Solvent engineering and other reaction design methods for favouring enzyme-catalysed synthesis

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Solvent engineering and other reaction design methods for favouring enzyme-catalysed synthesis

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December 2013

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Preface

This thesis presents the work carried out during my PhD project at Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark, from December 2009 to December 2013.

The work was carefully supervised by Professor Anne S. Meyer, Center for BioProcess Engineering, and co-supervised by Associate Professor Anders Riisager, Centre for Catalysis and Sustainable Chemistry, Department of Chemistry, Technical University of Denmark.

The PhD project was funded by the Technical University of Denmark, Department of Chemical and Biochemical Engineering.

First and foremost, I would like to thank Anne Meyer for her incomparable enthusiasm and encouragement, skilful supervision, and for first igniting my interest in enzyme technology back in 2006. Secondly, I want to thank Anders Riisager for valuable insight into the world of ionic liquids and helpful suggestions.

Thanks to my colleagues at Center for BioProcess Engineering for sharing the good times as well as the bad, and for making me want to go to work every day. I would like to thank Christian Nyffenegger for showing me how to perform circular dichroism measurements and for numerous constructive discussions and suggestions. I also want to thank Jianquan Luo for performing and reporting the membrane reactor experiments for Paper IV.

Thanks to Camilla Arboe Jennum for assisting me in the synthesis of iso-propyl ferulate, and to Karsten Jennum for helping me out with the curved arrows. Also thanks to several people at Centre for Catalysis and Sustainable Chemistry: to Andreas Jonas Kunov-Kruse for providing the COSMO-files and to Tim Ståhlberg, Saravanamurugan Shunmugavel, and Olivier Nguyen van Buu for preparation of the ionic liquids.

Finally, I want to thank my parents for never-ending love, trust, and support. Special thanks to Peter for your love, understanding, patience, valuable input, and scientific discussions and to Christian for always putting a smile on my face.

Birgitte Zeuner
Bispebjerg, December 2013
Summary

This thesis investigates different methods for improving reaction yields of enzyme-catalysed synthesis reactions. These methods include the use of non-conventional media such as ionic liquids (ILs) and organic solvents as main solvents or as co-solvents as well as the use of more classical reaction design methods, i.e. enzyme immobilization and the use of an enzymatic membrane reactor. Two different enzyme classes, namely feruloyl esterases (FAEs) and sialidases are employed.

Using sinapoylation of glycerol as a model reaction it was shown that both the IL anion nature and the FAE structure were important for FAE activity and stability in IL-buffer (15% v/v) systems. The quantum chemistry-based COSMO-RS method was applied for explaining the IL anion effect in terms of hydrogen bonding capacity. Furthermore, the usefulness of COSMO-RS and other thermodynamically based tools in solvent selection for FAE-catalysed acylation reactions was reviewed. FAE type A from Aspergillus niger and an FAE from a commercial preparation from Humicola insolens, Depol 740L, could not catalyse the esterification of arabinose or xylose with hydroxycinnamates in IL-buffer systems or in surfactantless microemulsion. However, both FAEs catalysed the feruloylation and/or sinapoylation of solvent cation C2OHMIm+, thus underlining the broad acceptor specificity of FAEs and their potential for future solvent reactions.

An engineered sialidase from Trypanosoma rangeli, Tr6, catalyses trans-sialylation but the yield is hampered by substrate and product hydrolysis. The formation of 3'-sialyllactose from lactose and casein glycomacropeptide was used as a model reaction. Addition of 20-25% (v/v) t-butanol improved the trans-sialylation yield 1.4-fold and the synthesis/hydrolysis ratio 1.2-fold. Using ILs as co-solvents, the synthesis/hydrolysis ratio was also improved, but the trans-sialylation yield decreased, probably due to destabilization of Tr6 caused by the ILs. Returning to the conventional aqueous medium, immobilization of Tr6 on magnetic nanoparticles improved the synthesis/hydrolysis ratio 2.1-fold and increased the biocatalytic productivity of 2.5-fold. However, the recyclability of the immobilized enzyme was low. Reusing Tr6 seven times in a membrane reactor increased the trans-sialylation yield on the limiting substrate 1.3-fold, emphasizing the importance of the continuous product removal. Furthermore, the biocatalytic productivity was increased more than 9-fold as a result of the enzyme recovery.

In conclusion, where the use of non-conventional media is required for catalysis, e.g. in the thermodynamically controlled FAE-catalysed esterification, careful selection of both solvent system and the FAE itself is required to obtain adequate reaction yields. In contrast, for Tr6 the most promising results were obtained when keeping the reaction in aqueous medium and employing other reaction design methods such as continuous product removal and enzyme immobilization.
Dansk sammenfatning

Afhandlingen undersøger forskellige metoder til at øge reaktionsudbyttet i enzymkatalyserede syntesereaktioner. Først undersøges brugen af ukonventionelle reaktionsmedier som ioniske væsker og organiske solventer, både som hovedsolvent og co-solvent. Til sidst undersøges brugen af mere klassiske metoder i reaktionsdesign såsom enzymimmobilisering og brug af en membranreaktor. To forskellige enzymtyper anvendes, nemlig ferulinsyreesteraser og sialidaser.


En modificeret sialidase fra *Trypanosoma rangeli*, Tr6, kan katalysere trans-sialylering, fx produktion af 3'-sialyllaktose fra laktose og kaseinglykomakropeptid (CGMP), men reaktionsudbyttet trækkes ned af substrat- og produkthydrolyse. Ved at tilsætte 20-25 % (v/v) t-butanol blev trans-sialyleringsudbyttet øget 1,4 gange, og forholdet mellem syntese og hydrolyse blev øget 1,2 gange. Forholdet mellem syntese og hydrolyse blev også øget ved at tilsætte ioniske væsker som co-solventer, men på bekostning af enzymaktiviteten. Tilbage i vandigt miljø kunne immobilisering af Tr6 på magnetiske nanopartikler øge forholdet mellem syntese og hydrolyse 2,1 gange og desuden give en 2,5 gange forøget biokatalytisk produktivitet (mg produkt pr. mg enzym). Desværre tabte de magnetiske nanopartikler en stor del af enzymaktiviteten i brug og kunne kun genbruges få gange. I stedet kunne frit Tr6 bruges i en membranreaktor med et 1,3 gange højere udbytte på sialyldonoren (CGMP) til følge; dette understreger, at kontinuerligt fjernelse af produktet via en membranreaktor er en brugbar metode til at undgå Produkthydrolyse. Stabiliteten af Tr6 var høj i membranreaktoren, og 7 ganges genbrug resulterede i mere end 9 ganges forøgelse af den biokatalytiske produktivitet.

Det kan konkluderes, at omhyggelig udvælgelse af både enzymstrukturer og solventsystemer nødvendig i reaktioner hvor ukonventionelle solventer kræves for at opnå et tilfredsstillende
reaktionsudbytte, fx i den termodynamisk kontrollerede esterificering katalysert af ferulinsyreesteraser. Med den modificerede sialidase Tr6 var resultaterne derimod mest lovende, når katalyseren fandt sted i vandigt reaktionsmedium hjulpet på vej af kontinueret fjernelse af produktet eller enzymimmobilisering.
List of publications

Paper I:

Paper II:

Paper III:

Paper IV:
List of abbreviations

3′-SL  3′-sialyllactose
AndFaeC  feruloyl esterase type C from *Emericella (Aspergillus) nidulans*
AnFaeA  feruloyl esterase type A from *Aspergillus niger*
αw  water activity
BcβGal  β-galactosidase from *Bacillus circulans*
[BMIm][BF₄]  1-butyl-3-methylimidazolium tetrafluoroborate
[BMIm][Cl]  1-butyl-3-methylimidazolium chloride
[BMIm][PF₆]  1-butyl-3-methylimidazolium hexafluorophosphate
[C₂OHMIm][BF₄]  1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate
[C₂OHMIm][PF₆]  1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate
[C₃OHMIm][PF₆]  1-(2-methoxyethyl)-3-methylimidazolium hexafluorophosphate
CaLB  lipase B from *Candida antarctica*
CD  circular dichroism
CE  carbohydrate esterase
CelB  β-glycosidase from *Pyrococcus furiosus*
CGMP  casein glycomacropeptide
C-Hex  51:46:3% (v/v/v) n-hexane-2-butanone-MES-NaOH buffer ‘microemulsion’
C-IL  51:46:3% (v/v/v) [BMIm][PF₆]-2-butanol-MES-NaOH buffer microemulsion
[CPMA][MeSO₄]  cocosalkyl pentaethoxy methylammonium methylsulfate
DMSO  dimethyl sulfoxide
DP  degree of polymerization
EAN  ethylammonium nitrate (also [EtNH₃][NO₃])
EFA  ethyl ferulate
[EMIm][EtSO₄]  1-ethyl-3-methylimidazolium ethylsulfate
EMR  enzymatic membrane reactor
[EtNH₃][NO₃]  ethylammonium nitrate (also EAN)
FA  ferulic acid
FA-Ara  feruloylated arabinose
FAE  feruloyl esterase (ferulic acid esterase)
FA-Xyl  feruloylated xylose
FoFaeC  feruloyl esterase type C from *Fusarium oxysporum*
FOS  fructooligosaccharides
GalNAc  N-acetyl-D-galactosamine
GH  glycoside hydrolase
GlcNAc  N-acetyl-D-glucosamine
GOS  galactooligosaccharides
HMO  human milk oligosaccharide
IDA  iminodiacetic acid
IL  ionic liquid
IMAC  immobilized metal affinity chromatography
IPFA  iso-propyl ferulate
\( k_D \) thermal inactivation constant
LacNAc \( N \)-acetyl-D-lactosamine
LLE liquid-liquid equilibria
MES 3-(\( N \)-morpholino)ethanesulfonic acid
MFA methyl ferulate
\([\text{MMIm}][\text{MeSO}_4]\) 1,3-dimethylimidazolium methylsulfate
MNP magnetic nanoparticle
MOPS 3-(\( N \)-morpholino)propanesulfonic acid
MSA methyl sinapate
\([\text{OMIm}][\text{Cl}]\) 1-octyl-3-methylimidazolium chloride
\([\text{OMIm}][\text{PF}_6]\) 1-octyl-3-methylimidazolium hexafluorophosphate
\( r_H \) rate of hydrolysis
\( r_S \) rate of synthesis
\( S \) (or \( S_c \)) selectivity factor
SA sinapic acid
SA-Ara sinapoylated arabinose
SA-Xyl sinapoylated xylose
SCD screening charge density
TcTS trans-sialidase from \( \text{Trypanosoma cruzi} \)
Tf\( _2\)N\( ^- \) bis(trifluoromethylsulfonyl)amide
\( T_m \) temperature at the midpoint of denaturation
Tr6 engineered sialidase from \( \text{Trypanosoma rangeli} \)
Tr6-MNP Tr6 immobilized on magnetic nanoparticle
TrSA sialidase from \( \text{Trypanosoma rangeli} \) (wild type)
TTP0042 \( \beta \)-galactosidase from \( \text{Thermus thermophilus} \) HB27
\( \sigma \) screening charge density (SCD)
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Chapter 1: Introduction

Enzyme-catalysed synthetic reactions

During the past few decades, interest in enzyme-catalysed synthesis reactions has increased dramatically, not least due to the high degree of regio-, stereo-, and enantioselectivity obtained in enzyme-catalysed reactions compared to chemical synthesis (Koeller and Wong 2000; Pollard and Woodley 2007; Hudlicky and Reed 2009). High enantio- and stereoselectivity is particularly useful in the pharmaceutical industry due to the demand for enantiopure chiral drugs (Pollard and Woodley 2007), whereas the high regioselectivity is required in modification of carbohydrates and other multi-hydroxyl compounds in order to achieve high yields of specific isomers without the need for the extensive protection and deprotection (van Rantwijk et al. 1999; Kennedy et al. 2006).

Esterases such as the feruloyl esterases (FAEs) studied in the current work catalyse both ester hydrolysis, esterification (i.e. reversed hydrolysis), and trans-esterification. In the reversed hydrolysis, the position of the thermodynamic equilibrium sets the limit for the achievable yield. In trans-esterification, the competition between trans-esterification and substrate hydrolysis is of major importance for maximal synthesis yields in this kinetically controlled reaction. At prolonged reaction times, trans-esterification will also become thermodynamically controlled due to product hydrolysis. The balance between the synthetic and hydrolytic reactions is largely governed by water activity ($a_w$): a decrease in $a_w$ shifts the reaction equilibrium from hydrolysis towards synthesis in thermodynamically controlled reactions, and in the kinetically controlled trans-esterification it also decreases the extent of undesirable hydrolysis (van Rantwijk et al. 1999; Yang and Wang 2004). Thus, (trans-)esterification is promoted by the use of non-conventional reaction media, e.g. organic solvents or ionic liquids (ILs), which lower the $a_w$. So far, most enzyme-catalysed (trans-)esterifications in non-conventional media have been carried out with lipases, which are generally both active and stable in these nearly anhydrous environments. Introducing FAEs for biocatalysis in synthesis-promoting media paves the way for expanding the range of applications of hydroxycinnamate derivatives. (Trans-)esterification of the antioxidative hydroxycinnamates with alcohols or saccharides will widen their application range due to changes in solubility and may also increase their anti-carcinogenic and antimicrobial potential (Vafiadi et al. 2006b; Katsoura et al. 2009; Shahidi and Chandrasekara 2010). Hence, the first part of this thesis explores the use of non-conventional media, especially ILs, for FAE-catalysed synthetic reactions. In doing so, the stability of enzymes in non-conventional media is assessed, since stability is an obvious requirement for successful catalysis, but also an issue especially in ILs; if not selected carefully, ILs – and the anion in particular – may destabilize enzymes through hydrogen bond interaction with the enzyme structure (Zhao 2010).
The second part of the thesis deals with glycoside hydrolases possessing trans-glycosidase activity, using a sialidase with trans-sialidase activity as an example. Reaction yields in the trans-glycosylation are governed by the competition between the accepting nucleophiles, i.e. the desired acceptor substrate and water. If the enzyme's selectivity for the desired acceptor substrate is good, much higher yields than the equilibrium yield can be obtained in this kinetically controlled reaction (Hansson et al. 2001). Again, a decrease in $a_w$ diminishes the extent of unwanted substrate and product hydrolysis (van Rantwijk et al. 1999). In aqueous media, trans-glycosylation yields can also be increased by increasing the acceptor:donor ratio and through continuous product removal to avoid product hydrolysis. Furthermore, ILs and organic solvents have been used as co-solvents in order to adjust the selectivity of glycosidases towards trans-glycosylation (Kaftzik et al. 2002; Lang et al. 2006; Sandoval et al. 2012a). Thus, a number of methods are explored to improve the feasibility of the synthesis reaction catalysed by an engineered sialidase with trans-sialidase activity.

For the enzyme classes studied in the current work, acceptor specificity is generally broad. In terms of increasing the versatility of enzymes for synthetic reaction, this promiscuity is beneficial as long as it does not compromise the regio-, stereo-, or enantioselectivity of the enzyme. Therefore, the acceptor specificity of the relevant enzyme classes is also outlined below.

**Reaction mechanisms, acceptors, and methods for suppressing hydrolysis**

Carbohydrate-active enzymes, including glycoside hydrolases (GH) and carbohydrate esterases (CE), have been divided into families based on amino acid sequences and three-dimensional structures in the CAZy database (www.cazy.org). Enzymes within the same family share the same basic protein fold and the same basic chemical reaction mechanism (Sinnott 2007). In the current work, two different classes of carbohydrate-active enzymes have been studied, namely:

- Feruloyl esterases (EC 3.1.1.73) belonging to the CE 1 family.
- Sialidases (EC 3.2.1.18) with trans-sialidase activity; the enzyme in question is a mutant of a sialidase with mutations based on a trans-sialidase, both belonging to the GH 33 family.

The following sections review the reaction mechanisms of these enzymes in relation to how their synthetic activity can be favoured over the hydrolytic one.

**Feruloyl esterases**

In nature, FAEs catalyse the hydrolysis of ester bonds between ferulic acid (FA) and carbohydrates in the plant cell wall. In the complex network that forms the plant cell wall, FA is bound to $O$-5 of arabinose in arabinoxylan and to $O$-2 of arabinose or $O$-6 of galactose in pectin,
its major role being the formation of diferulate cross-links strengthening the plant cell wall network (Colquhoun et al. 1994; Wong 2006). FAEs also show activity towards simpler alkyl esters (e.g. methyl ferulate (MFA)) and to a varying degree also towards esters of other hydroxycinnamates, i.e. sinapic acid (SA), caffeic acid, and p-coumaric acid (Wong 2006). The sub-type FAE nomenclature is based on the specific activity towards the methyl esters of these four hydroxycinnamates (Crepin et al. 2004).

**Reaction mechanism**

FAEs contain the classic Ser-His-Asp catalytic triad characteristic of serine protease and esterase families, which catalyse the hydrolysis of amide and ester bonds, respectively, using Ser as a nucleophile (Figure 1.1; Prates et al. 2001; Schubot et al. 2001; McAuley et al. 2004; Wong 2006).

![Figure 1.1](#)

**Figure 1.1.** Hydrolytic reaction mechanism of FAEs. The numbering of the catalytic Ser-His-Asp triad and the oxyanion hole is based on FAE type A from *Aspergillus niger* (AnFaeA). R1: carbohydrate moiety; R2: hydroxycinnamate moiety. Adapted from Wong (2006).

The nucleophilic Ser residue is located at the centre of the highly conserved consensus sequence GXSG, also found in all the FAEs used in the current work. This motif is called the ‘nucleophilic elbow’ and creates an unusually tight turn with the Ser sitting at the top (Schubot et al. 2001). The crystal structure of FAE type A from *Aspergillus niger* (AnFaeA), the FAE most widely used in
current work, revealed His247 in a position where the Nε atom forms a hydrogen bond to the Oγ atom of Ser133 (2.8 Å away) and the Nδ atom forms hydrogen bonds to the Asp194 (2.8 Å to Oδ1 and 3.0 Å to Oδ2) (McAuley et al. 2004). Positioned like this, His247 acts as a general base catalyst increasing the nucleophilicity of Ser133 by deprotonating it. The resulting positive charge on His247 is stabilized through interaction with the negatively charged Asp194. The alkoxide ion formed by deprotonation of Ser133 can now perform a nucleophilic attack on the carbonyl C in the substrate ester bond, yielding a tetrahedral transition state. The negative charge on the substrate carbonyl O in the tetrahedral intermediate is stabilized by the so-called oxyanion hole (also a common feature of serine proteases and esterases), in AnFaeA formed by the backbone NH groups of Thr68 and Leu134. Next, the tetrahedral transition state collapses to give a covalent acyl-enzyme intermediate accompanied by the release of the formed alcohol (in nature, the carbohydrate moiety) from the active site. In the following decacylation step, the ester bond between the hydroxycinnamate moiety and Ser133 (i.e. the acyl-enzyme intermediate) suffers a nucleophilic attack from a water molecule activated by general base catalyst His247, leading to formation of a second tetrahedral intermediate and subsequent release of the acid product (Figure 1.1; Schubot et al. 2001; McAuley et al. 2004; Wong 2006).

FAEs can however also catalyse esterification or trans-esterification of primary or, in some cases, secondary alcohols with hydroxycinnamates (Hatzakis et al. 2003; Vafiadi et al. 2008a; Vafiadi et al. 2009). Whereas the hydrolysis mechanism of FAEs is well-known as outlined above, little is known about the catalytic mechanism behind these reactions. Some insight into the (trans-)esterification mechanism can be obtained by studying the reaction mechanism of lipases, which catalyse both hydrolysis, esterification, and trans-esterification of their substrates using a Ser-His-Asp/Glu catalytic triad and stabilization of the tetrahedral intermediate in the oxyanion hole (Monecke et al. 1998; Kwon et al. 2007; Juhl et al. 2010; Xin et al. 2011; Toledo et al. 2012). Based on this, it can be assumed that the (trans-)esterification mechanism of FAEs is similar to the lipase ping-pong bi-bi mechanism (Cleland 1963). The mechanism is thus assumed to be identical to the one shown in Figure 1.1 except that a carbohydrate or an alcohol acts as acceptor instead of the water molecule and that R1 is typically an alkyl in the trans-esterification and H in the direct esterification. Putative carbohydrate binding sites close to the active site, which may double as acceptor binding site in the (trans-)esterification, have been identified (Schubot et al. 2001). Especially, it has been speculated that the C-terminal Trp260 of AnFaeA plays a role in carbohydrate binding, but this could not be proven (Faulds et al. 2005). Attempts to crystallize FAEs with a feruloylated carbohydrate (i.e. the natural substrate) bound in the active site have been made; however, the carbohydrate moieties were not visible in the crystal structures, indicating that tight carbohydrate binding is not a requirement for catalysis in the hydrolytic reaction (Schubot et al. 2001; Faulds et al. 2005). Possibly, this is also true for the (trans-)esterification reaction. At any rate, FAEs exhibit broad acceptor specificity in the (trans-)esterification as outlined in the following section.
**Acceptors and donors**

Trans-esterifications and direct esterifications catalysed by FAEs have been carried out using both simple alcohols and a wide range of saccharides (please refer to Tables 1A and 1B in Paper II for examples of the latter). Most FAEs are specific for primary alcohols (Mastihubová et al. 2006; Vafiadi et al. 2006b), but can also be promiscuous: catalysing the trans-esterification of 1- and 2-butanol with MFA, AnFaeA showed strong preference for 1-butanol, but did also feruloylate 2-butanol (Vafiadi et al. 2008a). Remarkably, the FAE-containing commercial blend Pentopan 500 BG from *Humicola insolens* was found to be specific for secondary alcohols (Hatzakis et al. 2003). For di- and oligosaccharides, feruloylation takes place at the non-reducing end (Vafiadi et al. 2007b).

Donor specificity is not similarly broad, most likely because the donor is more tightly associated with the active site as outlined above. It was shown that the length of the aliphatic chain forming the backbone of the hydroxycinnamate is crucial for the substrate to fit into the active site: lengthening or shortening the distance between the aromatic ring and the ester bonds resulted in complete loss of activity (Kroon et al. 1997; Topakas et al. 2005a). In the trans-esterification, the conversion was markedly higher when using MFA compared to other branched and unbranched alkyl ferulate esters, indicating that a short alkyl chain is a requirement for successful trans-esterification (Vafiadi et al. 2005). This is probably an effect of steric hindrances caused by larger alkyl chains. Specificity for the substitutions on the aromatic ring is determined by the FAE sub-type (Crepin et al. 2004).

Some studies have concentrated on trans-esterifications of alcohols and saccharides in non-conventional media (Vafiadi et al. 2005; Mastihubová et al. 2006; Vafiadi et al. 2006b; Vafiadi et al. 2007b), whereas others have used direct esterification for similar reactions with other FAEs (Tsuchiyama et al. 2006; Couto et al. 2010). In IL systems with 15% (v/v) aqueous buffer, Vafiadi et al. (2009) compared the reaction yields obtained in the two types of reactions and found that higher yields were obtained in the AnFaeA-catalysed direct esterification. In contrast, a number of lipases displayed higher activity and reaction yields in trans-esterification compared to esterification, probably due to decreased lipase stability in the presence of the acid substrate and the influence of water by-product on the reaction equilibrium in the nearly anhydrous media (Pirozzi and Greco 2006; Vosmann et al. 2006; Weitkamp et al. 2006). Possibly, the generation of methanol in trans-esterification with methyl esters may have a destabilizing effect on some enzymes, but it can also be used for driving the reaction forward by evaporation of methanol under reduced pressure (Compton et al. 2000; Kurata et al. 2010). Whether esterification or trans-esterification is to be preferred may depend on the given enzyme and the reaction system; comparative data are too scarce to give general indications for FAEs.
Methods for suppressing hydrolysis in feruloyl esterase-catalysed reactions

In order to lower $a_w$ sufficiently for appreciable FAE-catalysed (trans-)esterification to take place, these reactions can be carried out in organic solvent systems, e.g. pure organic solvent or surfactantless microemulsions, in IL systems, or – when the acceptor substrate is liquid – in (nearly) solvent-free systems (Topakas et al. 2005b; Mastihubová et al. 2006; Tsuchiyama et al. 2006; Vafiadi et al. 2009). Even if the reason for using these systems is to lower $a_w$, small amounts of water are required for successful catalysis. For enzymes in non-conventional media it is important to consider their so-called water activity profiles, i.e. enzyme activity vs. $a_w$ in the reaction mixture, which differ from enzyme to enzyme (Halling 2004). Since the water content required to obtain a given $a_w$ differs from solvent to solvent, it is now widely acknowledged that $a_w$ should be considered instead of water content (Valivety et al. 1992; Halling 2004). Indeed, Vafiadi et al. 2009 found the optimal water content in the AnFaeA-catalysed esterification of glycerol with SA in an IL system to be 15% (v/v), which is considerably higher than the approx. 3% (v/v) used in the optimal surfactantless microemulsion composition for catalysis by FAE type C from Sporotrichum thermophile (Topakas et al. 2005b). The issue of enzyme stability in these non-conventional media is further discussed below.

