DNA sequencing results with the previous toxicity tests of BMA towards each of the five mutant strains, some insight was gained into which genes and mutations may be responsible for the resultant BMA tolerance. Colonies 1-5 contained different sets of mutations, hence were considered as five separate mutant strains. The observed mutations in each strain are displayed in Table 30.

Table 30. The mutations found by whole genome sequencing analysis of wild type *E. coli* MG1655 and mutant strains

			Strain					
Gene	**Mutation**	**Protein length (amino acids)**	**Wild type**	**1**	**2**	**3**	**4**	**5**
soxR	R20H	154	-	+	+	+	+	+
ycbO	I87M	96	-	-	-	-	+	-
acrR	V29G	215	-	-	-	+	-	-
acrR	Deletion at 32aa	215	-	-	+	-	-	+

+/- The mutation is present/absent on comparison of sequencing results with the gene sequence on NCBI database

The sequencing of the five mutant strains revealed that there were four different mutations in three genes; *soxR*, *acrR* and *ybcO*. The *soxR* mutation was present in all five strains, a missense mutation in the *soxR* gene, where there was a substitution in the 20th amino acid of arginine to histidine (R20H). Colony 1 contained the mutation only, whereas colony 4 contained a mutation in *ybcO*, where methionine had replaced isoleucine at the 87th amino acid position (I87M). These mutants were less resistant to BMA compared with the other mutant strains. The more resistant strains, colonies 2, 3 and 5, contained the same *soxR* mutation, but also presented mutations in the *acrR* gene. Colonies 2 and 5 had the same mutation in *acrR*, which was a deletion of an adenosine at the 32nd amino acid, resulting in a truncated protein. Colony 3 had a missense mutation in *acrR*, where a valine was substituted by a glycine at the 29th amino acid position (V29G).

To the best of my knowledge, these mutations have never been reported in the literature in these combinations. As the discovery of these mutants came late in the stages of this project, there was little time to investigate the molecular biology in order to fully understand the relationship between the observed mutations and apparent BMA tolerance, thus a follow-on project is currently being carried out in order to develop the strain and further improve

its tolerance to BMA. However, it was possible to gain an initial understanding of the potential causes of BMA tolerance by reviewing the literature and gaining an understanding of the function of the three genes.

The *soxR* gene encodes a transcription factor which is activated in the presence of oxidative stress.[17] It is responsible for the regulation of expression of several stress response genes. In the case of this particular *soxR* mutation, a single mutation was observed, which replaces a wild type arginine with histidine. The location of this amino acid is in the DNA binding site of *soxR* and therefore this variation could potentially improve or reduce the ability of *soxR* to bind to DNA, resulting in an alteration of the gene expression regulation. This particular mutation has yet to be reported in the literature, however other mutations have been reported in the *soxR* gene which confer solvent and antibiotic resistance.[91,242]

AcrR is a transcription factor and repressor of the AcrAB-TolC efflux system and regulates the expression of other stress response genes, including *soxR*.[243] The AcrAB-TolC system acts as an efflux pump in *E. coli* with AcrB as the inner membrane transporter acting as the pump, AcrA being the membrane fusion protein and TolC being the outer membrane protein.[17] Expression of *acrAB* and related genes is modulated by the transcriptional regulators *marR*, *marA*, *soxR* and *soxS*, however the *acrAB* operon is also repressed by AcrR, as it binds to the promoter region of the *acrAB* operon.[243] The observed deletion in *acrR* in colonies 2 and 5 causes a frameshift which would result in the expression of a truncated inactive protein. No similar mutation has been reported in the literature. The lack of this repressor would, in theory, increase the expression of *acrAB*, and this may contribute to BMA tolerance. It has been reported in the literature that overexpression of *marA* and *soxS* increased the production of AcrA and TolC, which resulted in a higher cyclohexane tolerance due to an increased activity of the efflux pump.[244] To further support the relationship between the *acrAB* operon and solvent tolerance, a deletion of *acrAB* also resulted in the loss of tolerance to both hexane and cyclohexane. [244]

It has been reported that the SoxRS operon affects the expression of AcrAB and TolC. Therefore, the mutation observed in the *soxR* gene may be the only mutation which causes a tolerance towards BMA. However, as the colonies containing both *soxR* and *acrR* mutations exhibited the highest level of tolerance compared with the strains containing the *soxR* mutation alone, it could be suggested that the *soxR* mutation affects the expression of the membrane transporter, and the *acrR* mutations further enhances its expression, resulting in a more efficient exportation of BMA out of the cells. As colonies 2, 3 and 5 had similar growth rates, this suggests that the truncated AcrR found in colonies 2 and 5 improves the tolerance of the strains to BMA to a similar extent as the mutated AcrR found in colony 3 does, irrespective of the functionality of the protein. Various strains containing V29G *acrR* and R20H *soxR* mutations have been published as being linked to antibiotic resistance, however a combination of the two mutations in the same strain, along with a resistance to BMA, is yet to be reported.[242,245,246]

By comparing the toxicity results of the growth of each of the mutant strains in the presence of BMA, with the mutations observed in the genome sequencing, it was possible to identify the genes and mutations that are responsible for the improved tolerance to BMA. Colonies 1 and 4, which demonstrated significantly increased lag phases and a reduced tolerance towards BMA, contained the *soxR* mutation only, indicating that a missense mutation in *soxR* alone does not give rise to BMA tolerance within *E. coli* cells. Additionally, colony 4 had a missense mutation in ybcO. Colony 4 appeared to have the lowest tolerance of all the mutant strains and therefore it could be suggested that this mutation decreases the strains tolerance towards BMA, or simply has no effect at all with regards to BMA tolerance. YbcO is an uncharacterised protein that belongs to the DPL12 prophage. There are no publications on the role of this protein and therefore the effect of the mutation cannot be explained at this stage with regards to its effect, if any, on BMA tolerance. Colonies 2, 3 and 5 which showed the highest tolerance towards BMA contained the combination of both *soxR* and *acrR* mutations,

indicating that both mutations are needed for maximal resistance of *E. coli* to BMA.

In conclusion, the information from the identification the mutations and tolerance of the mutant strains towards BMA is promising, however further experiments are needed to investigate the biological function of the mutations and are being carried out in a follow-on project. A patent application is currently being prepared based on the identification of these BMA tolerance *E. coli* MG1655 mutants. The discovery of these mutants which can tolerate such high concentrations of BMA (20 %v/v) is extremely significant as this would be a commercially viable production concentration. However, it would be important to establish whether the cells are still resistant when the BMA is being produced inside the cells, instead of being added from the outside of the cell in the culture medium.

10 Discussion and Future work

10.1 Bioproduction of MAA

The work in this thesis has explored the scope for the development of a biosynthetic route to MMA, by solving the problems associated with the toxicity of the potential bioproducts. Investigation into the use of ILs as alternative low-toxicity solvents has offered a proof of concept that ILs do indeed perform exceptionally well compared to traditional organic solvents, by removing up to 93 % of MAA from aqueous systems, at pH 7.0. Not only did ILs recover high concentrations of MAA, but also the extraction efficiency was not significantly affected by a reduction in temperature or an increase in viscosity. This would be highly beneficial in an industrial scale process as it indicated that when using ILs as solvents, recovery of the product will not be limited by a reduction in mass transfer. Therefore, this process could be developed using organisms that have lower optimal growth temperatures, such as *S. cerevisiae*. Thus, MAA production could be achieved using host organisms that have a higher tolerance towards acids, which may remove the toxicity issues associated with MAA, without affecting the viability of the extraction process due to the reduced process temperatures. Further investigation into the physicochemical properties of ILs would be beneficial to help predict IL structures which could improve the extraction process even more. As the viscosity of ILs did not affect the extraction process, there is scope for investigation into the interfacial surface tension of these IL/aqueous systems, as there may be a correlation between a low interfacial surface tension and improved product recovery.

With regards to the investigation into discovering biocompatible ILs, this work has suggested that there is huge potential for the design of a wide range of non-toxic ILs, compared to a very limited number of biocompatible hydrophobic solvents. The number of non-toxic solvents was limited and the toxicity was generally only dependent on the logP value of the solvent. By contrast, there may be vast numbers of ILs which can be designed to be

biocompatible with the biocatalysts. The toxicity studies in this thesis highlight the potential to further tune ILs based on our current knowledge and understanding of their structure-toxicity relationship to produce more biocompatible task-specific ILs (TSILs). There is also scope for further investigation into the mechanism of toxicity of ILs. In chapter 8.2 it was suggested that the interactions between ILs and components within the growth medium could play a part in the resulting inhibitory effects that certain ILs cause. Particularly when the biocatalyst is grown in a minimal salt medium it could be speculated that ILs may have the ability to extract salts out of the aqueous phase, which, if essential for growth, may result in inhibition. The ability of ILs to extract metal salts from aqueous systems has been reported previously in the literature, and therefore this is a viable hypothesis.[111,231,247] One possibility would be to use ion chromatography (IC) to examine, in depth, whether or not anion exchange is occurring during the mixing of fermentation broths and ILs.

It was anticipated in the early stages of this project that for efficient MAA extraction, the system would have to be at pH < pKa to produce and extract the undissociated neutral acid. Unlike neutral molecules, *in situ* production of an organic acid is challenging because of the need for the acid product to be in its undissociated form to improve extraction into conventional molecular solvents. However, extraction of MAA using ILs is independent of pH, due to the improved intermolecular reactions between the negatively charged MAA and ILs. Even at pH 7.0, extraction efficiencies were around 90 %, compared to organic solvents which recovered a maximum of ~30 % of the MAA from the aqueous phase at this pH. Consequently, there is no need to put biocatalysts under additional acidic pH stress in order to recover higher concentrations of MAA. Issues remain with the process of ISPR of MAA using ILs, as the maximum extraction efficiency still resulted in toxic concentrations of MAA remaining in the aqueous phase. Therefore, further development of the producing strain would be necessary, with the aim of improving tolerance towards MAA

enough for the biocatalytic productivities to not be hindered during the *in situ* extraction process.

The mechanism of the strong interaction between ILs and MAA requires further investigation. This could be carried out using solvatochromic studies. The determination of Kamlet-Taft parameters, with regards to the HBD and HBA abilities of the anions, would offer insight into the interactions between MAA and the ILs that may be responsible for the high extraction efficiencies. This would also help to indicate the best methods for the recovery of MAA from ILs, for which an understanding of the interactions between MAA and ILs would be crucial. There are currently issues with recovery of the MAA from the IL phase once extracted due to the high affinity of the IL for MAA. If the process is to be considered as economically viable, investigation into establishing an effective method for MAA removal from the IL would be essential. There is the possibility of extraction of the MAA from the IL into a highly alkaline aqueous medium, followed by acidification of the solution to precipitate the MAA. However, there is the potential for anion/cation exchange with the IL and therefore detailed studies would need to be carried out to assess the viability of this product recovery option.

The distillation of solutes from ILs is generally considered as a simple process for product recovery.[114] As ILs exert no measurable vapour pressure and hence are challenging to distil themselves, product recovery from them is generally easy. MAA has a boiling point of 161°C and could therefore, in theory, simply be distilled from the ILs. However, acidification of the IL would first be necessary, as the conjugate base of MAA would be non-volatile and therefore could not be removed *via* distillation. The ease of distillation of MAA from an IL would be dependent on the pKa of the IL anion and the boiling point of the conjugate acid of the IL anion, relative to the pKa and boiling point of MAA. For example, if the IL anion was $[NTf_2]^-$, which has a low basicity, and the distillation of MAA was attempted after acidification of the solution, the MAA would likely remain protonated and be distilled off easily. However, if the IL anion had a high basicity, such as an acetate anion $[OAc]^-$, there is the

possibility that some acetic acid would be distilled off, as well as MAA, due to their similar pKa values. Hence, solvatochromic studies would not only help to understand the ability of the ILs to be extracted by ILs, but also how easily MAA can be distilled from the IL. Additionally, distillation at industrial scale is an expensive and energy intensive process and may not give complete separation due to the formation of azeotropes.[248] Therefore, distillation of MAA from an IL may not be as simple as would be expected.

A different approach to remove MAA from ILs could be *in situ* reaction followed by extraction, *via in situ* esterification or transesterification to form MMA. This has the potential to cut down the processing costs by carrying out both the extraction and esterification in one step.[249] This would eliminate the requirement for two separate processes, as the alcohol acts as both an extraction solvent and a transesterification reagent.[250] This could potentially remove the need for an IL all together by esterifying MAA *in situ* to form an alkyl ester, which would phase separate from the aqueous phase. However, in this case the biocatalyst would still need to tolerate high concentrations of both MAA and the methacrylate ester so this raises other toxicity issues. The *in situ* transesterification of biomass to produce biofuels has been investigated over recent years. Reports on its success include the *in situ* transesterification of algal biomass to produce fatty acid methyl esters (FAME) and also the *in situ* transesterification of lipids in microalgae to make crude biodiesel.[251,252] There is potential for this method to be implemented in this process for the recovery of MAA, whilst producing an alkyl methacrylate product. However, the feasibility of whether this would work would need to be practically assessed.

An alternative method to recover MAA from ILs would be to use membrane separation technology.[253,254] A separation process based on nanofiltration has recently been reported that is especially well suited for the isolation of non-volatile, charged compounds, by exploiting the selectivity of nanofiltration membranes towards size and charge of the components.[254] Therefore, either the MAA would be retained and the IL would pass through the membrane, or the IL would be retained and the MAA would pass through. This method may

also be used for an easy recovery and/or purification of ILs in order for them to be recycled in the process, which perhaps makes it the most appealing method for MAA recovery.[114] This point also raises questions about the recyclability of the ILs. It would be important to determine how long these ILs could be used for MAA extraction, before the efficiency of the process reduces. This would be an important factor in determining the economic viability of the process. Recycling the IL may require distillation of impurities and byproducts, which may accumulate in the IL during the process. However, distillation of ILs is difficult and generally has to be carried out under ultra-high vacuum, which some ILs have been reported to decompose under these conditions and therefore they would not be able to be recycled in the process.[255,256] Therefore, this option would not be feasible at industrial scale.

Overall, the most crucial next steps in the development of this process for the ISPR of MAA *via* liquid-liquid extraction using ILs would be to test the growth of the *E. coli* strain while it is producing MAA, in the presence of the best performing biocompatible ILs, such as $[P_{66614}][C_7COO]$ and $[P_{4448}][Sal]$. By monitoring MAA production and extraction it could be determined whether or not the current low production titres are due to toxicity of the MAA, or actually due to bottlenecks in the pathway.

10.2 Bioproduction of BMA

The bioproduction and extraction of MAA has many issues that need to be addressed, including suggested bottlenecks in the metabolic pathways, resulting in low production titres, high toxicity of MAA, and recovery of the MAA from ILs after extraction. Therefore, alternative bioproduction strategies were investigated. The bioproduction of alkyl methacrylates offered the benefit of phase separation above a specific concentration, simplifying the product recovery process. During assessment of the toxicities of MMA, EMA, iPMA, BMA, a BMA resistant mutant was discovered and isolated, as discussed in chapter 9. The most significant discovery was that the isolated mutant strains could tolerate and grow in the presence of extremely high

concentrations of BMA (20 %v/v). At this production titre, the process would be considered as sustainable and viable for scale up. Therefore, it is a significant step towards the development of an industrial scale biosynthetic route to BMA. Additionally, the discovery of this BMA resistant mutant offers the opportunity to design a process without the need for an extractant. Therefore, process costs would be significantly reduced and product recovery would be much simpler, compared to the MAA route.

Numerous reports have been published on the discovery and isolation of chemically resistant mutants. The improvement of chemical tolerance within microorganisms creates opportunity for the bioproduction of chemicals in amounts sufficient to compete economically with chemical syntheses.[19] The majority of publications to date have reported solvent tolerance in mainly *Pseudomonas*, *Bacillus* and *Rhodococcus* strains.[77,20719] Studies involving these organisms have developed strains which can tolerate various concentrations of toluene (50 - 90 %v/v), however the majority of publications are based on tolerance expressed by *Pseudomonas* strains.[62,257] Additionally, these strains also showed a tolerance towards increased levels of organic solvents including styrene, cyclohexane and xylene.[207,257] However, no publications have ever reported a tolerance in any organism towards methacrylate esters. It would be interesting to test the tolerance of the five *E.coli* mutants towards other, usually toxic organic solvents to establish if the chemical tolerance is BMA specific, or if a range of other solvents of similar logP value could be tolerated by the mutant strains.