For trans-glycosidases catalysing both trans-glycosylation and the competing hydrolysis it is well known that trans-galactosylation yields can also be improved by increasing the concentration (or activity) of the acceptor substrate resulting in a high acceptor:donor ratio (Hansson et al. 2001; Lang et al. 2006; Bridiau et al. 2010). The acceptor:donor ratios used for FAE-catalysed synthesis reactions differ dependent on whether the acceptor is a liquid, e.g. 1-butanol or glycerol, or a solid compound with limited solubility such as saccharides. For the sinapoylation of glycerol with AnFaeA in an IL system, an acceptor:donor ratio of 125 was reported to give optimal yields (Vafiadi et al. 2009), and for the trans-esterification of 1- and 2-butanol with AnFaeA in surfactantless microemulsions, the acceptor:donor ratio used was 139 (Vafiadi et al. 2008a). Acceptor concentrations in this range cannot be achieved when working with saccharides due to their relatively low solubility. Surprisingly, optimization studies have shown that acceptor:donor ratios of approx. 1:3 were optimal for trans-esterification and direct esterification of saccharides in surfactantless microemulsions (Vafiadi et al. 2005; Couto et al. 2011). In conclusion, it seems that decreasing the $a_w$ is the most important method for suppressing hydrolysis in FAE-catalysed reactions, keeping in mind that some water is required for optimal biocatalysis.

Glycoside hydrolases with trans-glycosylation activity

Enzymes that catalyse the transfer of a glycosyl group to water (resulting in glycoside hydrolysis) are known as glycoside hydrolases or just glycosidases. The transfer can take place with either retention or inversion of the configuration at the anomeric centre of the glycoside,
i.e. the enzyme can follow a retaining mechanism or an inverting mechanism as initially described by Koshland (1953). Sialidases and trans-sialidases catalyse the hydrolysis or transfer of N-acetylneuraminic acid – often and in the current work called just sialic acid – with retention of the anomic configuration (Amaya et al. 2004). Hydrolysis with retention of the anomic configuration is in most cases achieved through a two-step, double-displacement mechanism which involves a covalent glycosyl-enzyme intermediate (Rye and Withers 2000; Sinnott 2007). The glycosyl-enzyme intermediate in retaining glycosidases can be intercepted by other acceptors than water, leading to trans-glycosylation. This trans-glycosidase activity can be the main activity of the enzyme or a side activity (Sinnott 2007). Trans-glycosidases should not be confused with glycosyl transferases which are a different class of enzymes requiring activated glycosyl donors, the activating group typically being a nucleotide or a lipid-phosphate (Davies et al. 2005; Sinnott 2007). The requirement for expensive, activated glycosyl donors renders glycosyl transferases less useful for large-scale synthesis than glycosidases, even if the latter struggle with undesirable substrate and product hydrolysis (van Rantwijk et al. 1999; Crout and Vic 1998).

Like esterases, many glycosidases with trans-glycosidase activity (including trans-sialidases) have been reported to operate through a ping-pong bi-bi mechanism in the trans-glycosylation, which reduces to an ordered uni-bi mechanism in the competing hydrolysis since water is not considered a ‘real’ substrate (Cleland 1963; Cogoli and Semenza 1975; Peruffo et al. 1978; Suzuki et al. 2002; Damager et al. 2008). In the ping-pong bi bi mechanism, the glycosyl donor binds and forms a glycosyl-enzyme intermediate while the aglycone (or reducing end saccharide moiety) is released before the acceptor substrate binds to take part in the formation of the trans-glycosylated product.

**Trans-sialidase reaction mechanism**

In general, trans-sialidases catalyse the transfer of sialic acids from a terminal β-galactosyl donor to a terminal β-galactosyl acceptor with retention of the anomic α-configuration (other acceptors may occur; see below). The amino acid residues taking part in the catalysis are strictly conserved across the entire sialidase superfamily, indicating a similar catalytic mechanism in all enzymes with trans-sialidase activity (Amaya et al. 2004; Paris et al. 2005). The catalytic mechanism as well as the three-dimensional structure of the active site has been intensely studied in trans-sialidase from *Trypanosoma cruzi* (TcTS). Initially, a sequential mechanism, i.e. an ordered bi-bi mechanism (Cleland 1963), was proposed for TcTS in which the binding of a sialyl donor was followed by the binding of the acceptor molecule (Ribeirão et al. 1997). This would require the presence of two binding sites for β-galactosyl residues near the catalytic centre (Ribeirão et al. 1997), but this theory was not corroborated by the crystal structure of TcTS (Damager et al. 2008). Instead, there is now substantial evidence that TcTS follows a ping-pong mechanism: Firstly, the sialyl donor binds to the active site and a sialyl-enzyme
intermediate is formed, releasing the remaining $\beta$-galactosyl moiety of the donor molecule from the binding site. Secondly, the acceptor molecule is accommodated in the active site and the sialyl group is transferred to the acceptor (Figure 1.2; Amaya et al. 2004; Damager et al. 2008).

The sialyl donor is accommodated in the active site by hydrogen bond formation between an Arg triad (Arg35, Arg 245, and Arg314) and the substrate’s carboxylate group and between Asp96 and the substrate’s acetamide group (Buschiazzo et al. 2002; Amaya et al. 2004). The Arg triad and Glu357, which stabilizes Arg35, are strictly conserved in the sialidase superfamily (Paris et al. 2005). The $\beta$-galactosyl moiety of the sialyl donor is accommodated through stacking interactions with Tyr119 and Trp312 (Buschiazzo et al. 2002). Upon binding of the sialyl donor, Asp59 acts as an acid catalyst promoting a nucleophilic attack from Tyr342 on the anomeric centre in the sialyl donor, leading to formation of a sialyl-enzyme intermediate through a covalent bond to Tyr342 (Figure 1.2; Damager et al. 2008). The use of a Tyr residue as nucleophile is special to the sialidases and trans-sialidases of GH families 33 and 34 (Davies et al. 2005; Sinnott 2007). In most retaining glycosidases the covalent glycosyl-enzyme intermediate is formed through a covalent bond to a carboxylate nucleophile, i.e. an Asp or Glu residue (Rye and Withers 2000; Sinnott 2007). Tyr is a neutral nucleophile and requires a general base to enhance its nucleophilicity. It is believed that Glu230 acts as a general base catalyst assisting Tyr342 in the attack on the anomeric centre by transiently relaying its charge onto the nucleophile (Figure 1.2). It is hypothesized that the fact that Tyr works as the nucleophile in the trans-sialidases is a result of the need to minimize the electrostatic repulsion between a carboxylate nucleophile and the carboxylate of the sialyl substrate (Amaya et al. 2004). Asp59, Glu230, and Tyr342 are conserved throughout the sialidase superfamily (Paris et al. 2005).

Formation of the covalent sialyl-enzyme intermediate and the release of the $\beta$-galactosyl moiety induce conformational changes in the sialyl ring and a repositioning of Tyr119, giving rise to formation of new hydrogen bonds between the enzyme and the glycerol hydroxyl groups in the sialic acid molecule (O7-O9). The relaxation of the sialic acid sugar ring along with the stronger sialyl-enzyme interaction result in greater stabilization of the intermediate compared to the ground states, which increases the lifetime of the intermediate – a requirement for promoting trans-glycosylation rather than hydrolysis (Amaya et al. 2004). Furthermore, since Tyr119 and Trp312 also form the acceptor binding site by stacking interactions with the $\beta$-galactosyl acceptor, the repositioning of Tyr119 is also thought to be part of creating a binding site for the acceptor (Buschiazzo et al. 2002; Amaya et al. 2004; Damager et al. 2008). Transfer of the sialyl group from the intermediate to the acceptor takes place through attack on C2 in the sialyl-enzyme intermediate either by water or by the 3-OH group of a terminal $\beta$-galactosyl acceptor residue, which must first be deprotonated by Asp59, the acid catalyst (Figure 1.2; Amaya et al. 2004; Damager et al. 2008).
Figure 1.2. Reaction mechanism of trans-sialidase from *T. cruzi*, TcTS. The reaction mechanism is shown with a β-galactosyl acceptor since this is most common for TcTS. Water can also act as acceptor performing the nucleophilic attack on the sialyl-enzyme intermediate; this event leads to substrate hydrolysis. R₁ and R₂ are generally glycosides.
The sialidase with trans-sialidase activity employed in the current work is an engineered sialidase called Tr6. Since all amino acid residues taking part in the catalysis are conserved in the sialidase superfamily, and also between TcTS and Tr6, it is reasonably assumed that Tr6 possesses the same catalytic mechanism as shown for TcTS in Figure 1.2 (Paris et al. 2005). Tr6 is based on the wild type sialidase from *T. rangeli* (TrSA) which in contrast to TcTS has only low trans-sialidase activity but significant sialidase activity. These two activities share the catalytic mechanism in Figure 1.2, but differ in the choice of acceptor; if a β-galactosyl acceptor (or another glycosidic residue) is present, the sialyl group is transferred to this acceptor (trans-sialidase activity), but if water functions as the acceptor of the sialyl group from the sialyl-enzyme intermediate, hydrolysis takes place (sialidase activity). Indeed, major differences between TrSA and TcTS are found in the acceptor binding site: In TrSA, Trp313 (corresponding to Trp312 in TcTS) is found in a different conformation due to difference in a neighbouring residue (Pro283 in TcTS is substituted with Gln284 in TrSA), and instead of Tyr119 TrSA has a Ser residue (Ser120). ‘Correcting’ both the acceptor binding site and differences between TrSA and TcTS in the sialic acid binding pocket, Paris et al. (2005) increased the negligible trans-sialidase activity of TrSA to 10% of the TcTS trans-sialidase activity, forming the TrSA mutant (I37L, M96V, A98P, S120Y, G249Y, Q284P) called Tr6 in the current work. From their results, it is however evident that these six amino acid substitutions were far from sufficient to reach a ratio between trans-sialidase and sialidase activities as favourable as that displayed by TcTS (Paris et al. 2005; discussed further below and in Chapter 5).

**Acceptors for Tr6**

TcTS is specific for acceptors with a terminal β-galactosyl moiety at the non-reducing end (Vandekerckhove et al. 1992; Schenkman et al. 1994). Testing the acceptor specificity of a TrSA mutant (Tr6 with another seven amino acid substitutions) revealed promiscuous acceptor specificity of this mutant: the Tr6 mutant (Tr13) catalysed sialyl transfer to terminal α-galactosyl residues (melibiose), terminal α-glucosyl residues (maltose and isomaltooligosaccharides), and fucose as well as to terminal β-galactosyl moieties (lactose, lactulose, and galactooligosaccharides (GOS)) (Jers et al. 2013). It can be speculated that Tr6 also has broader acceptor specificity than TcTS, even if only acceptors with terminal β-galactosyl moieties have been reported, namely lactose, lacto-N-tetraoses, and lacto-N-fucopentaoses (Michalak et al. 2013). Obviously, water is also a good acceptor of the sialyl group from Tr6; indeed, this thesis is concerned with methods of reducing the use of water as acceptor by Tr6 as outlined in the next section.

**Methods for suppressing hydrolysis in glycoside hydrolase-catalysed reactions**

In reactions catalysed by glycosidases with trans-glycosidases activity, including sialidases with trans-sialidase activity, the trans-glycosylation yield is mainly determined by the competition
between the two acceptor nucleophiles, *i.e.* the (desired) glycosyl acceptor and water, and by the selectivity of the enzyme for the two acceptors. In kinetic terms, the yield is determined by the balance between (1) the rates of donor synthesis and hydrolysis, and (2) product hydrolysis, also known as secondary hydrolysis (Figure 1.3).

**Figure 1.3.** Scheme of the competing reactions catalysed by Tr6: The covalent sialyl-enzyme intermediate formed after the sialyl donor binds to the enzyme undergoes nucleophilic attack by a β-galactosyl acceptor (synthesis; trans-sialidase activity) or by water (hydrolysis; sialidase activity). The synthetic reaction results in the formation of a sialylated glycoside, while the hydrolysis yields free sialic acid. The sialylated glycoside product formed by the trans-sialidase activity is also a substrate for the sialidase activity and may undergo (secondary) hydrolysis to form free sialic acid.

Most of the theory on how trans-glycosylation can be improved at the expense of hydrolysis is based on β-glycosidases, but is easily transferred to sialidases with trans-sialidase activity since these are also glycoside hydrolases (van Rantwijk *et al.* 1999; Hansson *et al.* 2001; Lang *et al.* 2006). In order to maximize trans-sialylation yields, the ratio between the trans-sialylation rate ($r_S$) and the hydrolysis rate ($r_H$) (Figure 1.3) must be maximized:

$$\frac{r_S}{r_H} = S \cdot \frac{a_{\text{glycosyl acceptor}}}{a_w} = S_c \cdot \frac{[\text{glycosyl acceptor}]}{[\text{water}]} \quad (1)$$

The selectivity in the reaction is thus influenced both by the activities (or concentrations) of the competing nucleophiles and by the selectivity factor $S$ (or $S_c$ when working with concentration), which is enzyme-dependent (van Rantwijk *et al.* 1999; Hansson *et al.* 2001; Lang *et al.* 2006).

It follows from Equation 1 that an increase in the activity (or concentration) of the acceptor as well as a decrease in $a_w$ will favour the desired trans-sialylation over hydrolysis. However, glycosidases require fairly high $a_w$ for displaying activity compared to *e.g.* lipases (Ma *et al.* 2002). In a study of β-galactosidase from *Bacillus circulans* (BcβGal), trans-galactosylation of *N*-acetyl-D-glucosamine (GlcNAc) in aqueous media with organic co-solvents was maximized at
$a_w$ values between 0.92 and 0.99 with a mean of approx. 0.96 (Bridiau et al. 2010). Thus, $a_w$ must be fairly high, but lower than 1 to have a positive effect on trans-glycosylation yields. It is hypothesized that at low $a_w$ the enzyme becomes less flexible, thus favouring the smaller water molecule as acceptor (Partridge et al. 1998; Hansson et al. 2001; Bridiau et al. 2010).

It has been shown that the $r_S/r_H$ ratio (Equation 1) of β-glycosidases can be improved by addition of co-solvents including organic solvents (Bridiau et al. 2010; Giacomini et al. 2002) and ILs (Kaufzik et al. 2002; Lang et al. 2006; Bayón et al. 2013a). Some controversy exists regarding the mechanism by which IL and organic co-solvents alter enzyme selectivity. While Bridiau et al. (2010) reported a clear relationship between $a_w$ of the reaction mixtures and trans-galactosylation yields with organic co-solvents, Lang et al. (2006) showed that the effect of an IL co-solvent on β-glycosidase selectivity exceeded that which could be explained by the concomitant change in $a_w$. Some studies hypothesize that the improvement of enzyme selectivity is caused by alterations in the three-dimensional structure of the enzyme through interactions between the enzyme and the co-solvent (Sandoval et al. 2012a; Sandoval et al. 2013), especially because the effect was enzyme-dependent (Goldfeder et al. 2013). It is believed that increased enzyme flexibility makes glycosidases favour the larger acceptor molecules over water (Bridiau et al. 2010; Sandoval et al. 2013). However, another study found that the effect of the co-solvent on enzyme selectivity was acceptor-dependent and hypothesized that the co-solvent modifies the acceptor hydration (Lang et al. 2006). Furthermore, the effect on enzyme selectivity, i.e. the $r_S/r_H$ ratio, is also dependent on the co-solvent nature (Bridiau et al. 2010; Sandoval et al. 2012a; Sandoval et al. 2013; Bayón et al. 2013a; Goldfeder et al. 2013).

For oligosaccharide synthesis, saccharide concentrations as high as 80% (w/w) have been used to effectively increase reaction yields (Roberts and Pettinatti 1957; Rastall et al. 1992). At these high concentrations, $a_w$ is significantly lowered. For instance, concentrations of fructose and sucrose of 80% (w/w) give water activities of approx. 0.6 and 0.7, respectively (Baeza et al. 2010). Michalak et al. (2013) found significant improvement of trans-sialidase activity over sialidase activity when increasing the lactose acceptor concentration from 36.8 mM (1.3% (w/w)) to 117 mM (4% (w/w)). However, at 4% (w/w) sucrose $a_w$ is 1.0 (Baeza et al. 2010), and it is most likely that the same is true for the lactose solution. Consequently, this improvement in trans-sialidase activity is more likely linked to the corresponding increase in acceptor:donor ratio from 14 to 25. In the optimization of the reaction conditions for TcTS-catalysed trans-sialylation, Holck et al. (2013) reported an optimal acceptor:donor ratio of approx. 5. Increasing the acceptor concentration while keeping the acceptor:donor ratio constant increased trans-sialylation yield, but decreased the yield on the limiting donor substrate (Holck et al. 2013). An increase in acceptor:donor ratio also dramatically increased the yield of N-acetyl-D-lactosamine (LacNAc) in the trans-galactosylation of GlcNAc catalysed by BcβGal using o-nitrophenyl β-galactopyranoside as a donor (Bridiau et al. 2010). A high acceptor:donor ratio increases the probability of a nucleophilic attack on formed the sialyl-enzyme intermediate
from a β-glycosyl acceptor, thus increasing trans-sialylation yields even if the competing nucleophile, water, is always present in large excess (approx. 55.5 M).

In another study of BcβGal, it was found that increasing the concentration of acceptor molecules not functioning as donor substrates had a positive effect on $V_{\text{max}}$ through speeding up the liberation of the transferred β-galactosyl group from the active site. This acceptor acceleration was not observed with addition of inert carbohydrates, nor was it caused by a decrease in $a_{\text{w}}$. Furthermore, a decrease in $K_m$ was also observed with increasing concentrations of both inert and acceptor carbohydrates; this increase in enzyme affinity was hypothesized to be caused by molecular crowding where the enzymatic activity increases if the volume of the enzyme-substrate complex is smaller than the sum of the volume of the enzyme and the volume of the substrate ($V_{E+S} < V_E + V_S$). This molecular crowding effect was however much smaller than the effect of acceptor acceleration (Warmerdam et al. 2013). In essence, much seems to be gained from ensuring a high acceptor concentration as well as a high acceptor:donor ratio in trans-glycosylation reactions.

It follows from the trans-sialidase mechanism (Figure 1.2) that the sialylated product formed can also function as a donor substrate. In the case of TcTS, which has only negligible sialidase activity, such an event will not decrease the product yield, but it will not increase it either. However, for an enzyme such as Tr6 where sialidase activity is pronounced, measures must be taken to keep product hydrolysis at a minimum. Indeed, trans-glycosylation product yields often reach a maximum before decreasing due to product hydrolysis (Ismail et al. 1999; Hansson and Adlercreutz 2002; Kaftzik et al. 2002; Jers et al. 2013). Consequently, it is important to control reaction time of trans-glycosylation reactions since at extended reaction times the reaction becomes thermodynamically controlled and secondary product hydrolysis takes over (van Rantwijk et al. 1999; Figure 1.3). Furthermore, if possible based on the molecular weights of substrates, product, and enzyme, an enzymatic membrane reactor (EMR) can be used to facilitate continuous product removal, thus minimizing the extent of product hydrolysis.

Protein engineering was not pursued as a method for improving the ratio between trans-glycosylation and hydrolysis in the current work. However, an interesting observation was made in a recent study where Tr6 was rationally engineered to create a mutant containing 13 amino acid substitutions compared to the wild type TrSA, Tr13 (Jers et al. 2013). This enzyme had the same level of trans-sialidase activity as Tr6, but four times lower hydrolytic activity. Using an alignment between Tr6 and the efficient trans-sialidase TcTS a major difference between the two enzymes was observed in a seven amino acids long motif near the binding site. In Tr6 and TrSA, this motif (amino acids 197-203) is IADMGGR, but in TcTS it is VTNKKKQ. Thus, changing this motif in Tr6 to the one of TcTS to create Tr13 gave a net charge change from neutral to +3. The mutations were approx. 14 Å from the acceptor binding site, and it is thus unlikely that they affected acceptor binding directly. Instead, it was hypothesized that the – in
evolutional terms – highly unlikely +3 net charge change caused an alteration of the electrostatic field in the binding cleft, which may disrupt or even revert the water network in the active site. Hydrolysis requires a water network aligned with oxygen lone pairs pointing towards sialic acid. The strong positive charge and hydrogen donor tendency thus introduced at the edge of the binding cleft may have caused partial reversal of such a network and turned the oxygen lone pairs towards the three lysine residues, consequently impairing the nucleophility of the water in the active site and causing the 4-fold reduction of hydrolytic activity. This motif is however not conserved in all characterized trans-sialidases (in trans-sialidase from *T. congolese* the net charge is also +3, but in trans-sialidase from *T. brucei* the net charge is -1) and its beneficial effect may thus be context-dependent (Jers *et al.* 2013).

**Ionic liquids**

As mentioned above, the use of non-conventional media represent a means of suppressing hydrolysis for both FAEs and sialidases. This section introduces ILs, originally the non-conventional media of main importance in the current work due to their many interesting properties outlined below.

ILs are organic salts, which are liquid at room temperature thus allowing their use as reaction solvents or co-solvents. Being salts, ILs contain a cation and an anion which can both be selected or modified in order to tune the properties of the IL such as hydrophobicity, polarity, viscosity, solvent miscibility, and not least their effect on enzyme activity and stability, which is the main focus of the current work (Yang and Pan 2005; van Rantwijk and Sheldon 2007). Because of the strong ionic interactions, ILs have negligible vapour pressure, are non-flammable, and have high thermal stability (van Rantwijk and Sheldon 2007; Galonde *et al.* 2012). These properties along with a high polarity compared to traditional organic solvents has caused a huge interest in replacing organic solvents with ILs for enzymatic reactions which require low $a_w$, *i.e.* reactions where hydrolysis is competing with the desired, synthetic reaction. A major advantage of ILs is indeed their high polarity (usually 0.6-0.7 on the normalized polarity scale, similar to that of short-chain alcohols), which is beneficial for biocatalysis in terms of substrate and product solubility: unlike most organic solvents, ILs can dissolve a wide range of both polar and non-polar compounds (Yang and Pan 2005; van Rantwijk and Sheldon 2007; Galonde *et al.* 2012). It is however important that the IL is carefully selected based on the biocatalytic reaction it should function as a solvent for. Indeed, mixed results have been obtained using ILs for biocatalysis, not least due to enzyme stability issues.

Many enzymes have shown higher stability in selected ILs than in traditional organic solvents. Furthermore, increased catalytic efficiency as well as higher enantio- and regioselectivity are among the advantages of using ILs rather than traditional organic solvents for biocatalysis (Yang and Pan 2005; van Rantwijk and Sheldon 2007). In addition, the use of IL as co-solvents in
aqueous systems has been reported to improve the selectivity of β-galactosidases and tyrosinase (Kaftzik et al. 2002; Lang et al. 2006; Bayón et al. 2013a; Goldfeder et al. 2013).

**Enzyme stability in non-conventional media**

Enzyme stability is an important issue when working with non-conventional media, especially since extended reaction times are often used (cf. Paper II, Tables 1A, 1B, 2A, and 2B). Enzyme stability is often higher in organic solvents than in aqueous media (Zaks and Klibanov 1984). This is explained by the fact that the breaking and reforming of the hydrogen bonds, which to a large extent are responsible for enzyme structure, are slower in a non-hydrogen bond forming medium such as an organic solvent; the enzyme is so to speak kinetically trapped in the stable, active conformation in which it was found prior to transfer to the non-aqueous medium and cannot radically change its conformation due to high kinetic barriers (Zaks and Klibanov 1988). The increased stability has however only been observed in weakly interacting organic solvents and not in very hydrophilic ones such as dimethyl sulfoxide (DMSO) and formamide which dissolve and hence inactivate enzymes (Chin et al. 1994). Indeed, enzymes – which are generally only suspended, not dissolved, in organic solvents – do not tolerate strongly interacting solvents other than water. For FAEs in particular, Faulds et al. (2011) recently showed that the use of DMSO, acetone, and 1,4-dioxane as co-solvents in aqueous systems resulted in inactivation of four different FAEs including AnFaeA and Ultraflo (see Chapter 2), except at concentrations below 2-25%, depending on the enzyme and the solvent.

Unfortunately, the increased stability observed in organic solvents often comes at the price of a reduced reaction rate due to reactant stabilization, transition state destabilization, reduced enzyme flexibility, and/or suboptimal pH conditions (Klibanov 1997). The desire to improve the reaction rate has been a driving force in the introduction of ILs as alternative media with low $a_w$: their highly polar nature may remedy transition state destabilization and the loss of flexibility due to stripping of water molecules. However, just like organic solvents, ILs can also greatly affect enzyme stability and the IL used in a given reaction must thus be chosen carefully.