Before further developing these mutant strains, it is important to gain a better understanding of how the three mutations observed within the BMA mutant strains might affect the BMA resistance. Although *ybcO* is an uncharacterized protein, based on the expressed mutations within colonies 2, 3 and 5, and their resulting higher resistance to BMA than colonies 1 and 4, the mutations of *soxR* and *acrR* are the most likely to confer the observed resistance. Furthermore, the latter genes are already known to be involved in stress response mechanisms in *E. coli.* Therefore, future investigation into the effect

of these mutations would be vital to understand the mechanisms of BMA resistance and to develop further strategies to enhance BMA production. Additionally, it was suggested that the combination of *soxR* and *acrR* mutations was more advantageous than the other combinations of mutations. The colonies which showed the highest tolerance towards BMA contained the combination of both *soxR* and *acrR* mutations, indicating that both mutations are needed for maximal resistance of *E. coli* to BMA. To the best of my knowledge this combination of mutations has yet to be published or indeed associated with an expressed chemical tolerance within an *E. coli*. To move forward towards the development of a biosynthetic route to BMA in these mutant strains, it is crucial to gain an understanding of the resistance mechanisms in *E. coli*, which are affected and/or regulated by these mutations. The emerging market for the bioproduction of chemicals is yet to overcome these toxicity issues to achieve an economically viable bioprocess and be able to compete against the chemical processes currently used. Therefore, the crucial next steps would be to demonstrate the influence of each mutated gene on the apparent BMA resistance in *E. coli*. Additionally, it is extremely important to establish whether the mutant *E. coli* cells are still resistant when the BMA is being produced inside the cells instead of being added from the outside of the cell in the culture medium.

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12 Appendices

12.1 Toxicity tests

12.1.1 pH value

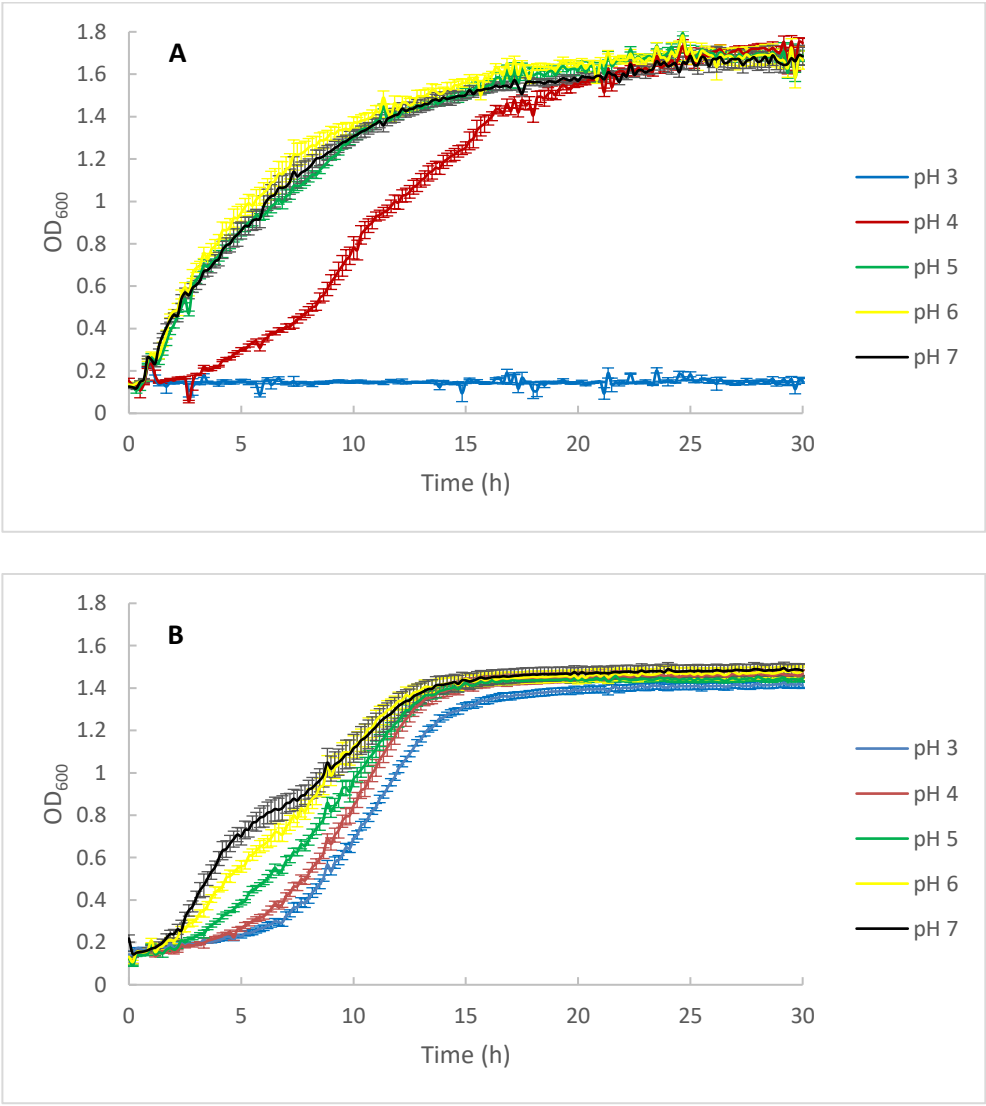


Figure 18. Effect of pH value on the growth of *E. coli* MG1655 and *S. cerevisiae* DSM 70449

E. coli MG1655 (A) and *S. cerevisiae* DSM 70449 (B) were grown in LB and YEPD medium buffered at various pH values. The growth was performed in 100 well plates and increase in

cell density was measured using a Bioscreen C MBR. Results and standard deviations are the average of quintuplicate experiments.