Stability and activity of enzymes in ILs are governed by a number of IL properties, namely hydrogen bond basicity, hydrophobicity, nucleophilicity, viscosity, and in aqueous IL systems also ion kosmotropicity as recently reviewed by Zhao (2010). Due to the complex nature of IL-enzyme systems, no universal prediction tool for IL-enzyme compatibility exists, but there is general consensus that enzyme stability in IL systems is favoured by low hydrogen bond basicity and nucleophilicity as well as an adequately high hydrophobicity (Zhao 2010). It is hypothesized that a hydrophobic solvent allows preservation of essential water surrounding the enzyme structure, thus protecting the enzyme from denaturing protein-ion interaction (Yang and Pan 2005; Fehér et al. 2007). In general, the anion has a larger effect on enzyme stability than the cation does (Kaar et al. 2003; Lau et al. 2004), conceivably because the cation is significantly
larger than the anion and thus has its positive charge delocalized over a larger molecule, whereas the anion is more compact and has a more localized negative ion charge (Klähn et al. 2010). Indeed, Klähn et al. (2011) showed that the interaction between lipase B from Candida antarctica (CaLB) and eight different ILs was clearly dominated by Coulomb interactions with anions, while van der Waals interactions with the cations played the second largest (but comparably minor) role. Correspondingly, it has been suggested that large anions with the negative charge delocalized on more atoms are indeed less destabilizing (Gorke et al. 2010; Klähn et al. 2011). Furthermore, it is believed that larger anions are also less destabilizing because they are sterically demanding, i.e. their larger size would require more hydrogen bonds in the protein matrix to be broken in order to form a few new ones, making the IL-enzyme destabilizing interaction less favourable (Lau et al. 2004). Although hydrophobicity, nucleophilicity, and hydrogen bond basicity are separate concepts that should not be confused with each other, the general picture is that the more hydrophilic anions are also the ones with the higher hydrogen bond basicity and nucleophilicity, making them more likely to interact with the positively charged sites in the enzyme structure thus causing it to change conformation – and vice versa. Typically, hydrophobic ILs with PF₆⁻ and Tf₂N⁻ anions stabilize enzymes, while hydrophilic ILs with NO₃⁻, EtSO₄⁻, lactate, and in some cases also BF₄⁻ destabilize enzymes (Lau et al. 2004; De Diego et al. 2004; Katsuora et al. 2009; Zhao 2010).

While not applicable to hydrophobic ILs or nearly anhydrous systems with hydrophilic ILs, the Hofmeister series may be used as a measure of how an IL will affect enzyme stability (Zhao 2005). In aqueous systems of hydrophilic ILs, e.g. when using hydrophilic ILs as co-solvents, where the IL is hydrated and dissociates into its individual ions, the importance of having a chaotropic cation and especially a strong kosmotropic anion has indeed been pointed out through a thorough literature review (Zhao 2005). Using hydrophilic ILs as co-solvents for formate dehydrogenase from Candida boidinii and BcβGal, Kaftzik et al. (2002) generally found that increasing the IL content from 0 to 75% (v/v) resulted in decreasing activity of the enzymes, except when using [MMIm][MeSO₄] (1,3-dimethylimidazolium methylsulfate) for the formate dehydrogenase. The MeSO₄⁻ anion has previously been found to be a borderline case since it is only mildly H-bond accepting (Lau et al. 2004). MeSO₄⁻ was found to have a less destabilizing effect on BcβGal than BF₄⁻, which is consistent with BF₄⁻ being very chaotropic (Kaftzik et al. 2002; Zhao 2005). Interestingly, Sandoval et al. (2012a) reported that low concentrations (0.08-0.5% (w/v)) of [CPMA][MeSO₄] (cocosalkyl pentaethoxy methylammonium methylsulfate) caused great loss of α-helix structure in β-galactosidase from Thermus thermophilus indicating enzyme unfolding and concurrent inactivation, whereas higher co-solvent concentrations (1.5-5% (w/v)) increased the α-helix structure thus having a less destabilizing effect on the enzyme. For tyrosinase from Bacillus megaterium, Goldfeder et al. (2013) reported an increase in activity upon increasing the content of EAN ([EtNH₃][NO₃]; ethylammonium nitrate) from 0 to 40% (v/v), whereas an increase in [BMIm][Cl] (1-butyl-3-methylimidazolium chloride) or [EMIm][EtSO₄] (1-ethyl-3-methylimidazolium ethylsulfate)
content from 0 to 20% (v/v) caused enzyme inactivation. This is not consistent with the Hofmeister series, which list the following anion kosmotropicity: \((\text{SO}_4^{2-} > \text{Cl}^- > \text{NO}_3^-)\). BMIm\(^+\) is expected to be a kosmotrope, whereas EMIm\(^+\) and EtNH_3\(^+\) are believed to be chaotropes (Zhao 2005). Thus, predicting enzyme stability in aqueous IL systems may not just be a matter of assessing ion kosmotropicity as also pointed out by van Rantwijk and Sheldon (2007).

Enzyme stability in non-conventional solvents is not only dependent on the solvent, but also on the enzyme and its general stability and structure. In general, thermostable enzymes more often remain active in non-conventional media (van Rantwijk et al. 1999; Reetz et al. 2010; De Winter et al. 2013). Studying a \(\beta\)-glycosidase and two \(\alpha\)-galactosidases, Ferdjani et al. (2011) reported a clear correlation between the thermal stability of these three enzymes in aqueous medium and their stability in an IL-buffer system containing 80% (v/v) \([\text{MMIm}][\text{MeSO}_4]\). Using \([\text{MMIm}][\text{MeSO}_4]\) as co-solvent, \(\text{Bc}\beta\text{Gal}\) had a residual activity of 74% in 25% (v/v) co-solvent and 14% in 50% (v/v) co-solvent (Kaftzik et al. 2002). In contrast, the hyperthermostable \(\beta\)-glycosidase from \textit{Pyrococcus furiosus} (CelB), retained 98% activity at 25% (v/v) \([\text{MMIm}][\text{MeSO}_4]\) and 30% at 60% (v/v) (Lang et al. 2006). Recently, Nordwald and Kaar (2013) proposed chemical modification of charged residues as a way of making enzymes more compatible with ILs to avoid enzyme destabilization through IL-enzyme interactions. Based on the general consensus that destabilization of enzymes in ILs is caused by IL anions interacting with the enzyme structure, they hypothesized that a decrease in the ratio between positive and negative charges in the enzyme would benefit enzyme stability: More negative charges cause greater anion repulsion, while fewer positive charges result in fewer IL anions being attracted to the enzyme. The chemical modifications included acetylation and succinylation of primary amines as well as cationization or neutralization of carboxylic acids. No change in \(\alpha\)-chymotrypsin activity was observed after these charge modifications. The acetylation of \(\alpha\)-chymotrypsin caused a decrease in primary amine:acid ratio from 1.17 for the wild type to 0.39, and brought about a 1.6- and 4.3-fold increase in half-life in 40% (v/v) \([\text{BMIm}][\text{Cl}]\) and 55% (v/v) \([\text{EMIm}][\text{EtSO}_4]\), respectively. Similarly, the half-lives of lipase from \textit{Candida rugosa} and papain were increased 4.0- and 2.4-fold, respectively, in 30-40% \([\text{BMIm}][\text{Cl}]\) upon chemical modification to lower the primary amine:acid ratio (Nordwald and Kaar 2013). Using this strategy or genetic modification to alter enzyme surface charge may thus turn out to be a viable method for improving enzyme stability in ILs, though care must be taken not to compromise enzyme activity.
Thesis outline and hypotheses

This thesis focuses on enzyme-catalysed synthesis reactions, believing that the importance of these reactions will only grow in the future. Applying enzymes of hydrolytic activity in synthetic reactions, e.g. FAEs and sialidases, requires that the hydrolytic activity be suppressed in order to achieve acceptable yields. As outlined above, several different methods can be employed to minimize hydrolysis, e.g.:

- Use of non-conventional solvents such as ILs, organic solvents, and surfactantless microemulsions to lower the \( a_w \).
- Use of co-solvents, e.g. organic solvents or ILs, to improve the synthesis vs. hydrolysis ratio of hydrolases.
- Control of reaction time and continuous product removal to avoid extensive product hydrolysis.

The main aims of the thesis are (1) to achieve an increased understanding of biocatalytic synthesis reactions as well as of the effect of the abovementioned methods for improving reaction yields, and (2) to gain insight into the influence and usability of ILs in non-lipase reactions. In order to achieve these aims, the following hypotheses have been formulated:

Hypothesis 1: Stability and reactivity of FAEs in IL-buffer systems vary depending on the IL nature and different FAEs may respond differently.

Hypothesis 2: Pentoses arabinose and xylose can be enzymatically esterified with hydroxycinnamoyl moieties in non-conventional solvents using compatible FAEs.

Hypothesis 3: Available thermodynamic models can assist in solvent screening in order to optimize the solvent selection for enzyme-catalysed acylation reactions in non-conventional media.

Hypothesis 4: Trans-sialylation can be enhanced relative to hydrolysis in aqueous media by using ILs or organic solvents as co-solvents.

Hypothesis 5: The biocatalytic productivity of an enzyme catalysing a synthesis reaction can be optimized by reaction time control through immobilization and/or by continuous product removal.
In order to test these hypotheses, the specific objectives of the thesis were:

- To assess the stability and activity of three different FAEs in a carefully selected series of ILs using the sinapoylation of glycerol as an example (Chapter 2).
- To elucidate the potential of the quantum chemistry-based COSMO-RS method for explaining and predicting FAE stability in IL systems (Chapter 2).
- To further investigate the potential of COSMO-RS and other thermodynamically based tools for solvent screening in the design of FAE-catalysed (trans-)esterification of saccharides (Chapter 3).
- To catalyse the (trans-)esterification of arabinose and xylose with hydroxycinnamates using carefully selected FAEs in IL systems and surfactantless microemulsions (Chapter 3).
- To elucidate the effect of ILs and organic co-solvents on the trans-sialylation vs. hydrolysis activity of an engineered sialidase (Chapter 4).
- To improve the biocatalytic activity of an engineered sialidase through enzyme immobilization or the use of a membrane reactor using the production of the human milk oligosaccharide model compound 3’-sialyllactose as an example (Chapter 5).

Two different enzyme classes are studied, namely FAEs and sialidases. Although they are not directly comparable, the intention is to widen the perspective into a more general study of synthetic, enzyme-catalysed reactions. Among the non-conventional solvents, ILs are of main interest in this thesis due to their much-coveted ability to improve enzyme activity, selectivity and in some cases also stability compared to organic solvents as well as their beneficial properties such as negligible vapour pressure and non-flammability, as outlined in the introduction. Nevertheless, organic solvents are also considered where ILs fall short.
Chapter 2: Stability of feruloyl esterases in IL-buffer systems and sinapoylation of glycerol

This chapter relates to Paper I:

Introduction

Several different enzyme classes including glycosidases, proteases, and lyases have been used for biocatalysis in IL systems, but lipases clearly dominate this field probably owing to their high stability and ability to work at interfaces. The use of esterases is limited, mainly because they are less tolerant to anhydrous media than lipases and in most cases have only little activity when \( a_w \) is low (van Rantwijk and Sheldon 2007). However, Vafiadi et al. (2009) recently optimized reaction conditions for AnFaeA (FAE type A from *A. niger*) in the esterification of glycerol with SA, finding that 15% (v/v) water gave the highest yield and initial reaction rate in an IL-buffer system comprising 1-(2-hydroxyethyl)-3-imidazolium hexafluorophosphate ([C2OHMIm][PF6]) and MOPS-NaOH buffer. This water content is high compared to the 0.5-4% often used in lipase reactions in IL systems (Ulbert et al. 2004; Shan et al. 2008; Yang et al. 2010), underlining the increased need for water in these non-conventional media exhibited by esterases compared to lipases. FAEs have also been found to catalyse a number of (trans-)esterifications in other non-conventional media including surfactantless microemulsions and pure organic solvents (cf. Chapter 3). To step further into the less explored field of FAE-catalysed synthesis in IL media, the first part of this thesis investigates the stability of three different FAEs in a carefully selected series of IL-buffer systems using the sinapoylation of glycerol as a model reaction.

Enzyme stability is an obvious prerequisite for successful biocatalysis in IL systems, and assessing enzyme stability is especially important when working with FAEs or other enzymes which are less stable in non-conventional media than lipases. Only few have studied enzyme stability in IL media at more than one temperature (Ulbert et al. 2005; Lou and Zong 2006). Hence, the knowledge of thermal stability of enzymes in IL media is scarce.

In the current work, it is hypothesized that:
- Stability and reactivity of FAEs in IL-buffer systems vary depending on the IL nature and different FAEs may respond differently (Hypothesis 1).
Based on this hypothesis, the objective of the work presented in this chapter was to elucidate the effect of the enzyme structure and IL nature on the thermal stability and activity of three FAEs in IL systems. This was done by determining the synthetic activity and thermal stability of AnFaeA, FAE type C from *Emericella (Aspergillus) nidulans* (AndFaeC), and the commercial FAE-containing blend from *H. insolens*, Ultraflo L, in IL-buffer systems. This was done using a carefully selected series of four ILs with pairwise similar anions and cations of varying hydrophobicity and polarity, namely [C$_2$OHMIm][PF$_6$], [C$_2$OHMIm][BF$_4$](1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate), [BMIm][PF$_6$] (1-butyl-3-methylimidazolium hexafluorophosphate), and [BMIm][BF$_4$] (1-butyl-3-methylimidazolium tetrafluoroborate). The choice of [C$_2$OHMIm][PF$_6$] was based on the results previously obtained with AnFaeA (Vafiadi *et al.* 2009); [BMIm][PF$_6$] and [BMIm][BF$_4$] were included because they have been widely used in biocatalysis research, albeit not with FAEs, whereas [C$_2$OHMIm][BF$_4$] was included to complete the series. Furthermore, the study aimed to explore the use of the quantum chemistry-based COSMO-RS method for explaining the effect of individual ILs on enzyme stability to provide a foundation for predicting the optimal IL for a given FAE-catalysed esterification reaction.

**Experimental**

For Materials & Methods, please refer to Paper I.

After publication of Paper I, circular dichroism (CD) measurements were performed on AnFaeA in aqueous medium as follows: CD measurements were carried out in an AVIV 410 CD spectropolarimeter (Lakewood, NJ, USA) equipped with a temperature control unit. The enzyme was assayed in 10 mM MES-NaOH buffer (pH 6.0). Far-UV CD spectra were recorded at 4°C, 25°C and 95°C with AnFaeA concentrations of 17.7 $\mu$M in a 1 mm cell. Control baselines were measured with buffer in the absence of AnFaeA. Secondary structure contents were estimated from the spectra using the K2D2 server (Perez-Iratxeta and Andrade-Navarro 2008). Thermal transitions were measured from 4°C to 82°C with an AnFaeA concentration of 0.9 $\mu$M in a sealed 10 mm cell with stirring. After reaching the desired temperature, AnFaeA was incubated for 2 minutes and data was collected for 1 minute. Thermal denaturation was followed at 222 nm where the change in ellipticity as a function of temperature ($[\theta]_T$) was most pronounced. Unfolding curves were fitted by Equation 2:

$$[\theta]_T = \alpha([\theta]_F - [\theta]_D) + [\theta]_D$$

where $\alpha$ is defined as the fraction of folded AnFaeA at any temperature, $[\theta]_F$ and $[\theta]_D$ describe the ellipticity of the fully folded and denatured form and were normalized to 1 and 0, respectively. The temperature at the midpoint of denaturation ($T_m$) was determined with $\alpha = 0.5$.  

Furthermore, the commercial FAE-containing enzyme blend Depol 740 L (Biocatalysts Ltd, Cardiff, UK) was also tested for activity in the esterification of glycerol with FA (Sigma-Aldrich, Steinheim, Germany) and SA in the [BMI][PF₆] and [C₂OHMIm][PF₆] systems for up to 24 hours following the same procedure as described in Paper I. Based on hydrolytic activity of methyl sinapate (MSA; Paper I), the dosage of Depol 740L was approx. 5 times lower than that of AnFaeA. These experiments were included for coherence since Depol 740L was later used as a benchmark enzyme in the experiments reported in Chapter 3.

Results & Discussion

FAE activity in IL-buffer systems

Of the four ILs tested, only [BMI][PF₆] is water-immiscible and formed a biphasic system, while the others formed monophasic solutions with buffer and glycerol (Table 2.1). In the biphasic system, enzyme activity was only found in the water-rich phase.

AndFaeC was completely stable for more than 2 hours in the buffer, but was rapidly inactivated in the aqueous IL systems: In the C₂OHMIm⁺-based systems inactivation was immediate, and in the BMI⁺-based systems inactivation of AndFaeC occurred within 10 minutes. Consequently, only negligible esterification activity (1.1% conversion) was observed and only in the [BMI][PF₆] system (Table 2.1). A similar inactivation pattern was seen for Ultraflo L, except that this enzyme was stable throughout the 30 minutes of reaction in [BMI][PF₆]. Even so, the conversion yield obtained was not significantly different from that of AndFaeC: 1.0% conversion of SA to glycerol sinapate was detected in [BMI][PF₆] (Table 2.1). These two FAEs were thus very sensitive to the IL environment. The fact that they were slightly more stable and/or active in [BMI][PF₆] can most likely be explained by them being protected from the IL in the water-rich phase of this biphasic system.

Table 2.1. Conversion (%) of SA to glycerol sinapate after 30 minutes of reaction in IL-glycerol-buffer (67:18:15% (v/v/v)) systems by AnFaeA, AndFaeC, and Ultraflo L. The number of phases in each IL-buffer system was determined by visual detection. Complete enzyme inactivation within the reaction time is indicated: i0: complete inactivation was immediate; i10: complete inactivation occurred within 10 minutes. Superscript letters a-c indicate significant difference ($p < 0.05$) between different ILs for each enzyme, while x-y indicate significant difference between enzymes for each IL.

<table>
<thead>
<tr>
<th>IL</th>
<th>No. of phases</th>
<th>AnFaeA</th>
<th>Ultraflo L</th>
<th>AndFaeC</th>
</tr>
</thead>
<tbody>
<tr>
<td>[BMI][PF₆]</td>
<td>2</td>
<td>13 ± 3%</td>
<td>1.0 ± 0.1%</td>
<td>1.1 ± 0.1%</td>
</tr>
<tr>
<td>[C₂OHMIm][PF₆]</td>
<td>1</td>
<td>21 ± 2%</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
</tr>
<tr>
<td>[BMI][BF₄]</td>
<td>1</td>
<td>0.9 ± 0.1%</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
</tr>
<tr>
<td>[C₂OHMIm][BF₄]</td>
<td>1</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
</tr>
</tbody>
</table>
In contrast, AnFaeA had significant esterification activity and catalysed the sinapoylation of glycerol to 13% conversion in [BMIm][PF₆] and 21% in [C₂OHMIm][PF₆] (Table 2.1). Interestingly, Vafiadi et al. (2009) who obtained 73% conversion after 24 hours in the [C₂OHMIm][PF₆] system reported that the reaction did not take place in the [BMIm][PF₆] system. The conversion yields obtained here are considerably lower than that of Vafiadi et al. (2009), but increasing the reaction time did not increase reaction yields. In fact, the SA conversion seemed to reach a steady state after 30 minutes of reaction; after this, glycerol sinapate yields did not increase further (data not shown). AnFaeA was completely stable throughout the reaction time in both PF₆-based systems, but was sensitive to BF₄⁻. Thus, only negligible activity (0.9% conversion) was obtained in [BMIm][BF₄], where the residual activity of the enzyme was only 36% after 10 minutes (Table 2.1; Figure 2 in Paper I). No esterification took place in [C₂OHMIm][BF₄], where AnFaeA was completely inactivated within 10 minutes (Table 2.1). The results indicate that the anion has a dramatic effect on AnFaeA activity.

The significantly lower esterification activity of AnFaeA observed in [BMIm][PF₆] compared to [C₂OHMIm][PF₆] (Table 2.1) may be explained by mass transfer limitations between the two phases in the [BMIm][PF₆] system. Furthermore, [BMIm][PF₆] has a higher viscosity (382 cP at 20°C (Tomida et al. 2006)) than [C₂OHMIm][PF₆] (149 cP at 20°C (Branco et al. 2002)) and even at a glycerol content of 18% (v/v), this difference may give rise to lower reaction rates also induced by increased mass transfer limitations in the more viscous system (Eckstein et al. 2002). Finally, water-miscible ILs generally exhibit a lower \(a_w\) at a given water content than water-immiscible ILs do (Garcia et al. 2004), and a lower \(a_w\) in [C₂OHMIm][PF₆] would favour esterification in this system. However, at this high water content (15% (v/v)) even the more hydrophilic BF₄⁻-based IL systems have an \(a_w\) of approx. 0.9 (Bou Malham et al. 2007), so a major difference between the (undetermined) \(a_w\) of the system with the more hydrophobic [C₂OHMIm][PF₆] and the \(a_w = 1\) of the [BMIm][PF₆] system (Anthony et al. 2001) is unlikely.

After publication of Paper I, the commercial FAE-containing blend Depol 740L was tested for its ability to catalyse the esterification of glycerol with FA and SA before being used alongside AnFaeA in the following experiments (Chapter 3). Depol 740L is an FAE-containing preparation from H. insolens (Faulds et al. 2008). According to Faulds et al. (2004), the induction of carbohydrate esterases and glycosyl hydrolases in H. insolens differs from strain to strain, and it is thus not to be expected that different commercial preparations contain the same FAEs. Nevertheless, Faulds and co-workers reported FAE activities in Depol 740L that were in agreement with the ones observed for Ultraflo L (Faulds et al. 2008), which has been reported to contain mainly type B FAEs and a small amount of type A (Faulds et al. 2002). Thus, commercial FAE-containing preparations from H. insolens are expected to have higher affinity for MFA than for MSA in the hydrolytic reaction, which was also the case for Depol 740L where activity on MFA was almost 7 times higher than the activity on MSA (data not shown).
Using the [BMIm][PF₆] system, 3% conversion was obtained in the sinapoylation of glycerol and 11% conversion in the feruloylation. In the [C₂OHMIm][PF₆] system, only 3% conversion was seen in the feruloylation, and no sinapoylation of glycerol was detected (Table 2.2). The dosage of Depol 740L was 5 times lower than that of AnFaeA when based on hydrolytic activity on MSA (higher dosage could not be achieved since Depol 740L was used undiluted). This may in part explain why the yields are generally low with Depol 740L compared to the ones obtained with AnFaeA (Tables 2.2 and 2.1), but since the reaction time much longer (24 hours compared to the 30 minutes used for AnFaeA) the results indicate that Depol 740L is less effective in catalysing esterification of glycerol with FA or SA compared to AnFaeA. The product concentration kept increasing over the 24 hours of reaction, so there is no indication that the lower product yields are caused by product hydrolysis (data not shown).

Table 2.2. Conversion (%) of SA and FA to glycerol sinapate or glycerol ferulate, respectively, catalysed by Depol 740L in IL-glycerol-buffer (67:18:15% (v/v/v)) systems. Based on the hydrolytic activity on MSA (Paper I), the dosage of Depol 740L was 5 times lower than that of AnFaeA (Table 2.1). Reaction time was 24 h (compared to 30 min. in Table 2.1). Superscript letters a-c indicate significant difference (one-way ANOVA; \(p < 0.05\)) between conversion yields.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>[C₂OHMIm][PF₆]</th>
<th>[BMIm][PF₆]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA + glycerol</td>
<td>0 ± 0%⁺</td>
<td>3 ± 0.8%ᵇ</td>
</tr>
<tr>
<td>FA + glycerol</td>
<td>3 ± 0.8%ᵇ</td>
<td>11 ± 0.1%ᶜ</td>
</tr>
</tbody>
</table>

The higher affinity for MFA than for MSA in the hydrolytic reaction exhibited by Depol 740L may explain why higher esterification activity is seen with FA compared to SA (Table 2.2). Ultraflo L reached 1% conversion after 30 minutes in [BMIm][PF₆] where it was stable for more than 2 hours, but was rapidly inactivated (within 30 seconds) in [C₂OHMIm][PF₆]. Similar to Ultraflo L, Depol 740L gives higher conversion yields in [BMIm][PF₆] compared to [C₂OHMIm][PF₆], where the conversion yield obtained with SA and glycerol is 0% and thus comparable to that obtained with Ultraflo L (Tables 2.2 and 2.1). Depol 740L is however not as unstable in [C₂OHMIm][PF₆] as Ultraflo L, since some conversion does take place in this IL with FA for which the affinity of Depol 740L is higher (Table 2.2), and the results obtained with arabinose and FA in IL-buffer systems (Chapter 3) indicate that Depol 740L is indeed stable in an IL-buffer system with [C₂OHMIm][PF₆] for up to 6 days. Thus, the results may rather suggest that Depol 740L prefers the biphasic system over the monophasic one. Possibly, higher yields may have been obtained with Ultraflo L in [BMIm][PF₆] catalysing feruloylation rather than sinapoylation, but AnFaeA, AndFaeC, and Ultraflo L were all dosed equally based on their hydrolytic activity on MSA in buffer.

The results presented in this section clearly corroborate the hypothesis that FAE reactivity is affected by the IL nature (Hypothesis 1).
Effect of enzyme structure on FAE activity in IL-buffer systems

Although the catalytic efficiency of Ultraflo L in [BMIm][PF₆] may have been improved by changing the substrates, it does not change the fact that both AndFaeC and Ultraflo L were unstable in the IL-buffer systems, while AnFaeA was not. In this respect, the most important difference between the three FAEs is found in their three-dimensional structure. When predicting the structure of AndFaeC by the homology modelling tool HHpred, the best match is the FAE domain of the cellulosomal xylanase Z (XynZ) from \textit{Clostridium thermocellum}. The FAE found in Ultraflo L also shows structural similarity to this domain, albeit less than AndFaeC (see Paper I for details). Comparing crystal structures, Hermoso \textit{et al.} (2004) reported that XynZ had poor homology in both sequence and structure to AnFaeA. Instead, AnFaeA is structurally similar to the lipases from \textit{Rhizomucor miehei} and \textit{Thermomyces lanuginosus} (Hermoso \textit{et al.} 2004), which have both been found to work well in several different ILs (De Diego \textit{et al.} 2009).

This structural similarity to IL-compatible fungal lipases is likely the reason for the superior stability of AnFaeA in aqueous IL systems observed in the current work, and it makes AnFaeA a good candidate for further synthetic reactions in non-conventional media. The results obtained here thus corroborate the second part of Hypothesis 1.

The differences in stability between the three enzymes could not be explained by large differences between their primary amine:acids ratio as hypothesized by Nordwald and Kaar (2013); cf. Chapter 1. The primary amines:acids ratios were 0.17, 0.16, and 0.13 for AnFaeA, AndFaeC, and Ultraflo L, respectively. Nordwald and Kaar (2013) explained that variations in the number of Arg and His residues between enzymes may affect the stability, but these are not accounted for in the ratio. AnFaeA has a total of 11 His and Arg residues, whereas Ultraflo L has 23 and AndFaeC has 13 plus 6 in the His-tag. This may in part account for the differences in stability in IL media, but the evidence is somewhat vague. It is more likely that the chemical modification strategy proposed by Nordwald and Kaar (2013) may be used for improving the stability of each enzyme compared to its wild type, and that the difference in stability observed between the FAEs are correlated with their three-dimensional structure as outlined above.

Stability of AnFaeA in aqueous medium

Of the three FAEs tested, only AnFaeA showed appreciable esterification activity in the selected IL-buffer systems. Therefore, only AnFaeA was subjected to stability studies as reported in this section and the following one.

Thermal stability of AnFaeA in buffer was kinetically determined as described in Paper I. Negligible values of the thermal inactivation constant, \(k_D\), were obtained at 30°C and 40°C, where AnFaeA was completely stable for at least 2 hours (see Table 2.4 below). At 50°C, AnFaeA
retained 84% of its activity after 2 hours in buffer \((k_0 = 0.0012)\), while at 60°C complete inactivation occurred after 1 hour \((k_0 = 0.0646;\) cf. Table 2.4 and Figure 2d in Paper I).

CD spectra and thermal transition of AnFaeA were recorded in aqueous medium (10 mM MES-NaOH buffer, pH 6.0). It was not possible to perform CD measurements in the in IL-glycerol-buffer (67:18:15% (v/v/v)) systems used in Paper I due to interference from the IL. The spectra obtained at 4°C and 25°C are similar (Figure 2.3a) and in agreement with that previously published for AnFaeA at 20°C (Benoit et al. 2006). The spectra are typical of a protein containing both α-helices and β-sheets. This is in agreement with the crystal structure of AnFaeA (PDB: 1USW (Hermoso et al. 2004)), which shows 36% α-helix and 24% β-sheet. From the data the K2D2 server (Perez-Iratxeta and Andrade-Navarro 2008) estimates a secondary structure content comprising 20% α-helix and 32% β-sheet, which may be within the range of what can be expected from this estimation tool. At 95°C, the spectrum clearly indicates unfolding of AnFaeA (Figure 2.3a).

![Figure 2.3. CD measurements on AnFaeA in 10 mM MES-NaOH buffer (pH 6.0). (a) CD spectra of AnFaeA at 4°C, 25°C, and 95°C. (b) Thermal transition of AnFaeA at 222 nm. The data are normalized to show the fraction of folded AnFaeA at a given temperature. The line is the fit used for determining the temperature at the denaturation midpoint, \(T_m\), fitted to the data with the Levenberg-Marquardt algorithm. \(T_m\) is 60.5°C.](image)

In relation to the enzyme stability discussed here, the thermal transition data are of greater importance than the CD spectra. From the thermal transition data, a denaturation midpoint temperature, \(T_m\) of 60.5°C was determined (Figure 2.3b). Benoit et al. (2006) used fluorescence measurements for determining a \(T_m\) of 56°C for AnFaeA. The thermal transition data (Figure 2.3b) agrees with the kinetic thermal stability data (Table 2.4 and Figure 2 in Paper I).
However, the stability data on AnFaeA in aqueous media obtained in this work does not quite agree with the temperature optimum for AnFaeA of 55-60°C determined by Faulds and Williamson (1994) based on hydrolysis of MFA over 30 minutes. The folded fraction was 0.94 at 55°C and 0.59 at 60°C (Figure 2.3b). After 30 minutes of incubation at 60°C, the residual activity of AnFaeA in buffer was 14%. Faulds and Williamson (1994) do mention that they changed the standard activity assay temperature from 50°C to 37°C to avoid thermal inactivation. For optimum activity, 50°C does however seem to be a good choice: At this temperature, AnFaeA retained 84% of its activity after 2 hours of incubation (cf. Figure 2c in Paper I) and the folded fraction was 0.999 (Figure 2.3b).

Effect of IL nature on AnFaeA stability

AnFaeA was assayed for thermal stability at 30-60°C in the four IL systems containing 15% (v/v) buffer. From the data, thermal inactivation constants, $k_D$, were calculated (Table 2.4). For a graphic view of the data, please refer to Figure 2 in Paper I.

Table 2.4. Thermal stability of AnFaeA in IL-buffer systems: Thermal inactivation constants, $k_D$, at 30°C, 40°C, 50°C, and 60°C when incubated in 100 mM MOPS buffer at pH 6.0 or in IL systems [C$_2$OHIm][PF$_6$], [BMIm][PF$_6$], [C$_2$OHIm][BF$_4$], and [BMIm][BF$_4$] containing 15% (v/v) buffer for up to 2 hours. Where no $k_D$ value is given, complete inactivation took place within 10 minutes of incubation and $k_D$ could thus not be calculated. $R^2$ values were generally high (above 0.8 for $k_D$ values above 0.002). Superscript letters a-c indicate significant difference between $k_D$ values ($p < 0.05$).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Buffer</th>
<th>[C$_2$OHIm][PF$_6$]</th>
<th>[BMIm][PF$_6$]</th>
<th>[C$_2$OHIm][BF$_4$]</th>
<th>[BMIm][BF$_4$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>0.0007$^a$</td>
<td>0.0001$^a$</td>
<td>0.0005$^a$</td>
<td>-</td>
<td>0.0174$^b$</td>
</tr>
<tr>
<td>40°C</td>
<td>0.0003$^a$</td>
<td>0.0007$^a$</td>
<td>0.0002$^a$</td>
<td>-</td>
<td>0.0179$^b$</td>
</tr>
<tr>
<td>50°C</td>
<td>0.0012$^a$</td>
<td>0.0105$^b$</td>
<td>0.0191$^b$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60°C</td>
<td>0.0646$^c$</td>
<td>0.1840$^d$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

At 30°C and 40°C, AnFaeA retained full activity for at least 2 hours in both [BMIm][PF$_6$] and [C$_2$OHIm][PF$_6$] as evidenced by the negligible $k_D$ values, and there was no significant difference from the stability in the buffer (Table 2.4). After 30 minutes of incubation at 50°C, the residual activity of AnFaeA was higher in [BMIm][PF$_6$] (75%) than in [C$_2$OHIm][PF$_6$] (58%). However, after 2 hours of incubation, the scenario was reversed (26% residual activity in [C$_2$OHIm][PF$_6$] against 11% residual activity in [BMIm][PF$_6$]) and this overall higher stability in [C$_2$OHIm][PF$_6$] was also reflected in a lower $k_D$ value for this system (0.0105) compared to that for the [BMIm][PF$_6$] system (0.0191); this difference was however not statistically significant (Table 2.4). Comparing to the $k_D$ value obtained in buffer (0.0012), the detrimental effect of the ILs at this elevated temperature is evident. At 60°C, thermal inactivation was severe in all systems: Complete inactivation took place within 1 hour in buffer ($k_D = 0.0646$), within 20 minutes in [C$_2$OHIm][PF$_6$] ($k_D = 0.184$), and within 10 minutes in [BMIm][PF$_6$].
While significant stability of AnFaeA was observed in the PF$_6$-based systems, at least at 30-40°C, AnFaeA was highly unstable in the BF$_4$-based systems. In [C$_2$OHMIm][BF$_4$], complete inactivation of AnFaeA was almost immediate and no $k_D$ values could be determined (Table 2.4). Even at 30°C and 40°C, inactivation of AnFaeA was significant in [BMIm][BF$_4$] as evidenced by the large $k_D$ values of 0.0174 and 0.0179, respectively (Table 2.4). At 30°C, the residual activity was 36% after 10 minutes and 8% after 2 hours, while at 40°C 31% residual activity remained after 10 minutes and 7% after 2 hours. At 50°C, complete inactivation of AnFaeA took place within 10 minutes in this system.

These results clearly emphasize the effect of temperature and not least the pronounced effect of the IL anion on enzyme stability, thus supporting Hypothesis 1. The effect of IL nature, i.e. hydrogen bond basicity, hydrophobicity, nucleophilicity, viscosity, and ion kosmotropicity, on enzyme stability in IL systems was reviewed in Chapter 1. From this it seems clear that the anion dominates the effect of the IL on enzyme stability, and that the effect is closely linked to the hydrogen bonding capacity of the anion.

**Potential of COSMO-RS for explaining and predicting enzyme stability in ILs**

COSMO-RS is a dielectric continuum solvation model; the name is short for COnductor-like Screening MOdel for Realistic Solvation. In contrast to the widely used group contribution models such as UNIFAC, which take their starting point in considering a fluid as a mixture of interacting functional groups rather than a mixture of specific molecules, the COSMO-RS approach considers the interactions between molecular surfaces computed through quantum chemical methods (Eckert and Klamt 2002). COSMO-RS can be seen as a two-level approach to thermodynamic modelling, combining electrostatic theory of locally interacting molecular surface descriptors (available from quantum chemistry computations) with statistical thermodynamics. At the first level, quantum chemistry calculations are performed for all compounds in the system of interest. For these calculations, the dielectric continuum solvation model COSMO is applied; it simulates the molecule being surrounded by a virtual conductor, in which the molecule induces a polarization charge density at the interface between the molecule and the conductor, also to be thought of as the molecule’s surface. A surface segment $i$ with the area $a_i$ thus has a polarization charge density, $\sigma_i$, which is a descriptor of the molecular surface polarity with the unit charge per area (e.g. e/nm$^2$). The polarization charge density is also called the screening charge density (SCD), and can be seen as a local measure of polarity for each molecule. Since the charges found at the molecular surface are those created by the molecule in the virtual conductor, these surface charges are the countercharges of those actually found in the functional groups of the molecule; thus, roughly speaking, a positively charged group will create a negative charge at the surface. For each molecule, a $\sigma$-profile showing the frequencies of screening charge densities, $\sigma$, on the molecular surface can be generated, providing a useful means of assessing the polarity profile of a molecule. At the second level of this two-level
method, a statistical thermodynamics approach is applied to the COSMO quantum chemistry calculations: the calculated screening charge densities are now used for quantification of the interaction energy of pairwise interaction between surface segments (Eckert and Klamt 2002; Klamt 2005).

The COSMO-RS method is applicable to almost the entire organic chemistry. A $\sigma$-surface can be calculated for virtually all molecules, because it requires a very limited number of element-specific parameters only, and therefore COSMO-RS has been successfully applied to a wide range of systems including IL systems (Diedenhofen and Klamt 2010; Kontogeorgis and Folas 2010). It has the advantage that it differentiates between e.g. primary, secondary, and tertiary hydroxyl groups because these differ in the underlying quantum chemistry calculations, and it can also handle isomers – problems at which other thermodynamic methods often fail. Importantly, COSMO-RS is a predictive model that does not require a high degree of parameterisation. It is therefore suitable for solvent screening and novel applications, especially where experimental data are scarce. Since COSMO-RS looks at the entire molecule and how functional groups interact rather than just adding the effects of the present functional groups like group contribution models (e.g. UNIFAC) do, it can handle rather complex molecules and solutions. Consequently, COSMO-RS often fails – or is simply outcompeted by other models, e.g. PC-SAFT – for very simple systems such as alcohols or glycols with alkanes (Kontogeorgis and Folas 2010). Furthermore, while COSMO-RS is often useful in a qualitative solvent screening, the quantitative results may not always be satisfactory. Unfortunately, the results cannot be further improved in these cases because there are no adjustable parameters in the model.

COSMO-RS is thus a relevant tool to use for ILs, which are considered to be highly complex systems with a low amount of available thermodynamic data. In the current work, its ability to explain the effect of the four different ILs on AnFaeA stability was tested by investigating the $\sigma$-profiles of the four ions BMIm+, C₂OHMIm+, PF₆⁻, and BF₄⁻ (Figure 2.5). The hydrogen bonding threshold is $\pm \sigma_{HB} = \pm 0.79 \text{ e/nm}^2$, but as hydrogen bonding is weak below $\pm 1 \text{ e/nm}^2$, only surface segments beyond $\pm 1 \text{ e/nm}^2$ are considered strongly polar and potentially hydrogen bonding (Klamt 2005). When $\sigma > 1$, that part of the molecule is considered a hydrogen bond acceptor. Conversely, $\sigma < -1$ indicates a hydrogen bond donor. From the $\sigma$-profiles it is seen that the peak SCD of BF₄⁻ is found outside the hydrogen bonding limit of 1 e/nm², whereas the peak SCD of the more hydrophobic PF₆⁻ is inside this limit (Figure 2.5). Thus, the destabilising effect of BF₄⁻ on AnFaeA can be explained by the tendency of BF₄⁻ to act as a hydrogen bond acceptor and thus interact with the hydrogen bond-based enzyme structure. It should also be noted that the SCD peak at $\sigma > 1 \text{ e/nm}^2$ is much larger for BF₄⁻ than for water, thus explaining why the IL anion destabilizes the enzyme while water does not (Figure 2.5). The fact that no major difference in the effect on AnFaeA stability was observed between BMIm⁺ and C₂OHMIm⁺ is consistent with the two cations having similar $\sigma$-profiles in the hydrogen bond donor range ($\sigma < -1 \text{ e/nm}^2$; Figure 2.5).
Figure 2.5. Sigma(σ)-profiles of BF₄⁻ (dark grey, solid), PF₆⁻ (black, dashed), C₂OHMIm⁺ (dark grey, dashed), and BMIm⁺ (conformer 0 (light grey, solid) and conformer 1 (light grey, dashed)). The σ-profile for water (black, solid) is included for comparison. The vertical lines indicate the hydrogen bonding limits at ±1 e/Å². σ-profiles are shown for two conformers 0 and 1 of BMIm⁺; COSMOOtherm estimates that in this IL-buffer system (15% (v/v) water), 85% of the BMIm⁺ molecules are in the conformer 0 form, while 10% are in the conformer 1 form. The two conformers generated for C₂OHMIm⁺ had almost identical σ-profiles; thus, only one is shown.

The COSMO-RS method proved to be a useful tool for explaining the effect of the four ILs studied in this work on AnFaeA stability in terms of hydrogen bonding capacity. Given the importance of hydrogen bonds in maintaining enzyme structure, the COSMO-RS method may thus prove to facilitate the prediction of useful ILs for enzymatic reactions in terms of stability. Figure 2.6 shows σ-profiles for the anions discussed in terms of stabilizing effects on enzymes by Zhao (2010), which were also available in the COSMO-RS library (COSMO-files for MeSO₄⁻ and NO₃⁻ were made as described in Paper I, but using BP86 and TZVP instead of B3LYP and 6-311++G(d,p) as recommended by COSMOlogic). In general, PF₆⁻ and Tf₂N⁻ stabilize enzymes, while BF₄⁻, MeSO₄⁻, NO₃⁻, and especially Cl⁻ destabilize them (Zhao 2010). This is corroborated by the σ-profiles in Figure 2.6, thus indicating the usefulness of COSMO-RS as a prediction tool in preliminary screening of ILs in terms of enzyme stability. Similarly, it has been reported that COSMO-RS could be used for screening ILs for flavonoid solubility, which also turned out to be anion dependent (Guo et al. 2007). However, as mentioned by Zhao (2010), a few studies have reported relatively high enzymatic activities in BF₄⁻- and MeSO₄⁻-based ILs, thus emphasizing the fact that while COSMO-RS can definitely assist in a preliminary solvent screening, actual experimental tests of enzyme stability in ILs are still required for successful catalysis.
Figure 2.6. Sigma(σ)-profiles of anions Tf2N− (black, solid), PF6− (dark grey, solid), BF4− (light grey, solid), MeSO4− (black, dashed), NO3− (dark grey, dashed), and Cl− (light grey, dashed). The vertical lines indicate the hydrogen bonding limits at ±1 e/nm². The two conformers for Tf2N had almost identical σ-profiles, so only one is shown.

Conclusions

Of the three FAEs tested here, only AnFaeA showed potential for being used for esterification in an IL-buffer system. Structurally, AnFaeA is more similar to fungal lipases than other FAEs and this seems to be determining for its IL compatibility. Furthermore, the stability of AnFaeA in IL-buffer systems was found to depend on the hydrogen bonding capacity of the IL anion. Thermal stability studies on AnFaeA showed that it can be used in PF6−-based IL-buffer systems at temperatures up to 40°C without losing activity for at least 2 hours. Whether the water-miscible [C2OHMIm][PF6] or the water-immiscible [BMIm][PF6] should be used depends of the nature of the desired system – in some cases a two-phase system may be preferable because it allows for easy recovery of IL, product, and enzyme, e.g. through centrifugation. COSMO-RS proved to be a useful tool for preliminary screening of ILs for enzyme stability.
Chapter 3: Feruloyl esterase-catalysed esterification of arabinose and xylose with hydroxycinnamates in non-conventional solvents

*This chapter relates to Paper II:*

**Introduction**

Chapter 2 and Paper I demonstrated the applicability of AnFaeA for direct esterification of glycerol with SA in IL-water systems due to its lipase-like structure, as well as the usefulness of COSMO-RS simulations for explaining the effect of IL anions on enzyme stability. (Trans-)esterification of various compounds with hydroxycinnamates introduces antioxidant potential to the compound (Vafiadi *et al.* 2009; Shahidi and Chandrasekara 2010) and synthesis of many new compounds for *e.g.* the food and cosmetics industries may be catalysed by FAEs. While vast amounts of glycerol are available as waste from the biodiesel production, this biodiesel waste is expensive to purify for use in food, pharmaceutical, and cosmetics industries (Santibáñez *et al.* 2011). In the bioethanol production major side streams containing pentoses arabinose and xylose are available for value addition in the biorefinery. Hence, the natural next steps would be to change the reaction from esterification of glycerol to esterification of pentoses, especially arabinose and xylose and to explore other thermodynamic models as tools for solvent design for these reactions.

In nature, FA is bound via O-5 bonds to arabinose in arabinoxylan, whereas pectins feature FA bound via O-2 bonds to arabinose and via O-6 bonds to galactose (Colquhoun *et al.* 1994). Feruloylated arabin-xylo-oligosaccharides have been extracted from wheat flour arabinoxylan with xylanase (Katapodis *et al.* 2003) and both feruloylated galactose disaccharides and feruloylated arabin-oligosaccharides have been extracted from sugar beet pectin treated with a carbohydrase mixture (Ralet *et al.* 1994). Furthermore, feruloylated arabin-oligosaccharides have been extracted and purified from sugar beet pectin using a combination of hydrophobic interaction and membrane filtration (Holck *et al.* 2011). However, these methods yield a mixture of feruloylated oligosaccharides with varying degree of polymerization (DP) rather than a specifically defined product. The feruloylated arabin-oligosaccharides and galactose disaccharides could be further degraded to feruloylated arabinose (FA-Ara) and feruloylated galactose, respectively, using mild acid hydrolysis (Ralet *et al.* 1994). However, yields were generally low since they are inevitably linked to the amount of hydroxycinnamates present in the biomass. Furthermore, only feruloylated saccharides have been extracted due to the
abundance of FA in nature, but other hydroxycinnamate derivatives may be equally interesting (Andreasen et al. 2001). Enzyme-catalysed synthesis of hydroxycinnamate saccharide esters provides a method for producing a uniform product at higher yields, but also serves as proof-of-concept for FAE biocatalysis in non-conventional media.

As part of this thesis it is hypothesized that:

- Pentoses arabinose and xylose can be enzymatically esterified with hydroxycinnamoyl moieties in non-conventional solvents using compatible FAEs (Hypothesis 2).
- Available thermodynamic models can assist in solvent screening in order to optimize the solvent selection for enzyme-catalysed acylation reactions in non-conventional media (Hypothesis 3).

Based on these hypotheses, the objective of the work reported in this chapter was to (1) investigate the potential of using thermodynamically based tools for predictive solvent screening for FAE-catalysed esterification of saccharides with hydroxycinnamoyl donors, and (2) catalyse feruloylation (and sinapoylation) of arabinose or xylose with two FAEs reported to have activity in non-conventional media.

**Literature review: Enzymatic saccharide acylation with hydroxycinnamic acids in non-conventional media**

The literature on enzymatic saccharide acylation with hydroxycinnamic acids in non-conventional media has been reviewed in Paper II. Enzymatic hydroxycinnamate saccharide ester synthesis has mainly been carried out in surfactantless microemulsions using FAEs or in pure organic solvents using FAEs or lipases. The enzymes exhibited broad acceptor specificity, (trans-)esterifying a wide range of saccharides and saccharide derivatives. Mainly FA or its esters have been used for saccharide (trans-)esterification (Tables 1A and 1B in Paper II). At the time of submission of Paper II, no enzyme-catalysed (trans-)esterification of saccharides with hydroxycinnamates in IL-containing systems had been reported. Paper II thus also reviewed the literature on FAE- and lipase-catalysed hydroxycinnamate (trans-)esterifications of alcohols in IL-containing systems. Comparing these results to the results obtained in organic solvent systems showed that using IL-containing systems generally gave higher yields and shorter reaction times. Thus, further research into the use of IL-containing solvent systems may improve enzymatic efficiency in reactions of this kind. Furthermore, the use of supersaturated saccharide-IL solutions may improve catalytic efficiency (Lee et al. 2008).

Synthesis of hydroxycinnamate esters can be catalysed by lipases and FAEs. Concluding on the literature reviewed in Paper II, the following points should be considered when designing an enzymatic saccharide acylation in order to maximize reaction rate, selectivity, enzyme stability and reaction yields:
- Saccharide solubility and enzyme stability should be accommodated through careful selection of (a mixture of) ILs or use of surfactantless microemulsions; a compromise between solubility and stability is required.
- A high ratio between product solubility and substrate solubility increases reactivity, but too low substrate solubility can have adverse effects on regioselectivity.
- Choice of enzyme class should be considered: For systems containing hydroxycinnamic acids FAEs may be the superior choice, while the more robust lipases may be advantageous when working with other aromatic acids.
- Enzymes successfully applied in surfactantless microemulsions and organic solvent systems should be tested in IL systems.
- Choice of hydroxycinnamic acid donor could be changed from the widely used FA to e.g. SA based on the affinity of the enzyme.

**Thermodynamically based tools for selecting solvents for enzymatic hydroxycinnamate saccharide ester synthesis**

Paper II also includes an assessment of how thermodynamic models may be used in designing or selecting solvents for enzymatic hydroxycinnamate saccharide ester synthesis. Application of thermodynamic models to these novel complex reaction systems turned out to be limited by the lack of data, but predictive thermodynamic tools do exist. The use of thermodynamic tools could ease the solvent design process and enhance the understanding of the reaction systems as follows:
- COSMO-RS may be used for predicting substrate and/or product solubility in a preliminary solvent screening, being aware of its quantitative inaccuracies.
- COSMO-RS may be used for predicting enzyme stability in ILs as part of a preliminary solvent screening (cf. Chapter 2).
- Activity coefficient models, UNIFAC, and/or COSMO-RS can be used to assess the liquid-liquid equilibria (LLE) of the solvent system depending on the availability of data and parameters for a given system.
- UNIFAC may be used for predicting $a_w$ in various solvents, except for very hydrophobic ones.

Further work is required before the usefulness of these tools can gain currency, not least due to their quantitative inaccuracies and the lack of data limiting their use. However, the foundation exists for Hypothesis 3 to become a reality.

**Feruloyl esterase-catalysed esterification of arabinose or xylose with hydroxycinnamates in non-conventional media**

It is evident from Table 1A in Paper II that numerous FAEs, albeit not including AnFaeA, have been used for (trans-)esterification of saccharides with hydroxycinnamates in organic
microemulsions. After Paper II was accepted for publication, Couto et al. (2011) published successful direct esterification of di- and oligosaccharides with FA using the FAE-containing commercial enzyme preparation from H. insolens Depol 740L in organic surfactantless microemulsions, albeit with lower yields than obtained with the monosaccharides (Couto et al. 2010). Furthermore, they tested the same reactions in IL-based microemulsions and in an IL-buffer system (Couto et al. 2011). Thus, a series of experiments were planned to investigate the potential of using AnFaeA, which had previously shown markedly better catalytic activity in IL-water systems than other FAEs (Chapter 2), in the (trans-)esterification of arabinose and xylose with FA/MFA and SA/MSA both in IL-water systems, organic microemulsions and IL-containing organic microemulsions. Depol 740L, which had been used by Couto and co-workers (Couto et al. 2010; Couto et al. 2011) was included as a reference enzyme. The rest of this chapter deals with the results obtained from this experimental work.