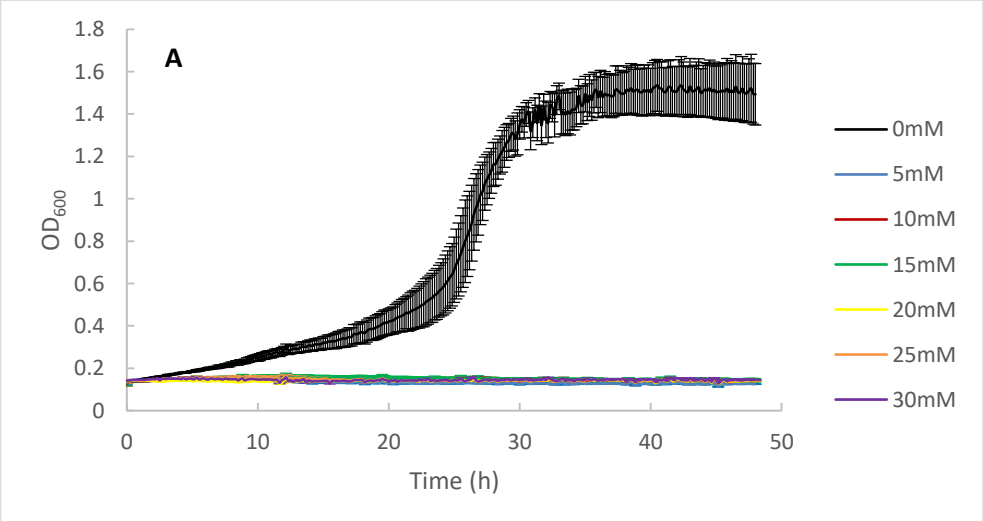
12.1.2 Methacrylic acid

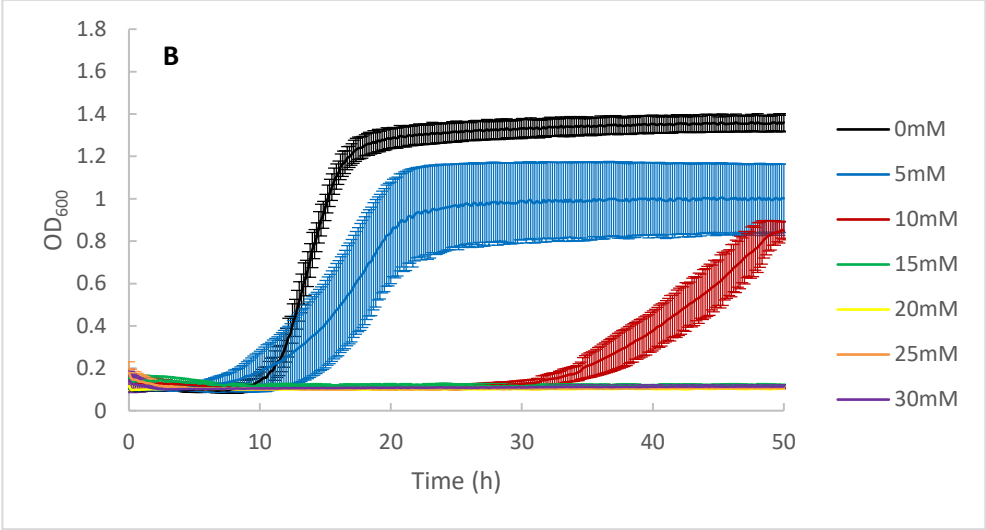
12.1.2.1 Preparation of MAA cultures

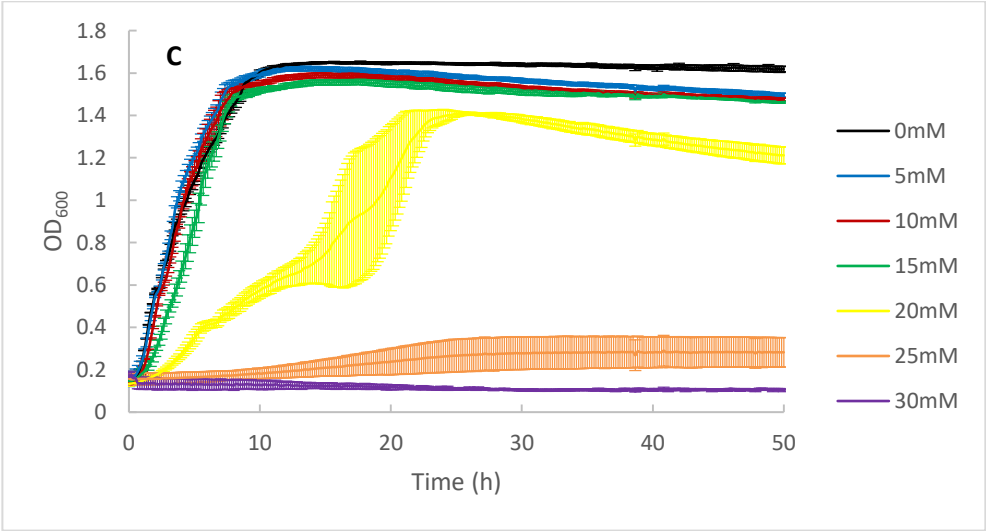
Table 31. The volumes of 1M MAA and growth medium required to produce each concentration of MAA

MAA Concentration (mM)	Volume of 1M MAA (µL)	Volume of medium (µL)
0	0	295.0
1	0.3	294.7
2	0.6	294.4
3	0.9	294.1
4	1.2	293.8
5	1.5	293.5
10	3.0	292.0
15	4.5	290.5
20	6.0	289.0
25	7.5	287.5
30	9.0	286.0

12.1.2.2 Growth curves




B
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
10
20
30
40
50
Time (h)
0mM
5mM
10mM
15mM
20mM
25mM
30mM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1.4
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0.8
0.6
0.4
0.2
0
OD600
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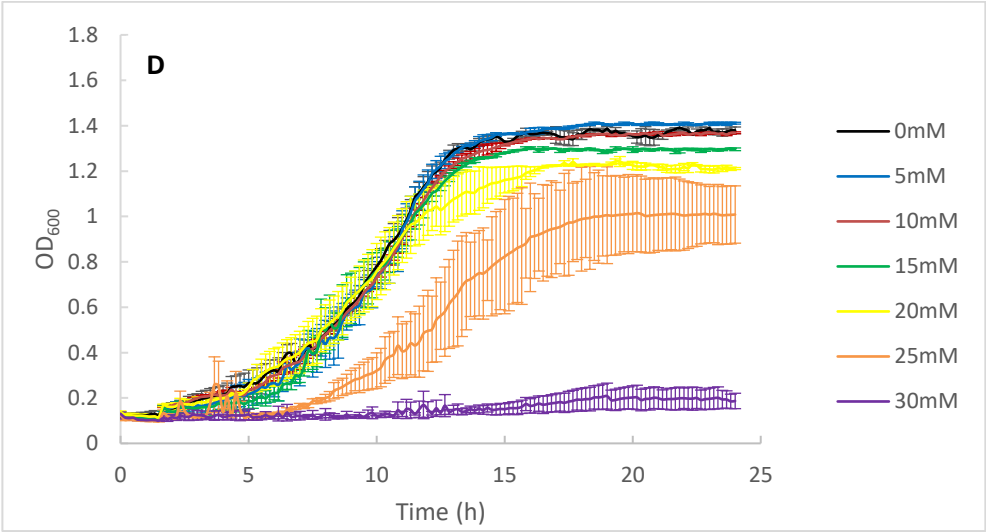
10
20
30
40
50
Time (h)
0mM
5mM
10mM
15mM
20mM
25mM
30mM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1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
5
10
15
20
25
Time (h)
0mM
5mM
10mM
15mM
20mM
25mM
30mM

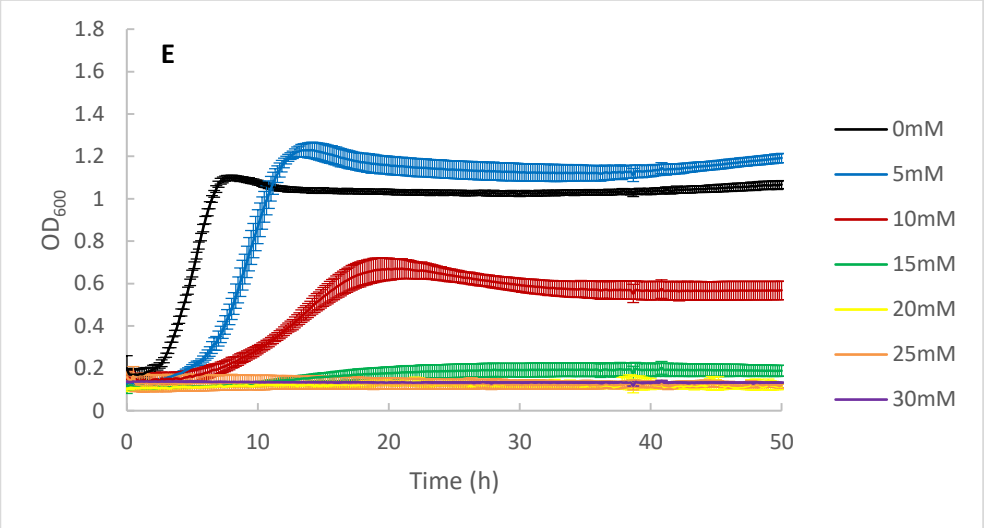
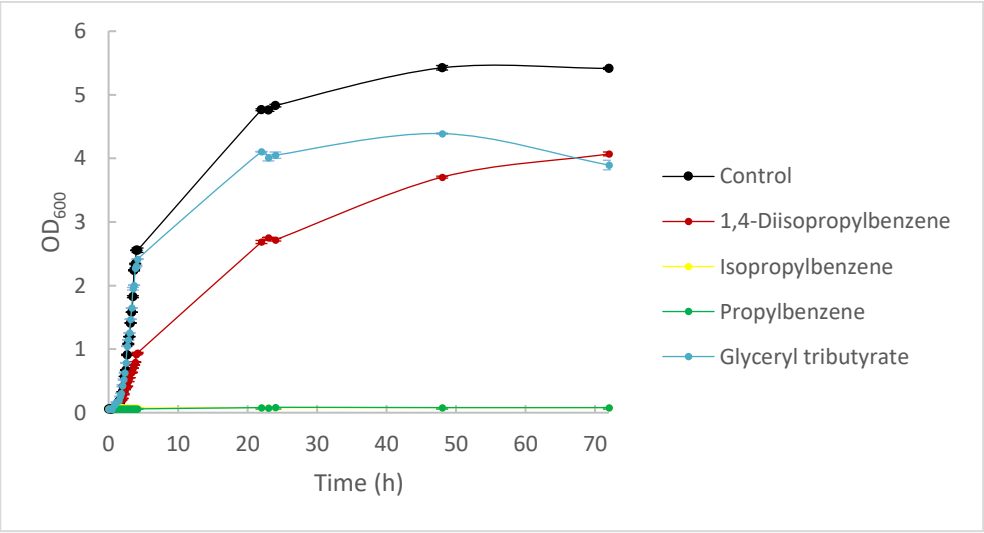
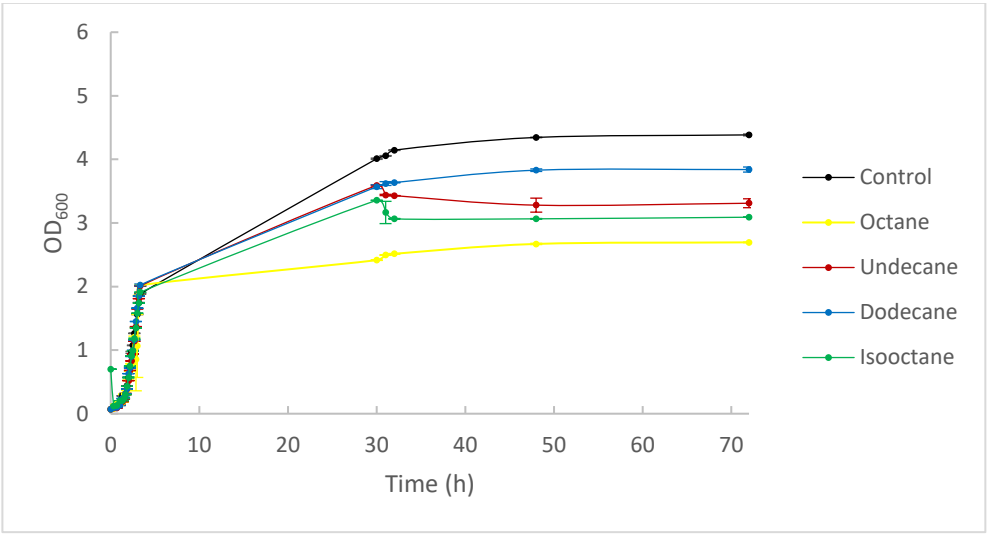


Figure 19. Effect of MAA on the growth of *E. coli* MG1655 and *S. cerevisiae* DSM 70449

E. coli MG1655 and *S. cerevisiae* DSM 70449 were grown in medium buffered to pH 4 or 7, containing various concentrations of MAA. The growth was performed in 100 well plates and increase in cell density measured using a Bioscreen C MBR. Results and standard deviations are the average of triplicate experiments. A: *E. coli* in LB at pH 4, B: *S. cerevisiae* in YEPD at pH 4, C: *E. coli* in LB at pH 7, D: *S. cerevisiae* in YEPD at pH 7, E: *E. coli* in MSX at pH 7.