**Experimental**

**Chemicals**

AnFaeA was provided by Novozymes A/S (Bagsværd, Denmark). Depol 740L was purchased from Biocatalysts Ltd (Cardiff, UK). AndFaeC was produced as described in Paper I. MFA (methyl 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate) was purchased from Alfa Aesar (Ward Hill, MA). MSA (methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoate) was purchased from Apin Chemicals (Abingdon, UK). All other chemicals including FA (3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoic acid), SA (3-(4-hydroxy-3,5-dimethoxyphenyl)-prop-2-enoic acid), ethyl ferulate (EFA; ethyl 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate), and IL [BMIm][PF₆] (1-butyl-3-methylimidazolium hexafluorophosphate) were purchased from Sigma-Aldrich (Steinheim, Germany).

**Preparation of [C₂OHMIm][PF₆], [C₃OMIm][PF₆], and iso-propyl ferulate**

ILs 1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate, [C₂OHMIm][PF₆], and 1-(2-methoxyethyl)-3-methylimidazolium hexafluorophosphate, [C₃OMIm][PF₆], were prepared according to the method described by Branco et al. (2002). The iso-propyl ferulate (IPFA; propan-2-yl 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate) standard was synthesized according to the conventional heating method described by Li et al. (2009).

**Esterification of arabinose in IL-buffer systems**

Esterification of arabinose with FA or SA was carried out in a 85:15% (v/v) IL-buffer system containing 20 mM FA or SA (solubilized in the IL from its solid form) and 20 mM L-arabinose (introduced in the aqueous phase, i.e. 100 mM MES-NaOH buffer, pH 6.0). The enzyme dosage of
AnFaeA was 0.9% (w/w) E/S, where S is FA. Depol 740L was dosed similarly, however the FAE activity in this preparation was 3.5 times lower than that of AnFaeA when measured on MFA. The total reaction volume was 500 µL, and the reaction took place in an Eppendorf tube kept at 40°C and 1400 rpm in a thermomixer for up to 168 hours (7 days). The reaction was stopped by extracting substrate and product with ethyl acetate (50 µL sample in 1 mL ethyl acetate) for 4 minutes at 40°C and 1400 rpm. After evaporation of the ethyl acetate extract, the remaining solids were re-dissolved in 0.5 mL of a 1:1 acetonitrile-water solution and analysed by RP-HPLC. Control samples without enzyme and without arabinose were included. Trans-esterification with MFA and MSA was carried out in a similar manner.

**Esterification of arabinose and xylose in microemulsions**

All the ternary systems listed in Table 3.4 were mixed in glass tubes and inspected visually to confirm whether one or two phases were formed. Stability of Depol 740L and AnFaeA was tested in all the monophasic systems and the \( n \)-hexane-2-butanone-buffer system used by Couto et al. (2010) (C-Hex), cf. Table 3.4. The enzymes were incubated in the ternary mixtures and samples were then taken out after 0, 1, 24, 72, and 120 hours of incubation and tested for activity in the standard FAE activity assay as described previously (Paper I). For feruloylation of arabinose and xylose, ternary systems A1, A4, B2, C1, C4, E1, E4, C-Hex, and C-IL were mixed according to Table 3.4 introducing FA in the alcohol/ketone phase and arabinose, xylose, and enzyme in the aqueous phase, giving final concentrations of 6 mM FA and 2 mM arabinose or xylose (acceptor:donor ratio 1:3, *i.e.* the optimum ratio established by Couto et al. (2011)). The enzymes were dosed as described for the IL-buffer systems. Samples were taken out after 30 seconds, 18 hours, 72 hours (3 days), and 120 hours (5 days), stopping the reaction with ethyl acetate as explained above. Control samples without arabinose or xylose as well as control samples without enzyme were included. In a series of follow-up experiments, SA and MFA were also tested as hydroxycinnamate donors (6 mM) using AnFaeA in A1, B2, and E1 (the latter only for MFA) following the same procedure as described above. Furthermore, AndFaeC was also tested with arabinose and FA or SA in A1 and B2.

**RP-HPLC analysis**

Quantitative analysis of FA, SA, MFA, MSA, FA-Ara, and sinapoylated arabinose (SA-Ara) was made by RP-HPLC using an Agilent Chemstation 1100 series equipped with a C18 column (ODS-L Optimal; 250 mm x 4.6 mm; 5 µm; serial no. ODS-L-OL-5-37538; Capital HPLC Ltd. (Edinburgh, UK)) with DAD detection of substrate and product with quantification at 316 nm. The eluent system comprised water with 5% (v/v) acetonitrile and 1 mM trifluoroacetic acid (A) and pure acetonitrile (B). Elution was conducted at 40°C starting with a linear gradient from 80:20 (% A:B) to 60:40 (% A:B) from 0 to 25 minutes followed by isocratic elution of strongly retained molecules at 100% acetonitrile from 25 to 28 minutes and finally re-equilibration of
the column at 80:20 (% A:B) from 28 to 30 minutes. SA eluted at 13.5 minutes, FA at 14.1 minutes, MSA at 28.1 minutes, and MFA at 29.1 minutes. A mixture of feruloylated arabino-oligosaccharides with a DP ranging from 2 to 10 (SFAOS; Holck et al. 2011) was used to give an estimate of the retention time of FA-Ara and SA-Ara due to lack of a proper standards. FA-Ara and SA-Ara were expected to elute at approx. 6 minutes. For proper detection of MFA by-product in the microemulsion experiments, elution was conducted at 40°C starting with a linear gradient from 80:20 (% A:B) to 0:100 from 0 to 48 minutes followed by isocratic elution at 100% acetonitrile for 5 minutes and re-equilibration of the column for 2 minutes. This gave baseline separation of FA (13.8 min.), MFA (22.6 min.), EFA (26.3 min.), and IPFA (29.6 min.).

Results & Discussion

Feruloyl esterase-catalysed esterification of arabinose with hydroxycinnamates in IL-buffer systems

AnFaeA is a type A FAE and has affinity for both MSA and MFA in the hydrolytic reaction, with the specific activity on MSA being 2.3 times as high as on MFA (Faulds and Williamson 1994). Therefore, AnFaeA-catalysed esterification of arabinose was tested with FA as well as SA. For Depol 740L, only FA was used based on the assumption that this commercial blend contains mainly type B FAEs (cf. Chapter 2).

AnFaeA and Depol 740L were tested in the direct esterification of arabinose with FA and/or SA in an IL-buffer system, containing 85% (v/v) IL in which the hydroxycinnamate is solubilized and 15% (v/v) MES-NaOH buffer (pH 6.0) in which arabinose and enzyme is introduced. The qualitative results are summarized in Table 3.1.

Table 3.1. Qualitative results (reaction/no reaction) in the FAE-catalysed esterification of arabinose (Ara) with FA or SA in an 85:15% (v/v) IL-MES-NaOH buffer (pH 6.0) system. Reaction time (days, d) is given in parenthesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate(s)</th>
<th>IL system</th>
<th>[C2OHIMim][PF6]</th>
<th>[BMim][PF6]</th>
<th>[C3OMim][PF6]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnFaeA</td>
<td>SA + Ara</td>
<td>Reaction (6 d)</td>
<td>No reaction (2 d)</td>
<td>No reaction (8 d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FA + Ara</td>
<td>Reaction (6 d)</td>
<td>No reaction (2 d)</td>
<td>No reaction (8 d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA (no Ara)</td>
<td>Reaction (7 d)</td>
<td>-</td>
<td>No reaction (8 d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FA (no Ara)</td>
<td>Reaction (7 d)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Depol 740L</td>
<td>FA + Ara</td>
<td>Reaction (6 d)</td>
<td>No reaction (6 d)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FA (no Ara)</td>
<td>Reaction (6 d)</td>
<td>No reaction (6 d)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>No enzyme</td>
<td>SA + Ara</td>
<td>No reaction (6 d)</td>
<td>-</td>
<td>No reaction (8 d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FA + Ara</td>
<td>No reaction (6 d)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
No reaction was observed in [BMIm][PF₆] or [C₃OMIm][PF₆]. However, in [C₂OHMIm][PF₆] both AnFaeA and Depol 740L catalysed a reaction regardless of the presence of arabinose, indicating that the product is not the desired FA-Ara (or SA-Ara). The product has not been characterized, but the UV spectrum clearly shows that it is an FA (or SA) derivative, most likely C₂OHMIm⁺-FA/SA or [C₂OHMIm][PF₆]-FA/SA since the cation contains a primary hydroxyl group. This would also explain why no reaction is observed in the other IL systems. When no enzyme was present, no reaction took place, indicating that this is not a spontaneous reaction. For both AnFaeA and Depol 740L, the product concentration increased rapidly within the first 10 minutes of reaction and then kept increasing linearly at a slower rate over the 6-7 days of reaction to a conversion yield of 40-55% for AnFaeA and 7% for Depol 740L, the reaction rate being unaffected by the presence of arabinose. This indicates that both enzymes were stable and active for at least 6-7 days in the [C₂OHMIm][PF₆]-buffer system. Interestingly, no reaction with the C₂OHMIm⁺ cation was observed in the control reaction without glycerol in Paper I; this may be explained by the fact that the enzyme dosage was 90 times lower in this study (0.01% (w/w) E/S vs. 0.9% (w/w) E/S) and the reaction time shorter (24 hours was the maximum time tested in the experiments conducted for Paper I).

The concentration of the C₂OHMIm⁺ cation is 4.6 M in the 85:15% (v/v) IL-buffer system, which is a large excess compared to the 20 mM arabinose. Therefore, it was attempted to increase the arabinose concentration to 0.4 M in order to achieve a acceptor:donor ratio of 20. However, while arabinose was soluble at this concentration in the buffer, it was not soluble in the IL-buffer mixtures and thus precipitated within 24 hours. This underlines the importance of meticulous solvent screening for saccharide esterification reactions as outlined in Paper II. Apart from not contributing to any formation of FA-Ara or SA-Ara, the precipitation of arabinose also caused much lower yields of the cation-FA/SA product, possibly due to co-precipitation of the enzyme. In the IL-buffer system used in Paper I the concentration of the C₂OHMIm⁺ cation was 3.6 M, whereas the glycerol concentration was 2.5 M. Glycerol does however contain two primary hydroxyl groups, meaning that the concentration of primary hydroxyl groups was 5 M, thus presenting an excess compared to the solvent cation. In this system, no reaction between the solvent cation and SA was observed. It is possible that glycerol is a better acceptor than arabinose, e.g. due a variation in binding energies or nucleophilicity. Indeed, Tsuchiyama et al. (2006) reported 23 times higher yields in the feruloylation of glycerol in a glycerol-rich system with DMSO as a co-solvent than in the feruloylation of arabinose and xylose in saturated sugar solutions with DMSO as a co-solvent when using an FAE from a commercial pectinase blend from A. niger, which was similar to AnFaeA (it has not been shown that the two enzymes are identical). The concentration of glycerol was only 2-4 times higher than the concentrations of arabinose or xylose (Tsuchiyama et al. 2006), indicating that glycerol may be a better acceptor than the monosaccharides. However, it can also be hypothesized that aₜ was higher in the saturated sugar solutions than in the glycerol system. Unfortunately, aₜ was not monitored (Tsuchiyama et al. 2006).
Furthermore, it was attempted to use the methyl esters MFA and MSA as donors in the 
[C$_2$OHIm][PF$_6$]-buffer system in case arabinose would be a better acceptor in a 
trans-esterification. A large number of FAE-catalysed trans-esterification of saccharides with 
ferulate alkyl esters have been reported (Table 1A, Paper II). However, Depol 740L has only 
been used in direct esterifications (Table 1A, Paper II; Couto et al. 2010; Couto et al. 2011), and 
AnFaeA has been shown to give higher yields in direct esterifications (Vafiadi et al. 2009). 
Indeed, the use of methyl ester donors did not alter the scenario: instead, the methyl esters were 
hydrolysed and the unwanted cation-FA/SA product formed at a rate comparable to the one 
observed when using FA or SA as donors (data not shown).

[C$_3$OMIm][PF$_6$] was included because it should be water-miscible like [C$_2$OHIm][PF$_6$] (Branco 
et al. 2002) but does not contain any hydroxyl groups. However, the produced [C$_3$OMIm][PF$_6$] 
turned out to be water-miscible only when water was present in small excess and not in the 
85:15% (v/v) system. Consequently, it was not possible to conclude whether the lack of reaction 
with arabinose was due to the fact that arabinose does not work as an acceptor or because the 
reaction could not take place in a two-phase system, where arabinose and the enzyme are 
assumed to be present in the aqueous phase while the hydroxycinnamate in the IL phase. 
Nevertheless, both AnFaeA and Depol 740L were active in the biphasic [BMIm][PF$_6$]-buffer 
system in the reaction with glycerol (cf. Chapter 2), indicating that the FAEs can work at 
interfaces, but then the solubility of glycerol in these two phases is expected to differ 
significantly from that of arabinose.

In conclusion, both AnFaeA and Depol 740L retain their activity in the IL-buffer systems, but no 
(trans-)esterification with arabinose takes place. This may be due to arabinose being a poor 
acceptor or a phase/solubility problem. In literature, only a single report of enzyme-catalysed 
saccharide (trans-)esterifications with hydroxycinnamates in IL-buffer systems exist: Recently, 
Couto et al. (2011) reported fairly successful feruloylation of arabinobiose, xylobiose, 
galactobiose, and raffinose with Depol 740L in a 97:3% (v/v) [BMIm][PF$_6$]-MES-NaOH buffer 
system, albeit with low yields (1-4% conversion), indicating that the 15% (v/v) buffer used here 
may only be an optimum for feruloylation/sinapoylation of glycerol in a system with the 
water-miscible [C$_2$OHIm][PF$_6$] using AnFaeA (Vafiadi et al. 2009; Paper I). Otherwise, these 
reactions have only been reported in surfactantless microemulsions, organic solvents, or nearly 
solvent-free systems (cf. Tables 1A and 1B, Paper II).

Importantly, the reaction with the solvent cation underlines the fact that the FAE acceptor 
specificity is broad in non-conventional media. Indeed, a number of saccharides and saccharide 
derivatives (Tables 1A and 1B in Paper II) and both primary and secondary alcohols (Table 2B in 
Paper II; Vafiadi et al. 2008a; Hatzakis et al. 2003) have been used as acceptors. Although not 
useful in the current context, the fact that both AnFaeA and Depol 740L readily catalyse the 
esterification of the solvent cation with hydroxycinnamates opens new possibilities for the use
of FAEs to synthesize new compounds from biomass hydroxycinnamates by direct solvent reaction. This of course requires that the acceptor can function as a solvent while at the same time forming a relevant product when reacted with the hydroxycinnamate. From a biorefinery point of view, direct esterification using FA or SA is to be preferred over the trans-esterifications with alkyl hydroxycinnamate esters performed by Vafiadi, Topakas, and co-workers (cf. Table 1A, Paper II). Using direct esterification, the hydroxycinnamates can be used directly from the biomass following either saponification or enzymatic release of the hydroxycinnamates from the biomass matrix. So far, no reports of direct transfer of hydroxycinnamates bound to the biomass matrix to a new acceptor (other than water) exist. In the sinapoylation of glycerol in IL-buffer systems, AnFaeA more readily catalysed direct esterifications than trans-esterifications.

Similarly, successful direct esterifications of arabinose, xylose, and galactose were reported with Depol 740L (Couto et al. 2010). In conclusion, these two FAEs should be included in future syntheses of new hydroxycinnamate derivatives in non-conventional media.

**Feruloyl esterase-catalysed esterification of arabinose or xylose with hydroxycinnamates in microemulsions**

With the discouraging results obtained in the IL-buffer systems, it was decided to turn to another non-conventional solvent system, namely the surfactantless microemulsions where numerous authors have reported successful FAE-catalysed (trans-)esterification of saccharides with hydroxycinnamates as reviewed in Paper II. Among the FAEs used is Depol 740L (Couto et al. 2010; Couto et al. 2011), but not AnFaeA.

**Surfactantless microemulsions**

As described in Paper II, surfactantless microemulsions (also called detergentless microemulsions) are thermodynamically stable, optically transparent, ternary mixtures of a hydrocarbon (typically n-hexane), an alcohol, and water or aqueous buffer. Aqueous microdroplets are stabilized in dispersion in a hydrocarbon-rich continuous phase by alcohol molecules adsorbed to their surface (Keiser et al. 1979; Khmelnitsky et al. 1988; Zoumpanioti et al. 2006). Several enzymes have been found to retain stability in these systems presumably because the enzyme become entrapped in the aqueous droplets and are thus protected from organic solvent contact (Zoumpanioti et al. 2006).

Figure 3.2 shows a general sketch of a ternary phase diagram for a mixture of hexane, water, and an alcohol, e.g. 1- or 2-propanol. There is a biphasic region (A), where a mixture of the three components forms an unstable macroemulsion which will split into two phases upon standing (the composition of each phase can be determined from tie lines usually present in real ternary phase diagrams). Region B is the monophasic region, which can be further divided into three regions, namely stable, transparent microemulsions (1), H-bonded aggregates of water and
alcohol dispersed in a hexane-rich medium (2), and simple ternary solutions (3). Testing mixtures of \( n \)-hexane, water, and 2-propanol, Smith et al. (1977) found that mixtures from region 1 (microemulsion) contain water-rich droplets dispersed in the hexane-rich medium, while the mixtures in regions 2 and 3 did not. These water-rich droplets have later been shown to be crucial for enzyme activity in microemulsions (Zoumpanioti et al. 2006). Thus, while transparent and indefinitely stable like the mixtures in regions 2 and 3, the microemulsions in region 1 are physically related to the mixtures in region A in that two distinct phases exist (Keiser et al. 1979).

\[ \text{Figure 3.2. A general sketch of a ternary LLE phase diagram showing the biphasic region (A) and the monophasic region (B), which is further divided into three regions namely stable, transparent microemulsions (1), H-bonded aggregates of water and alcohol dispersed in a hexane-rich medium (2), and simple ternary solutions (3). The sketch is based on existing phase diagrams of } n\text{-hexane-2-propanol-water (Keiser et al. 1979; Khmelnitsky et al. 1988), } n\text{-hexane-1-propanol-water (Zoumpanioti et al. 2006), and } n\text{-hexane-1-butanol-water (Sugi and Katayama 1977). Dashed lines are used between regions 1, 2, and 3 since these three regions are not universally recognized as separate phases according to the phase rule, even if they do represent compositions of physically distinct character (Smith and Barden 1982).} \]

Due to their composite nature, these microemulsions can dissolve both hydrophobic and hydrophilic substrates as well as accommodate the enzyme in the aqueous microdroplets. It is evident from Figure 3.2 that the water content in a microemulsion is low; it should be sufficient to accommodate the enzyme (Zoumpanioti et al. 2006), but too much water disrupts the microemulsion structure (moving from region 1 to regions A, 2 or 3 in Figure 3.2) and may also promote unwanted hydrolysis. The main advantage of using a surfactantless microemulsion compared to a surfactant-containing reverse micellar system is that it is possible to separate the phases simply by adding more water or hydrocarbon to create a two-phase system (displacing...
the system from region B to region A in Figure 3.2) from which the enzyme can be recovered from the aqueous phase and the product from either the organic phase or the aqueous phase (depending on its properties) without the interference of large amounts of surfactant (Khmelnitsky et al. 1988).

The alcohol is the structure-inducing component working as surfactant in the n-hexane-water mixture thus eliminating the requirement for an actual surfactant (detergent). Smith et al. (1977) studied the formation of microemulsions in water-hexane mixtures with a number of different alcohols using ultracentrifugation to test whether a monophasic system was indeed a microemulsion. Testing 1- and 2-propanol, 1-, 2-, and t-butanol, 1- and t-pentanol, 1-hexanol, and 1-heptanol for their ability to form microemulsions with water and n-hexane, only 1- and 2-propanol could stabilize a microemulsion on their own, while the rest of the tested alcohols required addition of the surfactant cetyltrimethylammonium bromide (CTAB). Clear, monophasic solutions were also obtained when mixing n-hexane and water with ethanol, 2- or t-butanol, but these were not microemulsions as tested by the centrifugation method. They explained this behaviour by the miscibility of the alcohol in n-hexane and water, respectively: While ethanol, t-butanol, 1- and 2-propanol are all infinitely miscible in both water and n-hexane (a key requirement for an alcohol to work as a surfactant creating a microemulsion), ethanol dissolves preferentially in water and t-butanol preferentially in n-hexane in the presence of both phases, thus not decreasing the oil-water interfacial tension sufficiently to create a stable microemulsion (Smith et al. 1977). Nevertheless, the surfactantless microemulsions used for enzyme-catalysed feruloylation of saccharides (cf. Table 1A in Paper II) consisted of n-hexane, buffer and either t-butanol, 1-butanol or even 2-butanone, but never 1- or 2-propanol. In a recent study, Couto et al. (2010) also reported successful enzyme-catalysed feruloylation of di- and oligosaccharides in surfactantless microemulsions of n-hexane and water emulsified with 1,4-dioxane or the IL [BMIm][BF4], the latter however giving very low yields. Replacing n-hexane with [BMIm][PF6] in a microemulsion with 2-butanone and buffer gave similarly low yields (Couto et al. 2011).

The choice of the alcohol (or ketone, ether, or IL) working as a surfactant in the surfactantless microemulsions may also be important in terms of solvent reactivity. Indeed, Vafiadi et al. (2008a) used n-hexane-1-butanol-buffer and n-hexane-2-butanol-buffer microemulsions for trans-esterification of 1-butanol and 2-butanol, respectively, with hydroxycinnamate esters, thus using the ‘surfactant’ as substrate. Remarkably, Couto et al. (2010) reported successful feruloylation of arabinose, galactose, and xylose using Depol 740L in a microemulsion comprising n-hexane, 1-butanol, and buffer and did not report any interference from the presence of 5 M 1-butanol, which would however be expected to be competing with the saccharides as acceptor. Since Depol 740L readily feruloylated xylose (Couto et al. 2010), which in its natural pyranose form contains only secondary hydroxyl groups, and AnFaeA has been shown to catalyse trans-esterification of 2-butanol with MSA, albeit at much lower yields than
1-butanol (Vafiadi et al. 2008a), it was decided to synthesize an IPFA standard and analyse for it in the 2-propanol containing microemulsions (see below). However, no IPFA was detected.

**Microemulsion compositions**

In the literature reviewed in Paper II, microemulsion compositions are given as volume percentages. However, ternary phase diagrams which may be a useful guidance when choosing which compositions to work with always give the composition as mole fractions. Hence, Figure 3.3 shows the molar compositions of the microemulsions used for enzyme-catalysed feruloylation of saccharides in literature (cf. Table 1A in Paper II; Couto et al. 2011).

![Figure 3.3. Molar compositions of ternary mixtures (microemulsions) used for enzyme-catalysed feruloylation of saccharides in literature. T: n-hexane-t-butanol-buffer system used by Vafiadi et al. (2005), Vafiadi et al. (2006a), Topakas et al. (2005b), Vafiadi et al. (2007a), and Vafiadi et al. (2007b) (references 32, 90, 97, 98, and 99 in Paper II). U: n-hexane-t-butanol-buffer system used by Vafiadi et al. (2006b) (reference 93 in Paper II). V: n-hexane-2-butanone-buffer system used by Couto et al. (2010) (reference 37 in Paper II) and Couto et al. (2011). W: n-hexane-1-butanol-buffer system used by Couto et al. (2010). X: n-hexane-[BMIm][BF₄]-buffer system used by Couto et al. (2011). Y: n-hexane-1,4-dioxane-buffer system used by Couto et al. (2011). Z: [BMIm][PF₆]-2-butanone-buffer system used by Couto et al. (2011). 1-6: The six molar compositions investigated in the current work (see below).](image)

Most of the compositions cluster in the region expected from the general ternary phase diagram sketch in Figure 3.2. Obviously, the actual phase diagrams for each of the six different systems shown in Figure 3.3 will not be identical, and the operational window (i.e. the microemulsion...
region) will differ from system to system. For the current work, six different systems were chosen based on the literature and a desire to replace $n$-hexane with an IL, namely $n$-hexane-2-propanol-MES-NaOH buffer, $n$-hexane-2-butanone-MES-NaOH buffer, $n$-hexane-$t$-butanol-MES-NaOH buffer, [BMIm][PF$_6$]-2-propanol-MES-NaOH buffer, [BMIm][PF$_6$]-2-butanone-MES-NaOH buffer, and [BMIm][PF$_6$]-$t$-butanol-MES-NaOH buffer. It was considered to perform UNIFAC simulations on these systems in order to model LLE phase diagrams with the aim of being able to predict the monophasic region in the ternary phase diagram. UNIFAC parameters exist for all the compounds including [BMIm][PF$_6$], where the parameters for the imidazolium group was determined by Alevizou et al. (2009), who did in fact use them for predicting LLE in ternary mixtures of e.g. [BMIm][PF$_6$], ethanol, and water. However, due to slow communication with colleagues skilled in the art and not least the fact that the microemulsion region (1 in Figure 3.2) could not be identified by such simulations these plans were abandoned. Instead, six different molar compositions were chosen in the region expected to give monophasic mixtures including microemulsions; see Figure 3.3 and Table 3.4. These 36 systems were mixed and visually inspected in order to determine whether one or two phases were formed (Table 3.4). Titration of the $n$-hexane-buffer and [BMIm][PF$_6$]-buffer mixtures with the alcohol/ketone may have been a more sensible method, but also far more time-consuming. Unfortunately, it could not be tested whether the monophasic mixtures were in fact microemulsions or rather simple ternary mixtures due to the lack of appropriate equipment (Smith et al. 1977; Keiser et al. 1979).

**Table 3.4.** Molar compositions ($X_{\text{hexane/IL}}, X_{\text{alcohol/ketone}}, X_{\text{buffer}}$) of and number of phases (1 or 2) observed in the 38 systems initially investigated in the current work. Using system abbreviations, the systems are named e.g. A1, B4, and F5 according to the table. C-Hex and C-IL designate the $n$-hexane-2-butanone-MES-NaOH buffer and [BMIm][PF$_6$]-2-butanone-MES-NaOH buffer systems, respectively, used by Couto and co-workers (Couto et al. 2010; Couto et al. 2011). Buffer is 10 mM MES-NaOH buffer, pH 6. The system numbers (1-6) correspond to the ones shown in Figure 3.3.

<table>
<thead>
<tr>
<th>System no.</th>
<th>Molar composition</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_{\text{hex/IL}}$</td>
<td>$X_{\text{alc/lut}}$</td>
<td>$X_{\text{buffer}}$</td>
<td>2-propanol buffer</td>
<td>2-butanone buffer</td>
<td>$t$-butanol buffer</td>
<td>[BMIm][PF$_6$]-2-propanol buffer</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.7</td>
<td>0.2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.6</td>
<td>0.2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>0.4</td>
<td>0.5</td>
<td>0.1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C-Hex</td>
<td>0.36</td>
<td>0.48</td>
<td>0.16</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-IL</td>
<td>0.27</td>
<td>0.55</td>
<td>0.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The $n$-hexane-2-butanone-MES-NaOH buffer and [BMIm][PF$_6$]-2-butanone-MES-NaOH buffer systems used by Couto and co-workers (Couto et al. 2010; Couto et al. 2011) were also included in the test. All tested systems comprising $n$-hexane-2-propanol-buffer (A), $n$-hexane-$t$-butanol-buffer (C), and [BMIm][PF$_6$]-2-butanone-buffer (E) were monophasic systems, whereas all [BMIm][PF$_6$]-2-propanol-buffer (D) and [BMIm][PF$_6$]-$t$-butanol-buffer (F) systems were
biphasic. For the \textit{n}-hexane-2-butanone-buffer (B) systems, the number of phases form depended on the composition: B2 was monophasic, whereas all the other systems – including the one apparently used by Couto and co-workers as a microemulsion – appeared to be biphasic. Based on the results in Table 3.4, it was decided to work with monophasic systems only, although still including the systems used by Couto \textit{et al.} (2011).

\textit{Stability of AnFaeA and Depol 740L in microemulsions}

Stability of Depol 740L and AnFaeA in the microemulsion mixtures was tested by incubating the enzymes in all the monophasic systems in Table 3.4 (and C-Hex). Enzyme samples were then taken out after 0, 1, 24, 72, and 120 hours of incubation and tested for activity in the standard FAE activity assay. The results were somewhat crude and are thus not included here. The tendency was however the same for all A, C, and E systems: The activity of both FAEs decreased over time, indicating that they were destabilized by incubation in microemulsions for up to 120 hours. Already after 1 hour of incubation, the FAE activity had decreased for all A, C, and E system incubations. Nevertheless, none of the systems caused complete inactivation of either enzyme during incubation for up to 5 days (120 hours). The remaining activity after 120 hours was comparable for the two enzymes, both having higher remaining activities in the E systems (approx. 40\%) than in the A and C systems (approx. 20\%). In contrast, both AnFaeA and Depol 740L activity remained constant throughout incubation for 120 hours in the B2 and C-Hex systems. This indicates that the combination of \textit{n}-hexane and 2-butanone has a stabilizing effect comparing to the other microemulsion systems. No clear pattern between system composition and enzyme stability was established. For both enzymes A1 and E1 gave higher stability compared to A5 and E6, respectively, indicating that high alcohol (‘surfactant’) content was beneficial for enzyme stability. This tendency was however not seen for the C systems. From these stability tests it seemed that the \textit{n}-hexane-2-butanone-buffer (B2 and C-Hex) and [BMIm][PF6]-2-butanone-buffer systems gave higher stability than the other ternary mixtures, indicating a possible effect of 2-butanone on FAE stability. However, when AnFaeA-catalysed hydrolysis of MFA was monitored for up to 120 hours in the microemulsions rather than in aqueous medium, a much higher hydrolysis rate was observed in A1 (16\% conversion after 120 hours) compared to B2 and E1 (0.6\% and 0.7\% conversion after 120 hours, respectively). This may however be intertwined with the effect of the solvents on hydrolysis and may not directly transfer to the esterification activity in the same media.

It was attempted to investigate the stability of AnFaeA in the microemulsions by CD, but the enzyme aggregated in the microemulsion at the concentrations required for performing CD analysis (800 times higher than the concentration used for reaction), making the analysis impossible. In this light, it was tested whether the enzyme also aggregated at the concentration used in the reaction in microemulsion systems A1, B2, C1, and E1. The test was performed by observing pellet formation upon moderate centrifugation (10000 \(g\) for 2 minutes). In C1 a visible
pellet was formed, indicating enzyme aggregation (and thus inactivation) in this system. In the other microemulsion systems (A1, B2, and E1), no enzyme aggregation was detected at reaction concentrations. Vafiadi et al. (2008b) reported problems with enzyme precipitation in an n-hexane-1-butanol-water microemulsion when using commercial FAE preparations Depol 740L, Depol 670L, and Ultraflo L.

**Activity of AnFaeA and Depol 740L in microemulsions**

Based on these preliminary experiments, a representative set of ternary systems – possibly microemulsions – were chosen for feruloylation of arabinose and xylose by AnFaeA or Depol 740L, namely A1, A4, B2, C1, C4, E1, E4, C-Hex, and C-IL. Samples were taken out after 30 seconds, 18 hours, 3 days, and 5 days. No feruloylated arabinose or xylose was detected in any of the reaction systems. This was surprising since Depol 740L has previously been shown to catalyse feruloylation of both arabinose and xylose in the n-hexane-2-butanone-buffer (C-Hex) microemulsion (which did however turn out to be a biphasic mixture in the current work) reaching conversion yields of up to 7% for arabinose and 21% for xylose after 6 days of reaction when using the same acceptor:donor ratio of 1:3 (Couto et al. 2010). The only compound detected apart from unreacted FA was MFA, which was present in the A1, A4, C1, C4, E1, E4, and C-IL systems when using AnFaeA, the amount generally increasing from 30 seconds to 18 hours, but then disappearing completely (probably due to FAE-catalysed hydrolysis) after 3 days. Conversion levels (based on FA) ranged from 1% to 34%, being highest in E1 and lowest in C4. Remarkably, no MFA was detected in the n-hexane-2-butanone-water systems (B2 and C-Hex). The origin of the MFA is unknown. MFA is not detected in all systems and along with the fact that the amount increases over time in the systems where it is detected this rules out the fact that it is present in the enzyme mixture. Furthermore, the fact that no MSA is formed in the reaction with SA renders it unlikely that the MFA stems from a reaction with a methanol group in the enzyme preparation. For all reactions, negative controls without enzyme were included. In all cases, no reaction was detected in the control samples.

Since the solubilities of FA and SA are different, the latter being more hydrophobic than the former, it was also tested whether AnFaeA would catalyse the sinapoylation of arabinose in systems A2 and B2. However, no product was formed within 5 days of reaction. AnFaeA-catalysed trans-esterification was also tested in A1, B2, and E1 using MFA and arabinose as substrates, but only hydrolysis took place. AndFaeC was also tested with arabinose and FA or SA in A1 and B2, but no product was formed within 5 days of reaction.

It is possible that AnFaeA is not able to catalyse the (trans-)esterification of arabinose and xylose with hydroxycinnamates. The only monocomponent FAEs used for this type of reaction are type C FAEs (see Table 1A, Paper II). However, since Depol 740L has been reported to successfully catalyse the esterification of arabinose, xylose, and galactose in the C-Hex system (Couto et al. 2010).
2010), it is striking that no such product formation was observed in the current work. The reaction times were comparable to the ones used previously (see Table 1A, Paper II). However, due to the method employed for terminating the reaction, the samples were diluted 10 times prior to analysis. Thus, the product concentration in the diluted sample may have been too low for detection. If that is the case, the yields were however so low that the reaction would not be feasible (the RP-HPLC detects FA-derivative concentrations as low as approx. 1 µM, giving a concentration of 10 µM, i.e. 0.5% saccharide conversion, in the concentrated sample). No FA-Ara standard was available, but based on the elution of a mixture of feruloylated arabino-oligosaccharides with a DP ranging from 2 to 10 (SFAOS; Holck et al. 2011), it was expected that FA-Ara would show up on the chromatogram with clear baseline separation from the FA and MFA peaks (see Materials & Methods above).

Recently, it was shown for FAE type C from Fusarium oxysporum (FoFaeC) that the trans-esterification activity increased and the hydrolytic activity decreased when using MOPS (3-(N-morpholino)propanesulfonic acid) buffer instead of a citrate-phosphate buffer (Thörn et al. 2013). Through molecular docking studies it was hypothesized that the MOPS molecule can bind in or near the active site, possibly inducing conformational changes which alter the selectivity of the enzyme. MOPS-NaOH buffer was used in the sinapoylation of glycerol reported in Chapter 2. However, for the studies reported in the current chapter, the buffer was changed to MES-NaOH because MES (3-(N-morpholino)ethanesulfonic acid; pKa = 6.15) is more appropriate than MOPS (pKa = 7.20) at pH 6.0. It is unknown whether MES has the same effect – or whether the reported effect of MOPS will also be observed in AnFaeA or Depol 740L – but the difference in chain length between the two buffer molecules may be important for binding since this is indeed the case for the FAE substrates (cf. Chapter 1; Kroon et al. 1997; Topakas et al. 2005a). It was not investigated whether changing the buffer to MOPS-NaOH had an effect on AnFaeA or Depol 740L activity in the esterification of arabinose or xylose, since the report on the importance of MOPS for FoFaeC trans-esterification activity (Thörn et al. 2013) was published after the work reported in the current chapter had been terminated. However, Couto and co-workers also used MES-NaOH buffer in their studies, indicating that at least for Depol 740L the buffer was not determining for the lack of esterification of arabinose or xylose observed in the current work (Couto et al. 2010; Couto et al. 2011).

Couto et al. (2011) tested a number of different microemulsion mixtures (all with the same volumetric composition, 51:46:3% (v/v/v)) for the Depol 740L-catalysed feruloylation of arabinobiose, xylobiose, galactobiose, and raffinose. For galactobiose, the highest yield (26.8%) was obtained in an n-hexane-1,4-dioxane-buffer microemulsion, whereas the C-Hex system was superior for the three other saccharides. They also tested IL-containing microemulsions, but no significant feruloylation was obtained in either n-hexane-[BMIm][BF₄]-buffer, [BMIm][PF₆]-2-butanone-buffer, or [BMIm][PF₆]-1,4-dioxane-buffer. The desire to replace one of the organic solvents in the microemulsion with an IL stems from the fact that higher yields and shorter
reaction times have generally been reported when replacing a pure organic solvent with an IL as outlined in Paper II (p. 262). In this case, the substitution was however unsuccessful. Reports on enzyme catalysis in IL-containing microemulsions are scarce. Previously, biocatalysis has only been reported in IL-based microemulsions containing a conventional surfactant, *e.g.* Triton-X, Tween 20, or AOT (dioctyl sodium sulfosuccinate) (Moniruzzaman *et al.* 2008; Pavlidis *et al.* 2009). Recently, increased activity of CaLB was reported in an aqueous solution of an IL which self-aggregates to form a micellar structure, *i.e.* an IL microemulsion (Ventura *et al.* 2012). The formation of a microemulsion of [BMIm][PF₆] and water using another IL, [OMIm][Cl] (1-octyl-3-methylimidazolium chloride), as surfactant has been reported; this surfactantless IL microemulsion has however not been used for enzyme catalysis (Safavi *et al.* 2010).

**Conclusions**

As reported above, the numerous attempts to catalyse (trans-esterification of arabinose and xylose with hydroxycinnamates using FAEs and non-conventional reaction media turned out to be unsuccessful and Hypothesis 2 could not be proven. It was therefore decided to terminate the work with the FAEs and move on to another enzymatic reaction, where non-conventional solvents (now used as co-solvents) may suppress hydrolysis in order to improve reaction yields. The following work focuses on a sialidase from *T. rangeli*, which has been engineered to improve its trans-sialidase activity. However, the degree of substrate and product hydrolysis catalysed by the engineered sialidase, Tr6, is still pronounced and must be suppressed in order to enhance trans-sialylation yields.
Corrections for Paper II

Figure 1 in Paper II unfortunately contains structural mistakes. The correct version of Figure 1 is shown below.

Furthermore, in Table 1A at the bottom of p. 257 the HC(ester) is methyl ferulate, not ferulate, in the reactions with L-arabinotriose, -tetraose, -pentaose, and -hexaose (reference 99).
Chapter 4: Improvement of trans-sialylation vs. hydrolysis activity of Tr6 with co-solvents

This chapter relates to Paper III:

Introduction

Human milk oligosaccharides (HMOs) are a family of glycans that are highly abundant in human milk, but rare in milk from other mammals including bovine milk, which is the basis of most infant formula. The diverse structures, biosynthesis, metabolism, and beneficial effects of HMOs as well as their concentration in milk from various sources were recently thoroughly reviewed by Bode (2012). HMOs are composed of five monosaccharides, namely glucose, galactose, GlcNAc, fucose, and N-acetylneuraminic acid, which is often (and in the current work) just called sialic acid since it is the predominant – if not the only – form of the N- and O-substituted derivatives of neuraminic acid called sialic acids present in HMOs (Bode 2012). From these five monosaccharides, the human mammary gland produces approx. 200 different HMO structures all containing β-lactose at their reducing end (Ninonuevo et al. 2006; Bode 2012). As a group, HMOs are believed to be prebiotic, function as anti-adhesive antimicrobials keeping pathogens from adhering to the mucosal surfaces thus preventing infection, be directly involved in the modulation of the immune response, and be involved in the brain development of infants (Bode 2012).

Infant formula does not provide the neonate with HMOs because (1) the oligosaccharides found in farm animal milk are less structurally complex and present in considerably lower concentrations than oligosaccharides in human milk and (2) no other natural sources to large amounts of HMOs exist. Currently, fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are added to infant formula because of their prebiotic effect, but do not give all the beneficial effects that HMOs do. For instance, FOS and GOS do not possess the negative charge introduced by sialic acid, which is necessary for some of the positive effects exerted by HMOs (Bode 2012). Sialylated compounds constitute approx. 16% of the total oligosaccharides in human milk (Ninonuevo et al. 2006) and are presumably involved in the brain development in infants (Wang et al. 2001; Wang et al. 2003). Moreover, 3'-sialyllactose (3'-SL), which is a HMO model case compound, has been shown to inhibit the binding of cholera toxin in vitro (Idota et al. 1995) and to induce growth of various Bifidobacterium strains (Frantz 2012). Thus, the interest in producing HMOs from cheap, abundant sources is huge. Dairy side streams are obvious candidates for large-scale production of 3'-SL and other sialylated compounds from lactose,
which is abundant in whey, and the sialylated CGMP (casein glycomacropeptide), which is the soluble glycosylated casein residue produced after chymosin action on κ-casein during cheese manufacture (Figure 4.1).

**Figure 4.1.** Generalized structure of CGMP. By convention, the numbering of the 64 amino acids is based on that of κ-casein. The two main genetic variants (A and B) differ in positions 136 and 148. Amino acids marked in light grey are O-glycosylation sites (obviously, position 136 can only be glycosylated if it is a Thr residue (variant A)). Five different types of glycosylations (chains A-E) have been identified and their relative distribution is indicated in parentheses. The glycosylations all contain N-acetyl-D-galactosamine (GalNAc) O-linked to Thr or Ser. GalNAc can then be decorated with galactose through β(1, 3)-linkages or with sialic acid through α(2, 6)-linkages. Galactose may in turn be decorated with α(2, 3)-linked sialic acid. The three (possible) phosphorylation sites are also indicated (Saito and Itoh 1992; Farrell et al. 2004; Thomä-Worringer et al. 2006).

Trans-sialidase activity catalyses the transfer of a sialic acid moiety from one glycosyl residue to another. In contrast to sialyl transferases, trans-sialidases do not require expensive sialylated nucleotides as sialyl donor substrates, but use sialylated glycosides among which CGMP is a cheap and abundantly available candidate (Buschiazzo and Alzari 2008). Recently, it was shown that an engineered sialidase from *T. rangeli*, Tr6, could be expressed at high yield in *Pichia pastoris* and used for production of 3′-SL in gram quantities using CGMP and lactose as substrates for the trans-sialidase reaction. In addition, the versatility of Tr6 with respect to transferring the sialyl group from CGMP to more complex HMO structures was demonstrated by
using lacto-N-tetraoses and lacto-N-fucopentaoses as acceptor substrates (Michalak et al. 2013). Tr6 possesses both sialidase activity and trans-sialidase activity (Figure 1.3). The enzyme is a mutant of TrSA containing six amino acid substitutions (I37L, M96V, A98P, S120Y, G249Y, Q284P), which significantly increased its trans-sialidase activity compared to the wild type. These point mutations were based on TcTS, which is similar to TrSA in amino acid sequence but has negligible hydrolytic activity (Paris et al. 2005). The interest in producing TrSA mutants rather than using wild type TcTS stems from the fact that *T. rangeli* is not pathogenic for humans, while *T. cruzi* causes the tropical parasitic disease Chagas disease (D’Alessandro and Saravia 1999; Bern et al. 2011). While the six amino acid substitutions that make Tr6 different from the wild type TrSA significantly increased the trans-sialidase activity, it did however also increase the $k_{\text{cat}}/K_m$ of the hydrolysis of 3'-SL 1.8-fold (Paris et al. 2005). Consequently, trans-sialylation yields can still be significantly improved by reducing both substrate and product hydrolysis.

For other glycosidases, strategies to improve trans-glycosylation yields include the use of high acceptor:donor ratios (Hansson and Adlercreutz 2002; Bridiau et al. 2010; Michalak et al. 2013), organic media (Ismail et al. 1999; Hansson et al. 2001; Mladenoska et al. 2008), and co-solvents such as organic solvents (Bridiau et al. 2010; Giacomini et al. 2002), glycerol-based bio-solvents (Bayón et al. 2013b; Sandoval et al. 2013), and ILs (Kaftzik et al. 2002; Lang et al. 2006; Sandoval et al. 2012a; Bayón et al. 2013a; De Winter et al. 2013). The Tr6-catalysed production of 3'-SL from CGMP and lactose has already been optimized in terms of the acceptor:donor ratio, which is as high as 25 at the optimal conditions (Michalak et al. 2013). Therefore, the current study investigates whether ILs and organic co-solvents can improve the trans-sialylation vs. hydrolysis ratio of Tr6 in the production of the HMO model compound, 3'-SL.

It has been shown that co-solvents can alter the ratio between trans-galactosylation and hydrolysis ($r_s/r_H$) both by affecting the $a_w$ of the reaction mixture (Bridiau et al. 2010), but also by effecting the selectivity of the enzyme for the competing nucleophiles (i.e. the desired acceptor and water), expressed as the selectivity factor $S$ (or $S_c$) in Equation 1 (cf. Chapter 1) (Lang et al. 2006). Some controversy exists when it comes to the mechanism by which the co-solvents alter enzyme specificity. Sandoval and co-workers hypothesized that the improved trans-galactosylation yields obtained with β-galactosidase from *Thermus thermophilus* (TTP0042) when using ILs or bio-solvents as co-solvents was linked to alterations in the enzyme’s secondary and tertiary structure (Sandoval et al. 2012a; Sandoval et al. 2013). Goldfeder et al. (2013) reported that the positive effect of some IL co-solvents on tyrosinase specificity was highly enzyme-dependent, also indicating interactions between the enzyme and the co-solvent to be responsible for the observed effect; this reaction is however not an example of a competition between synthesis and hydrolysis. In contrast, Lang et al. (2006) showed that the effect of an IL co-solvent on enzyme selectivity was acceptor-dependent and hypothesized that the IL removed hydrogen-bonded water from strongly hydrated acceptors, thus improving
the selectivity of the enzyme by modification of the acceptor hydration. In a study on BcβGal with IL co-solvents it was found that the addition of [BMIm][PF₆] to the aqueous medium decreased the distance between the reactive hydroxyl group on the acceptor and the nucleophilic Glu residue in the active site, thus increasing the reactivity with the glycosyl acceptor. In addition, the presence of [BMIm][PF₆] increased the binding energy between the enzyme and the substrate, particularly by increasing their electrostatic interaction (Bayón et al. 2013a). Furthermore, the effect on enzyme selectivity also depends on the co-solvent nature (Bridiau et al. 2010; Sandoval et al. 2012a; Sandoval et al. 2013; Bayón et al. 2013a; Goldfeder et al. 2013).

For the current work, it is hypothesized that:

- Trans-sialylation can be enhanced relative to hydrolysis in aqueous media by using ILs or organic solvents as co-solvents (Hypothesis 4).

Based on this hypothesis, the objective of the work reported in the current chapter was to apply both organic solvents and ILs as co-solvents in order to improve trans-sialylation yields in the Tr6-catalysed production of 3'-SL from CGMP and lactose, ideally by increasing the ratio between the trans-sialidase activity and the sialidase activity.

Using six different organic solvents as co-solvents, Bridiau et al. (2010) found that t-butanol had the least detrimental effect on BcβGal activity. In fact, t-butanol stabilized the enzyme when used in concentrations around 10% (v/v); this effect was not observed for DMSO, pyridine, 2-methyl-2-butanol, acetonitrile, or acetone. Therefore, t-butanol was included as an organic co-solvent in the current study. In addition, three different ILs were tested as co-solvents: EAN, which had positive effects on tyrosinase activity (Goldfeder et al. 2013), [MMIm][MeSO₄], which had positive effects on β-galactosidase selectivity (Kaftzik et al. 2002; Lang et al. 2006), and [C₂OHMMIm][PF₆], which had the least detrimental effect on FAE stability as shown in Chapter 2 (Paper I).

**Experimental**

For Materials & Methods, please refer to Paper III.

**Results & Discussion**

When using organic solvents or ILs as co-solvents for β-galactosidase-catalysed reactions, the co-solvents may also improve the regioselectivity (Bridiau et al. 2010; Sandoval et al. 2012a; Bayón et al. 2013; Sandoval et al. 2013). This is important for β-galactosidases which (in the studied formation of LacNAc from GlcNAc and a β-galactosyl donor) catalyse the formation of
LacNAc regioisomers (allo-LacNAc and lacto-N-biose) as well as donor self-condensation products (Bridiau et al. 2011; Sandoval et al. 2012b). The formation of donor self-condensation products is not an issue in the case of trans-sialylation where the transferred sialyl group cannot act as an acceptor (cf. Chapter 1). Furthermore, Tr6 transfers sialyl groups with high regiospecificity, catalysing the formation of α(2, 3)-bonds only (Michalak et al. 2013). Indeed, only 3'-SL and sialic acid were detected as reaction products.

**Effect of using t-butanol as co-solvent**

Using the optimal reaction conditions for Tr6-catalysed 3'-SL production determined previously (Michalak et al. 2013), up to 25% (v/v) t-butanol was added to the reaction mixture. Due to product hydrolysis, the 3'-SL concentration reached a maximum after 40-60 minutes. This is commonly observed in trans-glycosylation reactions (Ismail et al. 1999; Hansson and Adlercreutz 2002; Kaftzik et al. 2002; Jers et al. 2013). Upon addition of t-butanol, the concentration of 3'-SL increased, being significantly different from the level obtained without co-solvent after 40 minutes (the optimal reaction time) when using 20-25% (v/v) t-butanol (Figure 4.2; Table 4.3). The yield on the limiting substrate, i.e. sialic acid bound in CGMP, was 23% without co-solvent and increased to 32% when using 25% (v/v) t-butanol as co-solvent.

![Figure 4.2. Formation of 3'-SL (solid symbols, solid lines) and free sialic acid (SiA; open symbols, dashed lines) catalysed by Tr6 in the presence of 0-25% (v/v) t-butanol. The values are adjusted for t-butanol evaporation during reaction and sample preparation and influence on analysis.](image)
In contrast, no effect was observed on the level of sialic acid, indicating a higher total activity (Figure 4.2). A similar effect was observed by Bridiau et al. (2010), who reported increased BcβGal activity at t-butanol concentrations around 10% (v/v). Thus, the relative increase in synthesis vs. hydrolysis ratio (i.e. the [3'-SL]/[sialic acid] ratio with co-solvent compared to the [3'-SL]/[sialic acid] ratio without co-solvent) observed after 40 minutes was mainly a result of increased trans-sialidase activity (Table 4.3). In addition, the increase in synthesis vs. hydrolysis ratio observed after 100 minutes was a result of a less pronounced product hydrolysis caused by the addition of 10-25% (v/v) t-butanol (Figure 4.2; Table 4.3). Concentrations of t-butanol below 10% (v/v) had no effect on 3'-SL or sialic acid levels (data not shown).