12.1.3 Organic solvents





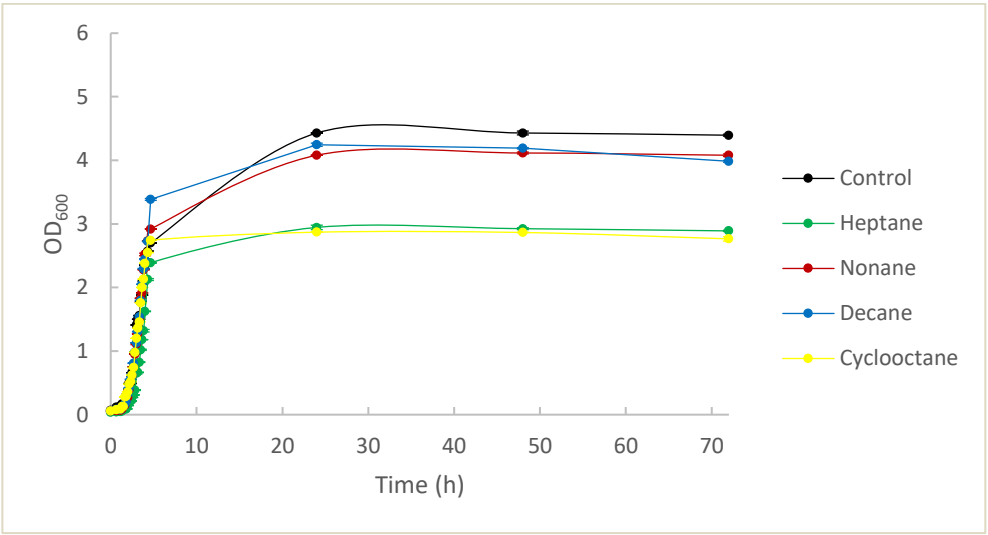
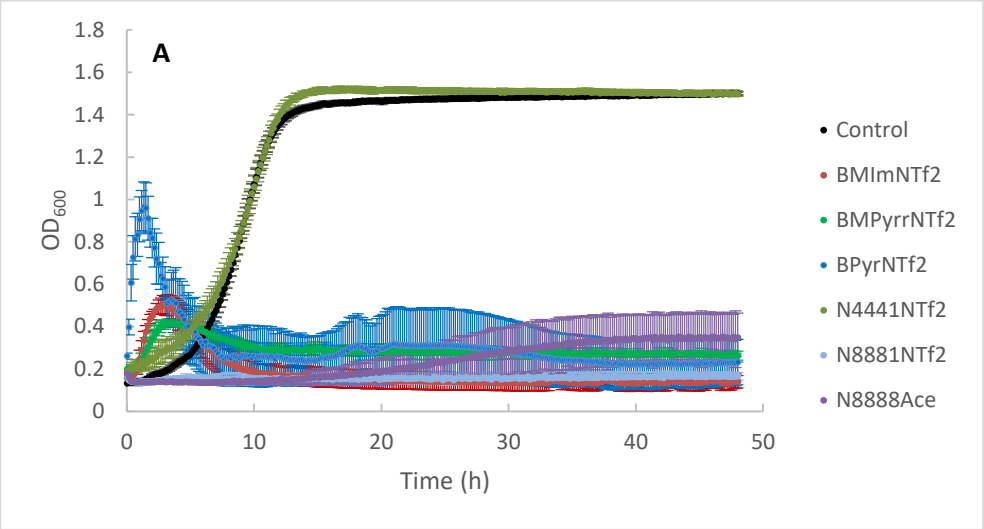
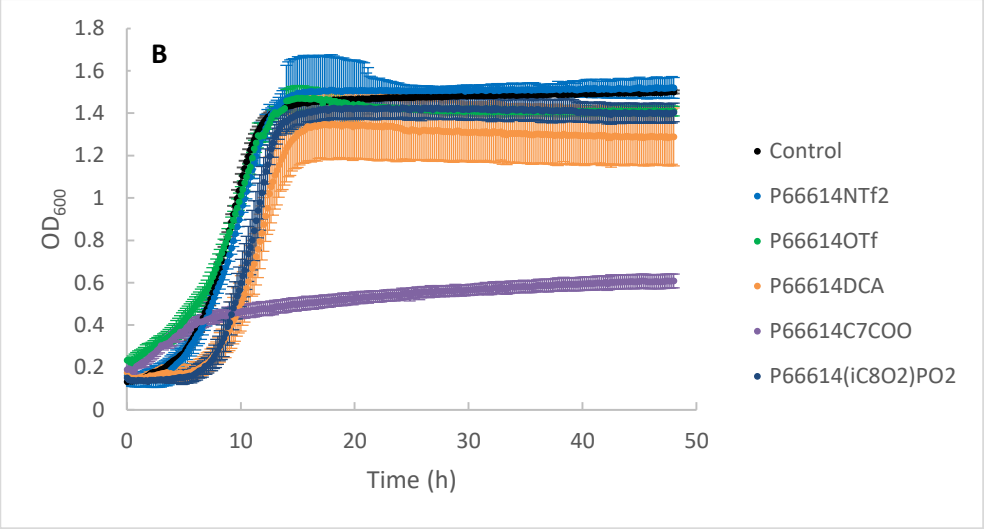


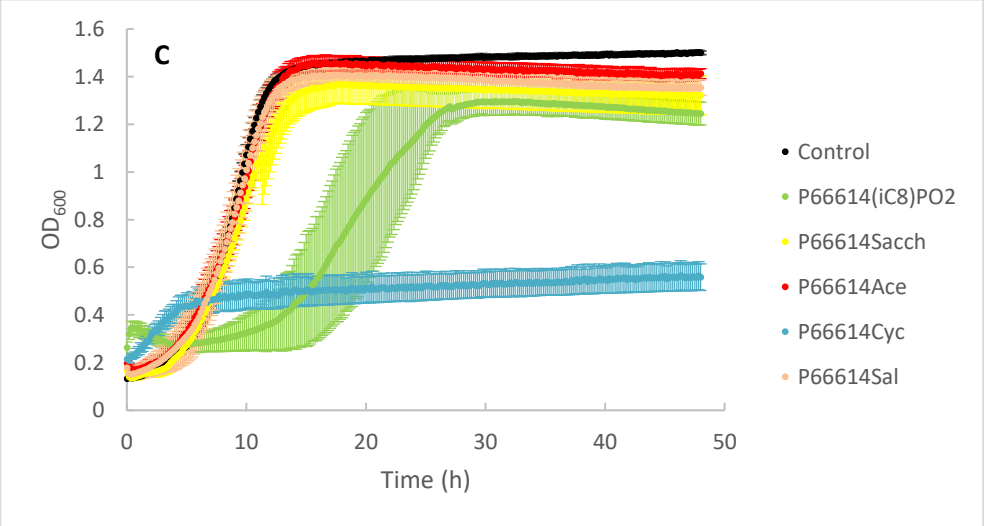
Figure 20. Effect of organic solvents in the growth of *E. coli* MG1655

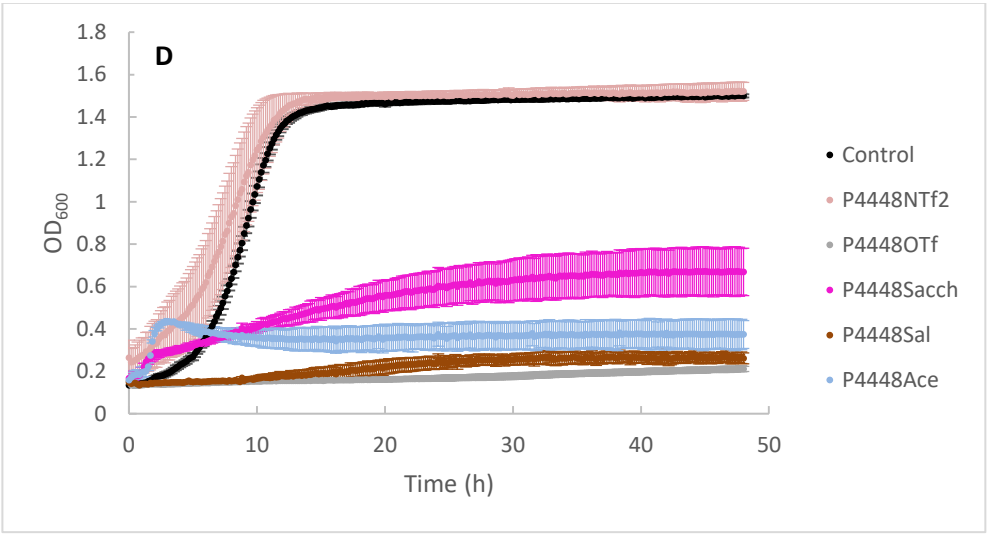
E. coli MG1655 was grown in LB medium in the presence of organic solvents (20%v/v). The growth was performed in 40mL Teflon sealed glass vials and increase in cell density was measured using a UV spectrophotometer. Results and standard deviations are the average of triplicate experiments.