Table 4.3. Relative values of the [3'-SL]/[sialic acid] ratio with 10-25% (v/v) t-butanol co-solvent compared to the the [3'-SL]/[sialic acid] ratio obtained in buffer after 10-100 minutes of reaction. Bold typeface indicates significant increase in [3'-SL]/[sialic acid] ratio within each reaction time (p < 0.05). The maximum 3'-SL concentration obtained at each t-butanol concentration is indicated relative to the maximum 3'-SL concentration obtained without co-solvent (mM/mM); bold typeface indicates significant increase in the 3'-SL concentration.

<table>
<thead>
<tr>
<th>Time</th>
<th>t-Butanol concentration (%) v/v</th>
<th>10%</th>
<th>15%</th>
<th>20%</th>
<th>25%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min.</td>
<td></td>
<td>1.0 ± 0.07</td>
<td>0.9 ± 0.14</td>
<td>0.8 ± 0.04</td>
<td>1.0 ± 0.08</td>
</tr>
<tr>
<td>20 min.</td>
<td></td>
<td>1.1 ± 0.09</td>
<td>1.1 ± 0.13</td>
<td>0.9 ± 0.11</td>
<td>1.1 ± 0.08</td>
</tr>
<tr>
<td>40 min.</td>
<td></td>
<td>1.0 ± 0.08</td>
<td>1.2 ± 0.06</td>
<td>1.2 ± 0.06</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>60 min.</td>
<td></td>
<td>1.1 ± 0.13</td>
<td>1.1 ± 0.15</td>
<td>1.1 ± 0.10</td>
<td>1.1 ± 0.11</td>
</tr>
<tr>
<td>100 min.</td>
<td></td>
<td>1.4 ± 0.17</td>
<td>1.6 ± 0.17</td>
<td>1.5 ± 0.15</td>
<td>1.5 ± 0.15</td>
</tr>
<tr>
<td>Relative 3'-SL conc.</td>
<td></td>
<td>1.2 ± 0.11</td>
<td>1.1 ± 0.09</td>
<td>1.3 ± 0.10</td>
<td>1.4 ± 0.11</td>
</tr>
</tbody>
</table>

Using 25% (v/v) t-butanol, a 1.4-fold increase in the 3'-SL yield is obtained (Table 4.3). This is much lower than the approx. 7-fold increase in LacNAc yield obtained with BcβGal at 20-30% (v/v) t-butanol (Bridiau et al. 2010). However, this difference may be explained by the fact that the trans-sialylation took place at an optimized acceptor:donor ratio of 25, whereas the acceptor:donor ratio used in the reported LacNAc production was 1, thus leaving more room for improvement. Indeed, the LacNAc yield obtained with BcβGal at 20-30% (v/v) t-butanol was comparable to that obtained without co-solvent at an acceptor:donor ratio of 7-8, but lower that that obtained at an acceptor:donor ratio of 10 (Bridiau et al. 2010). In fact, when using an acceptor:donor ratio of 9.6 in the BcβGal-catalysed synthesis of LacNAc, a 1.9-fold improvement in LacNAc yield was obtained with 30% (v/v) [MMIm][MeSO₄] (Kaftzik et al. 2002) – a result which is more comparable to the increase in yield obtained here.

The 1.2-fold improvement in [3'-SL]/[sialic acid] ratio obtained at the optimal reaction time (40 minutes) with 15-25% (v/v) t-butanol (Table 4.3) is comparable to the increase in synthesis vs. hydrolysis ratio previously obtained by Lang et al. (2006) with the hyperthermostable CelB using 45% (v/v) [MMIm][MeSO₄] as co-solvent for the trans-galactosylation of a number of hydroxylated acceptors. In the BcβGal-catalysed synthesis of LacNAc from GlcNAc and lactose
the ratio was improved 2.4 times by addition of 30% (v/v) [MMIm][MeSO₄] when comparing the synthesis vs. hydrolysis ratios at the reaction times which gave optimal yields (Kaftzik et al. 2002). However, a 20-fold increase was observed when the ratios were compared at the end of the reaction (after 90 minutes) because the IL co-solvent suppressed the secondary product hydrolysis more efficiently than t-butanol did in the Tr6-catalysed production of 3’-SL (Kaftzik et al. 2002; Figure 4.2).

Using a volatile co-solvent suggests that the co-solvent may be easily removed from the reaction mixture by evaporation. However, t-butanol forms an azeotrope with water and therefore it cannot be fully removed by simple distillation (Xu and Wand 2006). However, the current process employed for recovery of 3’-SL from the aqueous reaction mixture already includes an anion exchange chromatography step, which will also remove the t-butanol from the final product preparation (Michalak et al. 2013).

**Effect of IL co-solvents**

As mentioned above, [MMIm][MeSO₄] successfully improved trans-galactosylation yields and the synthesis vs. hydrolysis ratio of both BcβGal and CelB by altering the selectivity of the enzymes and/or by suppressing hydrolysis of substrate and product (Kaftzik et al. 2002; Lang et al. 2006). However, for Tr6 0.1-1% (v/v) [MMIm][MeSO₄] had a detrimental effect on the 3’-SL yield as well as on the hydrolysis vs. synthesis ratio (Table 4.4). Using EAN and [C₂OHMIm][PF₆], an increase in co-solvent concentration to 1-2.5% (v/v) had a positive effect on the synthesis vs. hydrolysis ratio at longer reaction times (60-100 minutes; Table 4.4), mainly because the 3’-SL concentration kept increasing throughout the reaction time (data not shown).

<table>
<thead>
<tr>
<th>Time</th>
<th>[MMIm][MeSO₄]</th>
<th>[C₂OHMIm][PF₆]</th>
<th>EAN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1%</td>
<td>1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>10 min.</td>
<td>1.0 ± 0.06</td>
<td>0.5 ± 0.05</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>20 min.</td>
<td>0.9 ± 0.12</td>
<td>0.4 ± 0.07</td>
<td>0.9 ± 0.12</td>
</tr>
<tr>
<td>40 min.</td>
<td>0.6 ± 0.05</td>
<td>0.3 ± 0.04</td>
<td>0.9 ± 0.12</td>
</tr>
<tr>
<td>60 min.</td>
<td>0.8 ± 0.07</td>
<td>0.4 ± 0.04</td>
<td>1.1 ± 0.11</td>
</tr>
<tr>
<td>100 min.</td>
<td>0.7 ± 0.04</td>
<td>0.3 ± 0.02</td>
<td>1.0 ± 0.05</td>
</tr>
</tbody>
</table>

Table 4.4. Relative values of the [3’-SL]/[sialic acid] ratio with 0.1-2.5% (v/v) IL co-solvents compared to the the [3’-SL]/[sialic acid] ratio obtained in buffer after 10-100 minutes of reaction. Bold typeface indicates significant increase in [3’-SL]/[sialic acid] ratio (p < 0.05). The maximum 3’-SL concentration obtained at each solvent condition is indicated relative to the maximum 3’-SL concentration obtained without co-solvent (mM/mM).

However, this increase in the synthesis vs. hydrolysis ratio came at the expense of trans-sialylation yields. At 1% [C₂OHMIm][PF₆], the 3’-SL concentration was only 57% of that
obtained without co-solvent (Table 4.4). For EAN the effect on 3’-SL concentrations was even more detrimental: increasing the EAN concentration to 1% (v/v) and 2.5% (v/v) caused decreases in the 3’-SL level to 36% and 9%, respectively, of the level obtained without co-solvent (Table 4.4). Thus, increasing the IL co-solvent further was irrelevant. In addition, a further increase in IL concentration hampered the HPAEC analysis.

Probably, the detrimental effect of the ILs on trans-sialylation yields is linked to destabilization of Tr6 induced by the ILs. In general, enzyme stability decreases with increasing co-solvent concentration (Lang et al. 2006; Bridiau et al. 2010; De Winter et al. 2013). Furthermore, careful selection of IL co-solvents is required to avoid enzyme inactivation caused by the tendency of ILs (and especially the IL anion) to form hydrogen bonds that disrupt the secondary structure of the protein (Kaftzik et al. 2002; Zhao 2010; Goldfeder et al. 2013). NO3⁻ and MeSO4⁻ are among the anions expected to destabilize enzymes, while PF6⁻ is not (Figure 2.6). However, since this difference between the anions is not reflected in the results obtained here and since both NO3⁻ and MeSO4⁻ have been used with success for other, more thermostable enzymes, the activity loss may be related to the inherent stability of Tr6.

As mentioned in Chapter 1, thermostable enzymes are more often stable in non-conventional media including ILs (van Rantwijk et al. 1999; Reetz et al. 2010; Ferdjani et al. 2011; De Winter et al. 2013). Indeed, higher stability in [MMIm][MeSO4] was obtained with hyperthermostable CelB than with BcβGal (Kaftzik et al. 2002; Lang et al. 2006). Consequently, thermostable β-glycosidases have often been preferred for trans-glycosylation reactions in systems with IL co-solvents as well as in organic media (Hansson and Adlercreutz 2002; Lang et al. 2006; Sandoval et al. 2012a). Previously, no negative effect on the stability of the hyperthermostable TTP0042 was found when using [CPMA][MeSO4] as co-solvent in the LacNAc synthesis. Even so, the effect of the IL co-solvent on the selectivity of TTP0042 was correlated with conformational changes in the enzyme’s secondary and tertiary structure (Sandoval et al. 2012a). IL co-solvents may exert a similar effect on Tr6, but due to the lower enzyme stability the conformational changes induced by the co-solvent apparently resulted in enzyme inactivation at low IL concentrations as reflected by the lower reaction yields (Table 4.4).

As part of the CD measurements on Tr6 reported in Chapter 5, it was also attempted to determine the effect of the three ILs on Tr6 stability. CD has previously been used for determining secondary structure of TTP0042 in the presence of [CPMA][MeSO4] or the bio-solvent 1,3-dimethoxy-2-propanol (Sandoval et al. 2012a; Sandoval et al. 2013). In agreement with the results in Table 4.4, which show no significant effect on 3’-SL yields with 0.1% (v/v) IL co-solvent, no decrease in Tr6 stability was observed at this co-solvent concentration (data not shown). Unfortunately, CD spectra and thermal transitions could not be recorded at higher co-solvent concentrations due to much noise from the IL signal in the
relevant wavelength range. This was also a problem from Sandoval et al. (2012a), who did however manage to measure up to [CPMA][MeSO₄] concentrations of 5% (v/v).

Conclusions

Using Tr6-catalysed formation of HMO model case compound 3'-SL as an example, Hypothesis 4 was partially proven: Trans-sialylation was enhanced compared to the hydrolysis when using t-butanol, [C₂OHIMIm][PF₆] or EAN as co-solvents, but when using the ILs this increase took place at the expense of trans-sialylation yields. The co-solvent approach has not previously been applied to sialidases.

Considering the results by Bridiau et al. (2010), who achieved a trans-galactosylation yield with 20% (v/v) t-butanol at an acceptor:donor ratio of 1 which was comparable to the yield obtained without co-solvent at an acceptor:donor ratio of 7-8, the results obtained here indicate that the need for a high acceptor:donor ratio in the trans-sialylation may be replaced by the use of t-butanol as a co-solvent. This is especially important when using expensive acceptors such as lacto-N-tetraoses and lacto-N-fucopentaoses. Of course, this approach requires that the co-solvent can be fully removed from the final product. To avoid such complications and to obtain a greener process, albeit without obviating the need for a high acceptor:donor ratio, the next chapter investigates the potential of classical enzyme technology methods such as enzyme immobilization and the use of a membrane reactor to improve the biocatalytic productivity of Tr6 in order to enhance 3'-SL yields.
Chapter 5: Optimizing the biocatalytic productivity of Tr6 for 3'-sialyllactose production

This chapter relates to Paper IV:

Introduction

The reaction conditions for Tr6-catalysed production of 3'-SL from CGMP and lactose were optimized by Michalak et al. (2013); this included the using a high acceptor:donor ratio of 25, which significantly improved the trans-sialylation yield and lowered the extent of hydrolysis. However, as detailed in Chapter 4 the Tr6-catalysed synthesis of 3'-SL from dairy side stream components CGMP and lactose is still hampered by a high degree of substrate and product hydrolysis. As shown in Figure 4.2, the 3'-SL concentration reaches a maximum after 40-60 minutes after which it decreases due to product hydrolysis. Thus, it is crucial to control reaction conditions, not least reaction time, to minimize substrate and product hydrolysis. This chapter presents another strategy to improve trans-sialylation yields without the need for organic solvents or ILs as co-solvents. Instead, classical reaction design methods such as enzyme immobilization or the use of a membrane reactor are used to control reaction time and ensure continuous product removal – both in order to avoid extensive product hydrolysis. Using these two methods for enzyme recovery, this chapter sets out to increase the biocatalytic productivity of Tr6, i.e. mg 3'-SL per mg Tr6, with the aim of reducing enzyme cost and optimizing the reaction for future large-scale use.

It is hypothesized that:
- The biocatalytic productivity of an enzyme catalysing a synthesis reaction can be optimized by reaction time control through immobilization and/or by continuous product removal (Hypothesis 5).

Based on this hypothesis, the objective of the work presented in this chapter was to improve the biocatalytic productivity of Tr6 by enzyme recycling, i.e. by enzyme immobilization or by use of an EMR. Three different approaches to enzyme immobilization were tested, namely calcium alginate encapsulation of cross-linked Tr6, immobilization of Tr6 on Cu^{2+}-iminodiacetic acid (IDA)-functionalized carbon-coated magnetic nanoparticles (MNPs) via the His-tag, and membrane immobilization. Furthermore, the thermal stability of Tr6 and its stability at the
reaction temperature for extended reaction times were determined, since stability is a requirement for high biocatalytic productivity.

**Experimental**

For Materials & Methods, please refer to Paper IV.

**Results & Discussion**

**Calcium alginate encapsulation of cross-linked Tr6**

After cross-linking with glutaraldehyde using 1 mL glutaraldehyde per g purified Tr6 the retained trans-sialidase activity was only 33%. It is unknown whether this activity loss was due to extensive aggregation blocking the active site or a result of mass transfer limitations in the encounter between the enzyme aggregates and the relatively large CGMP donor. After encapsulation of the cross-linked Tr6 in calcium alginate only 0.1% of the original trans-sialidase activity was retained, possibly due to the fact that CGMP is too large to enter the calcium alginate network in order to gain access to Tr6. In conclusion, this was not a viable immobilization method for Tr6 in the current reaction.

**Immobilization of Tr6 on carbon-coated magnetic nanoparticles**

Superparamagnetic nanoparticles are emerging as carriers for enzyme immobilization, and a wide range of immobilization strategies have been proposed (Netto *et al.* 2013). The MNPs allow for easy separation of the immobilized enzyme from the reaction mixture, simply by applying an external magnetic field, *i.e.* a strong magnet, to the outside of the reaction vessel. In this work, the 6xHis-tag used for enzyme purification with immobilized metal affinity chromatography (IMAC) was exploited for Tr6 immobilization on carbon-coated MNPs, which were functionalized with IDA residues and loaded with Cu$^{2+}$. His-tag-facilitated immobilization of enzyme provides high specificity in the immobilization given that only one His-tagged protein is present (Herdt *et al.* 2007; Wang *et al.* 2011). It has been reported that higher activity and significantly better reusability could be obtained when using Cu$^{2+}$-IDA-functionalized silica-coated MNPs for lipase immobilization compared to Cu$^{2+}$-IDA-functionalized silica gel or silica-coated MNPs without the Cu$^{2+}$-IDA functionalization (Kim *et al.* 2009).

One requirement for successful His-tag-facilitated immobilization is that the His-tag is not positioned so that immobilization will block the active site. In Tr6, both N- and C termini are located far away from the active site (cf. PDB entries 1WCS, which is identical to Tr6 apart from a single amino acid residue (Paris *et al.* 2005) and 1N1Y, where sialic acid is bound in the active
site of the wild type sialidase from *T. rangeli* (Amaya *et al.* 2003), and it is thus unlikely that immobilization through the His-tag will block the active site entry. In Tr6, the His-tag is attached to the C-terminal. His-tag-facilitated immobilization is also a means of avoiding unspecific multipoint attachment of the enzyme to the carrier. Multipoint binding between the enzyme and the carrier surface often increases stability, but it may also significantly decrease activity due to decreased structural flexibility (Bolivar *et al.* 2007; Tardioli *et al.* 2006).

Uncoated MNPs tend to aggregate due to magnetic dipole attraction (Kim *et al.* 2009). Therefore, MNPs are usually coated *e.g.* with silica (Kim *et al.* 2009), epoxy polymers (Wang *et al.* 2011), or graphitic carbon (Grass *et al.* 2007) to create a core-shell structure. The coating furthermore makes the MNPs chemically inert towards air or acids and stable at high temperatures while at the same time providing groups for functionalization (Grass *et al.* 2007; Kim *et al.* 2009). The silica layer used for coating by Kim *et al.* (2009) was approx. 15 nm, resulting in a severe decrease in saturation magnetization value from approx. 60 emu/g to 2 emu/g. Although they were still able to separate the silica-coated MNPs from solution using an external magnet, it is obvious that such a coating will limit the separation distance and thus decreases the large-scale potential since the magnetic field of the external magnet also decreases with increasing distance. The MNPs used in this study are coated with a layer of graphitic carbon of approx. 1 nm (Grass *et al.* 2007). It is unknown whether this coating aids the MNP dispersion as well as the silica coating, but it is likely that the decrease in saturation magnetization value will be much smaller using this very thin coating. Other advantages of carbon coating over silica are that the covalent attachments used to functionalize the MNPs (in this case with IDA) are less prone to hydrolysis and that the carbon coating is more stable at harsh chemical conditions (Herrmann *et al.* 2009).

The main limitation of using MNPs for enzyme immobilization at large scale is indeed the fact that the magnetic field strength decreases with increasing distance, consequently reducing the separation efficiency then the size of the reactor increases. Thus, it is recommended to use a long reactor with a small diameter, *e.g.* a serpentine tube in which the reaction mixture is circulated. The Tr6-MNPs can then be drawn to the side of the reactor by an external magnetic field upon reaching the desired reaction time, thus being withheld in the reactor while the reaction mix containing produced 3'-SL is removed and replaced with fresh substrate solution.

### 3'-sialyllactose production with Tr6-MNPs

Purified Tr6 was immobilized on the Cu²⁺-IDA-functionalized carbon-coated MNPs with an immobilization efficiency of 94%. In a reaction time study, the 3'-SL production catalysed by Tr6 MNPs in a 35 mL working volume was followed for up to 2.5 hours and compared to the 3'-SL production achieved with free Tr6 (Figure 5.1). The dosage of free Tr6 corresponded to an immobilization efficiency of 100%, since this makes the enzyme usage similar assuming that non-immobilized Tr6 is not recycled.
Figure 5.1. Production of 3'-SL (solid symbols) and the undesired hydrolysis product, sialic acid (open symbols), with Tr6 immobilized on MNPs (circles) and free Tr6 (triangles) for up to 2.5 hours. The reaction volume was 35 mL. Reaction conditions were based on the optimization performed by Michalak et al. (2013): 117 mM lactose, 4.6 mM sialic acid bound in CGMP, pH 5.5, and 25°C.

The initial trans-sialidase rate was 2.4 times higher for free Tr6 compared to the Tr6-MNPs, giving higher 3'-SL yields at reaction times up to 20 minutes with free Tr6 (Figure 5.1). However, the rate of hydrolysis was also much higher for free Tr6 as evidenced by the higher yields of free sialic acid (Figure 5.1). Consequently, the level of 3'-SL produced with free Tr6 reached a maximum of 1.30 mM after 40 minutes before decreasing due to product hydrolysis (Figure 5.1). In contrast, the 3'-SL level obtained with the MNP-immobilized Tr6 kept increasing until reaching a maximum at 1.85 mM before decreasing due to product hydrolysis after 60 minutes of reaction (Figure 5.1). In fact, the ratio between the transfer reaction rate (trans-sialidase activity) and the hydrolysis rate (sialidase activity) was significantly higher for the Tr6-MNPs than for the free enzyme, resulting in a more desirable product composition and higher yields of the desired product, 3'-SL. Comparing the [3'-SL]/[sialic acid] ratio at the optimal reaction time for the Tr6-MNPs (60 minutes) to the ratio obtained at the optimal reaction time with free Tr6 (40 minutes), a 2.1-fold increase was achieved. The optimal reaction time was shorter for free Tr6, but since the product yield was higher for the Tr6-MNPs, no statistically significant difference was found between the biocatalytic productivity rates, i.e. mg 3'-SL/(mg Tr6 ∙ minute). Moreover, since Tr6 is an expensive enzyme the objective was to increase biocatalytic productivity rather than reduce reaction time. In conclusion, MNP-immobilized Tr6 was more efficient in the 3'-SL production than free Tr6 without even being reused: The biocatalytic
productivity was 33.5 mg 3'-SL/mg Tr6 for free Tr6 and 47.6 mg 3'-SL/mg Tr6 for the Tr6-MNPs after 40 minutes and 60 minutes of reaction, respectively (see Table 5.4 below).

**Recyclability of Tr6-MNPs**

Theoretically, only negligible amounts of Tr6 should be released from the carrier due to strong interactions between the His-tag and the Cu$^{2+}$-IDA complex as well as between Cu$^{2+}$ and IDA (Shnek *et al.* 1994; Wang *et al.* 2011). Using the same immobilization strategy, retained activities of 70-80% after 6-8 reaction cycles have been obtained (Kim *et al.* 2009; Wang *et al.* 2011). Unfortunately, the recyclability of Tr6 was not comparable when tested in seven consecutive 60-minute reaction runs. Already in the second reaction run, the 3'-SL level reached only 46% of the level in the first reaction run. The trans-sialidase activity of Tr6 kept decreasing over the 7 hours of reaction, and in the seventh reaction run the 3'-SL yield was merely 7% of that obtained in the first 60-minute reaction run (see Figure 3 in Paper IV for a graphic view of the data). Between each reaction run, the reaction mixture was replaced with fresh substrate while the Tr6-MNPs were withheld in the batch reactor with an external magnet. Although no MNPs were removed between the reaction cycles, it is possible that some Tr6 may have been lost from the carrier during the reaction, thus being removed at the end of each reaction cycle. To test this hypothesis, the experiment was repeated now including a wash of the Tr6-MNPs between each reaction run. 3-7% of the initial trans-sialidase activity was detected in each of the washing water batches, the amount of activity lost decreasing over time. This does not explain the entire loss of activity, but it is plausible that some Tr6 is also lost in the reaction mixture. To keep the pH at the optimal condition (5.5) a citrate buffer was used in all the reactions. IDA is a much stronger chelator of Cu$^{2+}$ than citrate (Nesterenko and Jones 1997), but the use of citrate may still account for some of this loss. Thus, replacing the buffer may improve the recyclability of the Tr6-MNPs. Nevertheless, the Tr6-MNPs significantly improved the yield of 3'-SL compared to free Tr6 already in the first reaction run (Figure 5.1), indicating that it may be worthwhile to look for other immobilization methods giving a more stable immobilization of Tr6.

**Integration of His-tag-facilitated purification and immobilization**

His-tag-facilitated enzyme immobilization offers the possibility of integrating the immobilization with the IMAC purification. This procedure was successfully used for immobilizing epoxide hydrolase from a cell-free *P. pastoris* extract on poly(glycidyl methacrylate)-coated MNPs (Wang *et al.* 2011). The immobilization on carbon-coated MNPs of crude Tr6, *i.e.* Tr6 from an ultrafiltered cell-free *P. pastoris* extract, and IMAC purified Tr6, respectively, was compared in the current work. The purification yield in the IMAC purification was 84%, meaning that 1.2 times more enzyme is used when immobilizing purified enzyme. However, the trans-sialidase activity obtained when immobilizing crude Tr6 was about half of that obtained with purified Tr6 (data not shown). The decay in immobilized activity over seven consecutive reaction runs was
comparable for the two enzyme preparations (data not shown). Thus, it follows that it is not advisable to integrate enzyme purification and immobilization when using the enzymatic reaction system employed in the current work. Possibly, components in the cell-free extract block the immobilization, even if His-tag-facilitated immobilization is known to be highly specific (Herdt et al. 2007; Wang et al. 2011). Indeed, the immobilization efficiency obtained in these tests was approx. 70% for crude Tr6 and approx. 100% for purified Tr6.

**Tr6 in an enzymatic membrane reactor**

EMRs allow continuous or semi-continuous product removal whilst retaining the enzyme in the reactor and have successfully improved the biocatalytic productivity in reactions where product inhibition severely reduces product yields (Andric et al. 2010; Gavliga et al. 2013). Similarly, a reduction in 3'-SL yield due to product hydrolysis by Tr6 (Figure 1.3) may be avoided by continuously removing 3'-SL from the EMR while confining Tr6 to the reactor thus enabling biocatalysis for extended reaction times, which will in turn improve the biocatalytic productivity. Tr6 can be free in the reactor or it can be immobilized on the membrane. The latter often results in enzyme stabilization (Rios et al. 2004; Luo et al. 2013).