12.1.4 Ionic liquids





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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
10
20
30
40
50
Time (h)
Control
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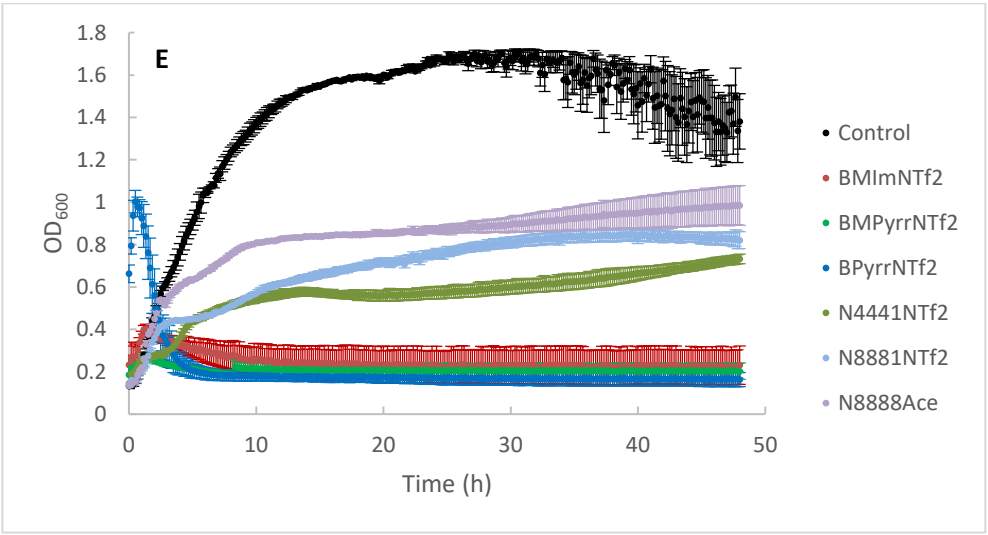
P4448OTf
P4448Sacch
P4448Sal
P4448Ace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OD600
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10
20
30
40
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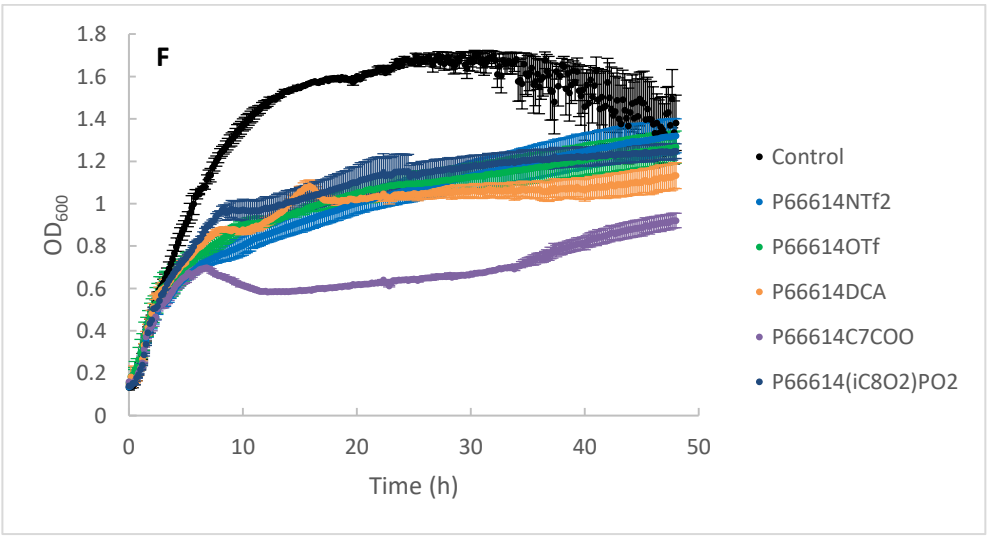
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10
20
30
40
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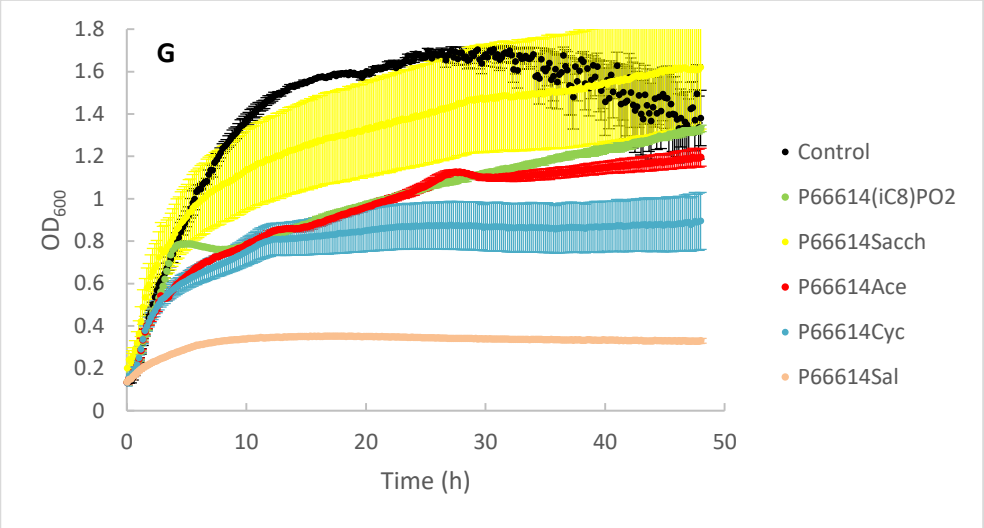
P66614OTf
P66614DCA
P66614C7COO
P66614(iC8O2)PO2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OD600
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20
30
40
50
Time (h)
Control
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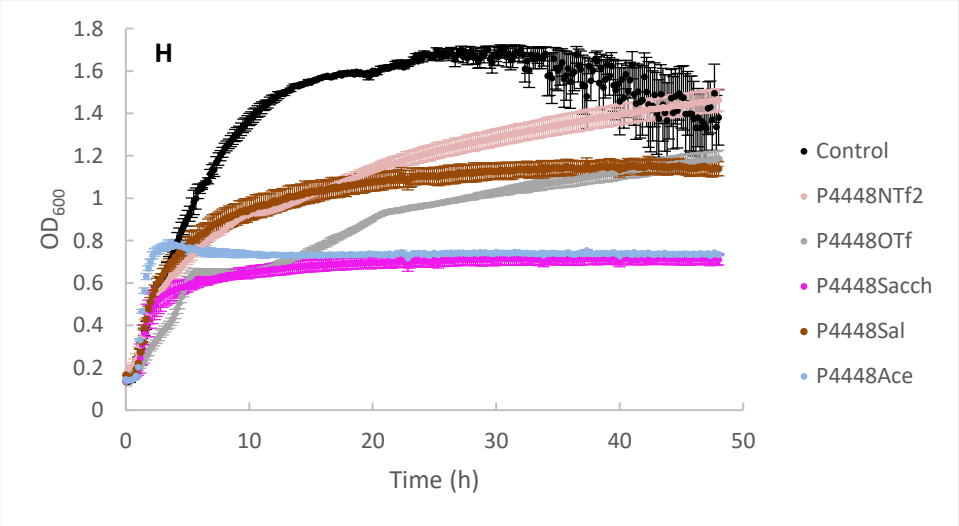
P66614Sacch
P66614Ace
P66614Cyc
P66614Sal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50
Time (h)
Control
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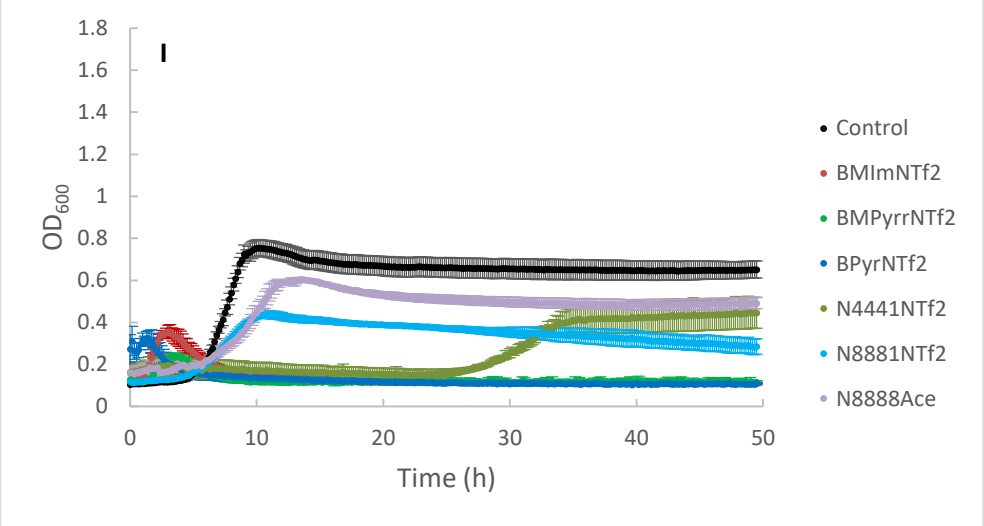
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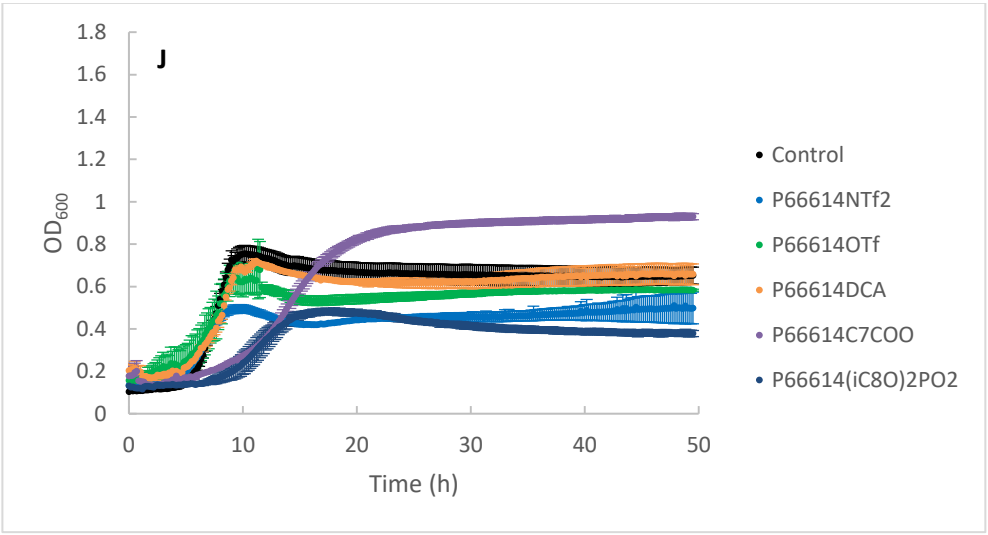
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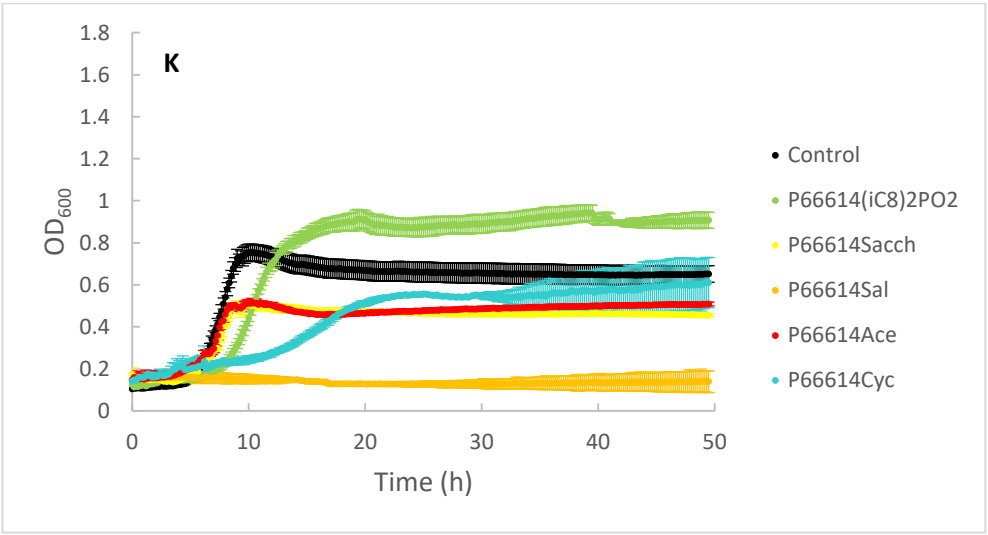
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Time (h)
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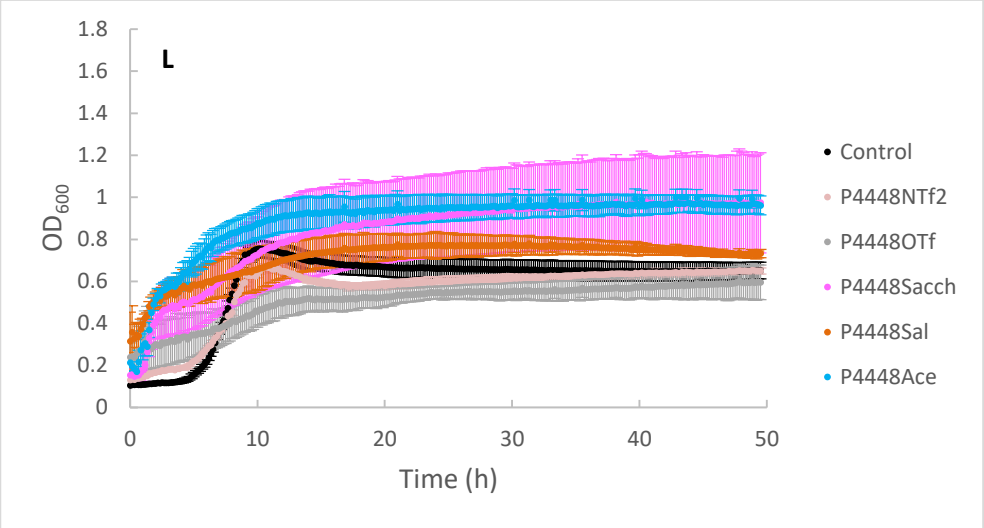

Figure 21. Effect of ILs on the growth of *E. coli* MG1655 and *S. cerevisiae* DSM 70449 in the presence of ILs

E. coli MG1655 and *S. cerevisiae* DSM 70449 was grown in various medium in the presence of ILs (20%v/v). The growth was performed in 100 well plates and increase in cell density was measured using a Bioscreen C MBR. Results and standard deviations are the average of triplicate experiments. A-D: *S. cerevisiae* in YEPD, E-H: *E. coli* in LB, I-L: *E. coli* in MSX.

12.1.5 Methacrylate esters

12.1.5.1 Preparation of alkyl methacrylate cultures

Table 32. The calculated volumes of each liquid component required for the methacrylate ester toxicity tests

		30°C		37°C	
Ester	**Solubility Limit (%)**	**Ester (µL)**	**Medium (µL)**	**Ester (µL)**	**Medium (µL)**
MMA	5	8	7992	11	7989
	10	15	7985	22	7978
	15	23	7977	34	7967
	20	31	7969	46	7956
EMA	5	3	7997	4	7996
	10	6	7994	8	7992
	15	9	7991	12	7988
	20	12	7988	16	7984
iPMA	5	2	7998	3	7997
	10	4	7996	6	7994
	15	6	7994	9	7991
	20	8	7992	12	7988
BMA	5	0.1	7999.9	0.2	7999.8
	10	0.2	7999.8	0.4	7999.6
	15	0.4	7999.6	0.6	7999.4
	20	0.5	7999.5	0.8	7999.2

Table 33. Effect of methacrylate esters on the growth of *S. cerevisiae* DSM70449

	OD_{600} (%)			
% Solubility limit	**MMA**	**EMA**	**iPMA**	**BMA**
5	81 ± 1.7	95 ± 1.9	87 ± 0.7	141 ± 1.3
10	28 ± 0.9	92 ± 1.8	79 ± 0.9	101 ± 8.2
15	8 ± 0.1	83 ± 1.3	63 ± 2.9	93 ± 12.9
20	8 ± 0.1	57 ± 1.9	62 ± 2.1	88 ± 2.9
20%v/v	9 ± 0.1	9 ± 0.6	8 ± 0.4	9 ± 0.4

Table 34. Effect of methacrylate esters on the growth of *E. coli* in LB medium

	OD_{600} (%)			
% Solubility limit	**MMA**	**EMA**	**iPMA**	**BMA**
5	79 ± 1.1	92 ± 1.9	95 ± 0.7	96 ± 3.2
10	67 ± 1.3	69 ± 2.0	79 ± 0.3	87 ± 1.9
15	54 ± 1.2	45 ± 2.0	67 ± 1.5	81 ± 3.3
20	18 ± 0.4	22 ± 0.9	60 ± 2.4	75 ± 2.6
20%v/v	17 ± 0.9	10 ± 0.9	5 ± 0	16 ± 0.2

Table 35. Effect of methacrylate esters on the growth of *E. coli* in MSX medium

	OD_{600} (%)			
% Solubility limit	**MMA**	**EMA**	**iPMA**	**BMA**
5	93 ± 1.6	97 ± 1.4	88 ± 0.9	90 ± 1.4
10	72 ± 1.7	94 ± 1.2	89 ± 0.8	92 ± 1.3
15	59 ± 0.9	82 ± 0.7	74 ± 0.8	92 ± 1.1
20	6 ± 0.1	75 ± 1.0	51 ± 0.4	89 ± 1.7
20%v/v	8 ± 0.1	6 ± 0	6 ± 0.1	79 ± 2.7

12.1.6 BMA

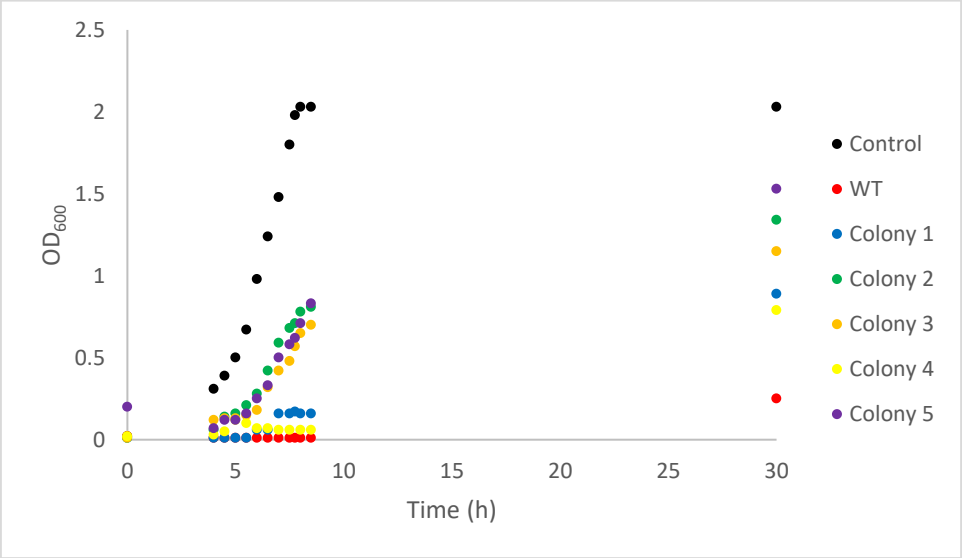

Figure 22. Effect of BMA (20%v/v) on the growth of wild type *E. coli* and the five BMA tolerant mutant strains in MSX medium

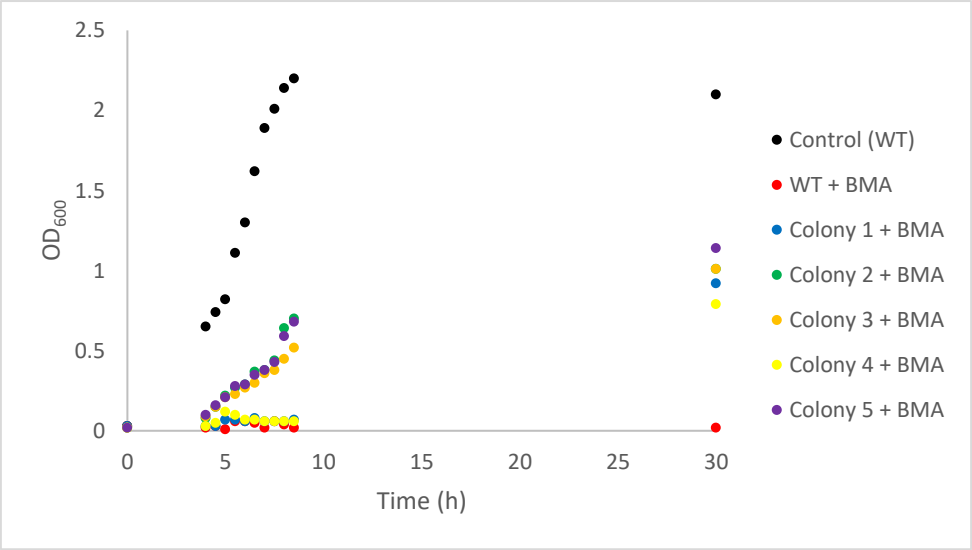

Figure 23. Effect of BMA on the growth of wild type *E. coli* and the five BMA tolerant mutant strains, originally cultured in MSX medium, then cultured in LB medium in the presence of BMA (20% v/v)

12.2 MAA extraction tests

12.2.1 HPLC calibration curves

To calculate the concentration of MAA extracted from the aqueous medium, HPLC standard curves were generated using known concentrations of MAA. This was achieved by the addition of a specific volume of 1M MAA to a specific volume of water. The peak area for each concentration of MAA was measured and used to plot standard curves of the peak area against MAA concentration. Samples taken from the extraction experiments were diluted to ensure the concentration fell within the range of the calibration curve. The MAA calibration curve used to calculate final MAA concentrations all extraction experiments can be seen in Figure 24.

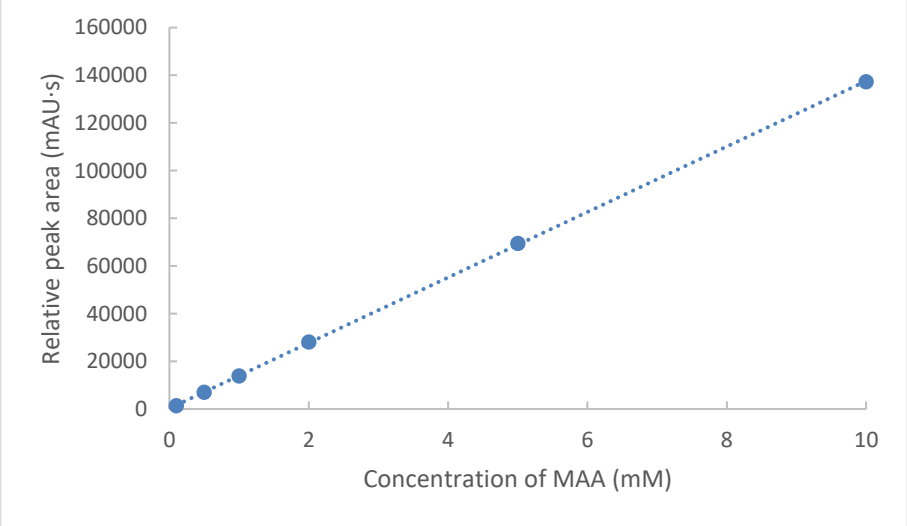

Figure 24. HPLC calibration curves of MAA in MSX medium using a UV detector (210nm)

12.2.2 Enthalpy of extraction calculations

Table 36. Change in enthalpy of extraction with increasing temperature

Solvent	**Change in enthalpy (ΔH) ($KJmol^{-1}$)**
Tributyrin	11.97
Isopropylbenzene	12.19
n-propylbenzene	12.19
Cycloheptane	20.29
Hexane	50.99
Heptane	24.72
Cyclooctane	24.72
Isooctane	7.65
1,4-diisopropylbenzene	28.10
Octane	31.93
Nonane	52.10
Decane	13.08
Undecane	44.89
Dodecane	0