**Figure 5.2.** Concentrations of 3'-SL (dark grey) and free sialic acid (light grey) in permeate after each of the seven consecutive 60-minute reaction cycles and in the retentate after the seventh cycle when using free Tr6 in the EMR equipped with a 10 kDa regenerated cellulose membrane. Fresh substrate was added between each cycle. New permeate was collected during each run, i.e. the yields are not cumulative. Average pressure applied in each cycle to keep the reaction times and the permeate volumes identical in each reaction cycle is indicated (triangles; right-hand axis). Lowercase letters indicate significant difference between permeate 3'-SL levels ($p < 0.05$).
Free Tr\textsubscript{6} was used in a small EMR equipped with a 10 kDa regenerated cellulose membrane at a working volume decreasing from 6 mL to 1 mL over each of the seven consecutive 60-minute reaction cycles. After each cycle, 5 mL fresh substrate was added to the reactor. The concentration of 3'-SL in the permeate was 1.33 mM in the first 60-minute cycle, but then increased to approx. 1.8 mM in the third cycle and remained constant at this level throughout the seven cycles (Figure 5.2). The 3'-SL yields were thus comparable to that obtained in the first reaction run with the Tr6-MNPs, but higher than that obtained with free Tr6 in a batch reactor (Figure 5.1), indicating that the continuous product removal employed in the EMR has a marked effect on product yields.

Since CGMP was retained in the reactor while lactose was not, the acceptor:donor ratio decreased over time in each reaction cycle thus gradually favouring substrate hydrolysis. Furthermore, fresh substrate was added to the reactor between each cycle, leading to a build-up of CGMP in the reactor. This is reflected by the permeate concentration of free sialic acid, which kept increasing throughout the seven hours of reaction, and by the high concentration of free sialic acid in the retentate (Figure 5.2). Consequently, the filtration resistance exerted by CGMP was severe as evidenced by the increase in required, applied pressure (Figure 5.2). Even so, the concentration of 3'-SL in the retentate was desirably low, indicating that 3'-SL was not rejected by the CGMP layer on the membrane (Figure 5.2). Build-up of CGMP may indeed be the most important problem to overcome in large-scale production of 3'-SL or other sialylated compounds. Fortunately, the CGMP was easily removed by simple washing, and the membrane regained full permeability. Furthermore, CGMP fouling may be decreased in a different filtration setup, e.g. cross-flow filtration. Preferably, the EMR must be operable for extended reaction times since recovery of Tr6 from the reaction mixture will require a new IMAC purification.

**Membrane immobilization of Tr6**

Recently, Luo et al. (2013) investigated fouling-induced enzyme immobilization and found that using the membrane with the support layer up, i.e. reverse mode, gave a much higher enzyme loading than the normal mode, i.e. skin layer up. The reverse mode was tested for the current work in a preliminary study, but CGMP fouling was so severe that reasonable amounts of permeate could not be obtained within the desired reaction time. Consequently, reverse mode membrane immobilization could not be used in the current reaction. Employing fouling-induced immobilization of Tr6 on the 10 kDa regenerated cellulose membrane in normal mode, the immobilization efficiency was only 35%. Furthermore, as expected from the results obtained by Luo et al. (2013) immobilization in normal mode was reversible, i.e. some of the enzyme diffused back into the bulk solution during reaction. Thus, the main advantage of normal mode membrane immobilization of enzymes compared to the use of free enzyme in the EMR is that the enzyme may be stabilized on the membrane (Rios et al. 2004; Luo et al. 2013). However, as reported below, Tr6 is quite stable. Therefore, membrane immobilization is not to be favoured
over the use of free Tr6 in the EMR, not least due to the low immobilization efficiency (35%) and the requirement of an extra process step (immobilization).

**Stability of Tr6**

The fact that the 3'-SL yield was stable during the seven hours of reaction in the EMR indicated that free Tr6 was stable throughout the reaction. Indeed, it was found that free Tr6 retained full trans-sialidase activity for at least 24 hours at the reaction temperature, 25°C. Furthermore, thermal stability of Tr6 was tested by incubation for up to 100 minutes; the results showed that Tr6 was also completely stable at 45°C for at least 100 minutes as indicated by \( k_0 = 0 \) (Table 5.3; see Figure 5 in Paper IV for a graphic view of the data). At 55°C, the remaining activity after 100 minutes of incubation was 16% \( (k_0 = 0.019) \), and at 65°C the remaining activity was only 2% after 5 minutes of incubation \( (k_0 = 0.8057) \). The temperature at the midpoint of denaturation, \( T_m \), where half of the enzyme molecules are unfolded, was determined by CD to 57.2°C (cf. Figure 6 in Paper IV for CD spectra and thermal transition of Tr6; CD data on Tr6 have not previously been published).

**Table 5.3.** Thermal stability of Tr6: Thermal inactivation constants, \( k_0 \), of Tr6 when incubated in 10 mM citrate buffer (pH 5.5) at 45°C, 55°C, 60°C, and 65°C for up to 100 minutes. See Figure 5 in Paper IV for a graphic view of the data. \( R^2 \) values were larger than 0.91 where \( k_0 \neq 0 \). Superscript letters indicate significant difference between the values \( (p < 0.05) \).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>( k_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>45°C</td>
<td>0\textsuperscript{a}</td>
</tr>
<tr>
<td>55°C</td>
<td>0.0190\textsuperscript{b}</td>
</tr>
<tr>
<td>60°C</td>
<td>0.1339\textsuperscript{c}</td>
</tr>
<tr>
<td>65°C</td>
<td>0.8057\textsuperscript{d}</td>
</tr>
</tbody>
</table>

In conclusion, the thermal stability of Tr6 suggests that the reaction temperature could be elevated to increase reaction rates. This is corroborated by the results obtained by Michalak et al. (2013) in the optimization of Tr6 reaction conditions, where 25°C was in fact the highest temperature included in the optimization study. These results obtained by Michalak et al. (2013) for Tr6 are in contrast to the ones obtained for TcTS by Ribeirão et al. (1997), who found that increasing the reaction temperature above 13°C decreased the ratio between the trans-sialidase activity and the hydrolytic activity for TcTS. Consequently, the effect of increasing the reaction temperature of Tr6 above 25°C needs to be tested in order to ensure maximal 3'-SL yields.

**Biocatalytic productivity and molar yield on sialic acid donor**

Biocatalytic productivities were calculated for MNP-immobilized Tr6 – the most promising of the immobilization methods – and for free Tr6 in the EMR and compared to the biocatalytic productivity of free Tr6 in the batch reactor (Table 5.4). Immobilizing Tr6 on the MNPs

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increased the biocatalytic productivity 2.5-fold from 33.5 to 84.1 when using the Tr6-MNPs for seven consecutive runs. However, due to the loss of Tr6 from the MNPs, the biocatalytic productivity after seven reaction runs was not significantly higher than that obtained after four runs (Table 5.3). Confining free Tr6 in the EMR caused a dramatic increase in biocatalytic productivity, as Tr6 was completely stable at the reaction temperature and kept producing a high level of 3'-SL throughout the seven consecutive reaction runs, resulting in a biocatalytic productivity of 305.6 (Table 5.3). Thus, using free Tr6 in the EMR gave a more than 9-fold increase in biocatalytic productivity.

In the current reaction, CGMP – or rather the sialic acid covalently bound to CGMP – is the limiting substrate. The molar yield on the limiting substrate was 37% after seven consecutive 60-minute reaction cycles in the EMR, i.e. the reactor setup giving the highest biocatalytic productivity in the current study, which represents a significant increase from the 28% obtained in the batch reaction. As mentioned in the introduction, the mutations introduced in the wild type TrSA to increase its trans-sialidase activity (thus yielding Tr6) were based on the amino acid sequence of TcTS. This trans-sialidase catalyses the transfer of α(2, 3)-bound sialic acid, but does not transfer α(2, 6)-bound sialic acid. Furthermore, Tr6 D363E (i.e. a mutant of Tr6 with a single amino acid substitution compared to the parent enzyme, Tr6) exhibited hydrolytic activity on 3'-SL, but not on 6'-sialyllactose (Jers et al. 2013). Based on this, it can be speculated that Tr6 is also specific for α(2, 3)-bound sialic acid only. If that is the case, the molar yields on the limiting substrate, the sialic acid donor, can be doubled since only 50% of the sialic acid present in CGMP is α(2, 3)-bound (Saito and Itoh 1992; Figure 4.1). In other words, the molar yield on α(2, 3)-bound sialic acid in CGMP was 74% with free Tr6 in the EMR.

**Table 5.4.** Biocatalytic productivity (mg 3'-SL/mg enzyme) and molar yield on sialic acid in CGMP obtained with free Tr6, MNP-immobilized Tr6 (Tr6-MNPs), and free Tr6 in the EMR. The enzyme loading was 1.4% E/S (w/w) based on sialic acid bound in CGMP for free Tr6 and approx. 1.3% E/S (w/w) for Tr6-MNPs (immobilization efficiency: 94%). The reaction time was 40 minutes for free Tr6 in batch reactor and 60 minutes in each run for both Tr6-MNPs and free Tr6 in the EMR. Adjusted for purification yield (84%). Superscript letters indicate whether the numbers are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Biocatalytic productivity [mg 3'-SL/mg Tr6]</th>
<th>Molar yield on sialic acid donor [mol 3'-SL/mol sialic acid in CGMP]·100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Tr6</td>
<td>33.5\textsuperscript{a}</td>
<td>28%\textsuperscript{f}</td>
</tr>
<tr>
<td>Tr6-MNPs (1 run)</td>
<td>47.6\textsuperscript{b}</td>
<td>40%\textsuperscript{c}</td>
</tr>
<tr>
<td>Tr6-MNPs (4 runs)</td>
<td>73.3\textsuperscript{c}</td>
<td>15%\textsuperscript{h}</td>
</tr>
<tr>
<td>Tr6-MNPs (7 runs)</td>
<td>84.1\textsuperscript{c}</td>
<td>10%\textsuperscript{i}</td>
</tr>
<tr>
<td>Free Tr6 in EMR (1 run)</td>
<td>34.3\textsuperscript{a}</td>
<td>29%\textsuperscript{f}</td>
</tr>
<tr>
<td>Free Tr6 in EMR (4 runs)</td>
<td>164.7\textsuperscript{d}</td>
<td>35%\textsuperscript{c}</td>
</tr>
<tr>
<td>Free Tr6 in EMR (7 runs)</td>
<td>305.6\textsuperscript{e}</td>
<td>37%\textsuperscript{c}</td>
</tr>
</tbody>
</table>


Conclusions

The results reported in this chapter clearly corroborate Hypothesis 5: The biocatalytic productivity could be improved by immobilization of Tr6 and especially through the use of free Tr6 in an EMR allowing continuous product removal to minimize product hydrolysis.

Encapsulation of cross-linked Tr6 in calcium alginate resulted in immediate loss of enzyme activity, and membrane immobilization suffered from low immobilization efficiency. In contrast, Tr6 was immobilized via its His-tag on carbon-coated MNPs with high efficiency. Remarkably, this immobilization improved the ratio between the trans-sialidase activity and the sialidase activity of Tr6, thus giving higher 3’-SL yields already in the first reaction run. Unfortunately, the recyclability of the Tr6-MNPs was low due to loss of enzyme from the carrier.

Using free Tr6 in the EMR turned out to be superior to the use of immobilized Tr6, not least due to the high stability of free Tr6. Furthermore, using EMRs is simple and no immobilization step is required. In the EMR, a biocatalytic productivity of 306 mg 3’-SL per mg Tr6 was obtained after seven consecutive 60-minute reaction cycles, corresponding to a more than 9-fold increase compared to the use of free Tr6 in a batch reactor. This underlines the importance of continuous product removal in a reaction catalysed by a trans-glycosidase which also hydrolyses its own product, as well as the importance of enzyme recovery in enzymatic reactions in general.
Chapter 6: General conclusions and perspectives

Main results: Activity and stability in enzyme-catalysed synthesis

In the quest to investigate various methods for improving activity in enzyme-catalysed synthesis reactions, varying activity results were obtained with FAEs and Tr6 in the many different reaction systems tested (Table 6.1).

Table 6.1. Overview of the enzyme activity results obtained. Maximum yield on donor (i.e. CGMP or hydroxycinnamate) obtained at optimal conditions in each system is indicated. Ch. is short for chapter.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Reactions</th>
<th>Results</th>
<th>Yield on donor</th>
<th>Ch.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnFaeA</td>
<td>IL-buffer-glycerol</td>
<td>Sinapoylation of glycerol (esterification)</td>
<td>Significant esterification in PF$_6$ systems (higher in [C$_2$OHMIm][PF$_6$] than in [BMIm][PF$_6$]); inactivation in BF$_4$ systems</td>
<td>21%</td>
<td>2</td>
</tr>
<tr>
<td>AndFaeC</td>
<td>IL-buffer-glycerol</td>
<td>Sinapoylation of glycerol (esterification)</td>
<td>Rapid inactivation in all systems</td>
<td>1%</td>
<td>2</td>
</tr>
<tr>
<td>Ultraflo L</td>
<td>IL-buffer-glycerol</td>
<td>Sinapoylation of glycerol (esterification)</td>
<td>Negligible activity in [BMIm][PF$_6$]; rapid inactivation in all other systems</td>
<td>1%</td>
<td>2</td>
</tr>
<tr>
<td>Depol 740L</td>
<td>IL-buffer-glycerol</td>
<td>Sinapoylation of glycerol (esterification)</td>
<td>Low activity in [BMIm][PF$_6$], none in [C$_2$OHMIm][PF$_6$]</td>
<td>3%</td>
<td>2</td>
</tr>
<tr>
<td>Depol 740L</td>
<td>IL-buffer-glycerol</td>
<td>Feruloylation of glycerol (esterification)</td>
<td>Higher activity in [BMIm][PF$_6$] than in [C$_2$OHMIm][PF$_6$]</td>
<td>11%</td>
<td>2</td>
</tr>
<tr>
<td>AnFaeA</td>
<td>IL-buffer</td>
<td>Feruloylation or sinapoylation of arabinose ((trans)-esterification)</td>
<td>No FA/SA-Ara formed; esterification of C$_2$OHMIm$^+$; in trans-esterification, only methyl ester hydrolysis occurred</td>
<td>0% (40-55% with solvent)</td>
<td>3</td>
</tr>
<tr>
<td>Depol 740L</td>
<td>IL-buffer</td>
<td>Feruloylation of arabinose ((trans)-esterification)</td>
<td>No FA-Ara formed; esterification of C$_2$OHMIm$^+$; in trans-esterification, only methyl ester hydrolysis occurred</td>
<td>0% (7% with solvent)</td>
<td>3</td>
</tr>
<tr>
<td>AnFaeA</td>
<td>Micro-emulsions</td>
<td>Feruloylation or sinapoylation of Ara or Xyl (esterification)</td>
<td>No FA/SA-Ara or FA/SA-Xyl formed</td>
<td>0%</td>
<td>3</td>
</tr>
<tr>
<td>Depol 740L</td>
<td>Micro-emulsions</td>
<td>Feruloylation of Ara or Xyl (esterification)</td>
<td>No FA-Ara or FA-Xyl formed</td>
<td>0%</td>
<td>3</td>
</tr>
<tr>
<td>AndFaeC</td>
<td>Micro-emulsions</td>
<td>Feruloylation or sinapoylation of arabinose (esterification)</td>
<td>No FA/SA-Ara or FA/SA-Xyl formed</td>
<td>0%</td>
<td>3</td>
</tr>
<tr>
<td>Tr6</td>
<td>Aqueous, t-butanol co-solvent</td>
<td>Trans-sialylation of lactose (donor: CGMP)</td>
<td>Increased trans-sialylation; increased synthesis/hydrolysis</td>
<td>32%</td>
<td>4</td>
</tr>
<tr>
<td>Tr6</td>
<td>Aqueous, IL co-solvent</td>
<td>Trans-sialylation of lactose (donor: CGMP)</td>
<td>Increased synthesis/hydrolysis; lower activity</td>
<td>18%</td>
<td>4</td>
</tr>
<tr>
<td>Tr6</td>
<td>Aqueous</td>
<td>Trans-sialylation of lactose (donor: CGMP)</td>
<td>Trans-sialylation improved by continuous product removal in EMR or by immobilization</td>
<td>37%</td>
<td>5</td>
</tr>
</tbody>
</table>
Furthermore, both FAEs and Tr6 have been analysed for stability in various solvent systems. As shown in Table 6.1, the stability of AndFaeC and Ultraflo L was low during the reaction in IL-buffer systems. Therefore, only AnFaeA was thoroughly analysed for thermal stability in the IL-buffer systems in Chapter 2. Furthermore, AnFaeA stability in aqueous medium (buffer) was determined both kinetically ($k_0$) and thermodynamically ($T_m$) (Chapter 2). In addition, crude results of AnFaeA and Depol 740L stability in microemulsions were obtained in Chapter 3. Finally, kinetic and thermodynamic stability of Tr6 was determined in aqueous medium (Chapter 5).

Among the results obtained with FAEs, the results of main importance are (1) that AnFaeA could catalyse sinapoylation of glycerol in both monophasic and biphasic systems, (2) that AnFaeA showed full stability in PF$_6$- systems for more than 2 hours at 30-40°C (mainly due to its lipase-like structure), and (3) that the acceptor specificity of both AnFaeA and Depol 740L is so wide that they appear to catalyse the esterification of the solvent cation C$_2$OHMIm$^+$ in the absence of a better acceptor such as glycerol. From the results it is clear that in order to use FAEs for synthesis reactions, both the FAE as well as the $\alpha$-lowering reaction system must be selected carefully in order to achieve adequate enzyme activity and stability. Thermodynamically based tools such as COSMO-RS may assist in this selection, even if the use of these tools in general is limited by lack of data and/or their quantitative inaccuracies.

With Tr6 the novelty value is higher because less work has been done with this class of enzymes and with this engineered enzyme in particular. The results of main importance are (1) that trans-sialylation could be improved by addition of 20% (v/v) $t$-butanol, (2) that immobilization improved the synthesis/hydrolysis ratio of Tr6 as well as the trans-sialylation yield and the biocatalytic productivity, and (3) that the most promising enzyme recovery method was the confinement of Tr6 to an EMR, which increased the trans-sialylation yield compared to the batch reactor and improved the biocatalytic productivity of Tr6 more than 9-fold.

Where the use of non-conventional media is required for catalysis, e.g. in the thermodynamically controlled FAE-catalysed esterification, careful selection of both solvent system and the FAE itself is required to obtain adequate reaction yields (Chapters 2-3). In contrast, for Tr6 the best results were obtained when keeping the reaction in aqueous medium and employing other reaction design methods such as continuous product removal and enzyme immobilization (Chapters 4-5).

**Usability of ILs in non-lipase systems**

While reports of successful reactions catalysed by proteases, glycosidases, esterases, peroxidases and laccases in IL systems exist, most of the successful biocatalytic reactions performed in IL systems have been catalysed by lipases (Yang and Pan 2005; van Rantwijk and...
Lipases are generally very tolerant to anhydrous solvent systems and do not require as high a $a_w$ for activity as glycosidases do (Ma et al. 2002; van Rantwijk and Sheldon 2007). Furthermore, lipases naturally work at interfaces (Reis et al. 2009) and are thus well suited for mixtures of water and water-immiscible solvents such as hydrophobic ILs, while other enzymes may experience interfacial inactivation (MacRitchie 1978). Thus, lipases are ideal for working in non-conventional media. However, in order to widen the field of applications, this thesis studied the use of IL systems for both FAEs and sialidases. Indeed, AnFaeA was superior in the IL systems, probably due to its structural similarity to IL-compatible fungal lipases (Chapter 2). The engineered sialidase, Tr6, did not tolerate the ILs well; in this case, t-butanol was a better co-solvent (Chapter 4). Although not an option here, the use of hyperthermostable glycosidases does however seem to minimize stability issues in IL-containing media (cf. Chapter 1; Ferdjani et al. 2011). Recently, a thermostable mutant of AnFaeA was produced exhibiting a more than 267-fold increased half-life at 55°C (Zhang et al. 2012). Possibly, this AnFaeA mutant will be even more suitable for use in non-conventional media than the wild type AnFaeA.

Sheldon and co-workers have reported numerous examples of the potential of cross-linked enzyme aggregates (CLEAs) for increasing enzyme-stability in non-conventional media including IL systems (López-Serrano et al. 2002; van Rantwijk et al. 2006; Toral et al. 2007). CLEAs are formed by precipitating enzyme by addition of salts (e.g. ammonium sulfate), water-miscible organic solvents (e.g. methanol, ethanol, or t-butanol), or non-ionic polymers (e.g. poly(ethylene glycol), PEG) and subsequently cross-linking the precipitated enzyme with a polyaldehyde, most often glutaraldehyde (Cao et al. 2000; Sheldon 2007; Vafiadi et al. 2008a). For AnFaeA catalysing sinapoylation of glycerol in an IL system with 15% (v/v) buffer similar to the one used in the current work, CLEA formation did not affect activity, but the CLEAs could be reused in five consecutive 24-hour reaction cycles before losing all activity (Vafiadi et al. 2009). However, using AnFaeA in an n-hexane/1-butanol/water microemulsion, the conversion yield was lowered compared to that obtained with free AnFaeA. The AnFaeA-CLEAs were easily recovered by brief centrifugation, but could only be re-used once (Vafiadi et al. 2008a). The use of CLEAs of commercial FAE preparations Ultraflo L, Depol 740L, and Depol 670L dramatically improved the conversion yields obtained in a similar microemulsion system, mainly because the enzyme precipitation observed with the free enzymes in this system was circumvented (Vafiadi et al. 2008b). Possibly, the CLEA technology can also be used for increasing the stability of glycosyl hydrolases in IL co-solvent systems. Indeed, it was recently shown that immobilized sucrose phosphorylase had a markedly increased stability in a system with 20% (v/v) IL co-solvent compared to free sucrose phosphorylase, especially when immobilized with the CLEA technique (De Winter et al. 2013).

In conclusion, IL systems are not as easily applicable to FAEs and glycosidases as to lipases, mainly because these enzymes are less tolerant to non-conventional media. However, with careful selection of both solvent and enzyme successful catalysis can be achieved.
Product recovery

While this thesis is more concerned with the synthesis reaction themselves than with their products, some consideration must be given to product recovery from the various reaction systems investigated.

Product recovery from IL-buffer systems is heavily influenced by whether the IL is water-miscible or water-immiscible. Water-immiscible ILs can be recovered from the reaction mixture by simple centrifugation (Sandoval et al. 2012a). However, commonly used water-immiscible ILs are however slightly soluble in water (maximum solubility of [BMIm][PF₆] is 33.5 mM, for [OMIm][PF₆] it is 5.94 mM (Sandoval et al. 2012a)), so some IL contamination of a water-soluble product must be expected. If products are non-polar they can be extracted from the IL with classical organic solvent extraction. However, if the product has a higher polarity, more polar solvents must be used, and this may result in the IL dissolving in the extraction solvent, thus contaminating the product (Wong et al. 2006; Van Doorslaer et al. 2010). For such polar, non-volatile components, nanofiltration has been suggested as a means of separating the product from the IL, reaching IL rejections of up to 96% (van Doorslar et al. 2010). Even so, it seems that complete avoidance of IL contamination of the reaction product is not easily accomplished. Although generally less toxic than organic solvents, ILs may however not be as ‘green’ and harmless as they have been claimed to be based on their negligible vapour pressure. For many ILs, biodegradability is low and ecotoxicity is high (van Rantwijk and Sheldon 2007; Galonde et al. 2012). Thus, care should be taken when choosing ILs for large-scale biocatalysis. ‘Greener’ ILs are currently being developed (Gorke et al. 2010) along with methods for reducing the required amount of IL (Fischer et al. 2011). The latter is not only relevant in terms of ecotoxicity, but also because ILs are generally expensive, further emphasizing the need for a processes to recover not only the product, but also the IL.

Surfactantless microemulsions of water, n-hexane, and an alcohol can be transformed to a biphasic system by addition of water or n-hexane as described in Chapter 3 (Khmelnitsky et al. 1988). This will produce a hexane-rich phase and a water-rich phase. The reaction products and the enzyme will partition in each phase based on their properties. Thus, in terms of product recovery, this system is particularly useful for products with a strong preference for either the water-rich phase or the hexane-rich phase. The composition of each phase may be determined from tie lines in an LLE phase diagram, and it is evident that even if the product is found in the water-rich phase only, some purification from n-hexane and the alcohol acting as surfactant is still required.

As mentioned in Chapter 4, 3’-SL is currently purified from the reaction mixture by anion exchange chromatography. While this may still work with t-butanol as co-solvent, it is obvious that this method is not ideal for product recovery from an IL-containing mixture since the IL will
interfere with the anion exchange column. As shown by Michalak et al. (2013), the anion exchange purification of 3’-SL is also operable above lab-scale. Consequently, the results obtained in aqueous medium in Chapter 5 are the ones which are most easily transferred to an industrial process.

**Outlook**

Among the FAE-catalysed reactions investigated here, only sinapoylation or feruloylation of solvent components was successful. However, as they took place with broad acceptor specificity, FAEs must be kept in mind for future applications. Both AnFaeA and Depol 740L tolerated the PF₆⁻-based IL-buffer systems well, and especially AnFaeA showed appreciable conversion yields in the solvent reactions.

For Tr6, the results obtained here suggest several new routes to be pursued. For instance, it may be investigated whether the use of t-butanol as co-solvent can be used to achieve acceptable trans-sialylation yields with lower acceptor:donor ratios – an approach which may be useful when using more expensive acceptors than lactose. In addition, it may be valuable to look for alternative immobilization strategies for Tr6 in order to obtain higher recyclability while maintaining the beneficial synthesis/hydrolysis ratio obtained with the Tr6-MNPs. Finally, the Tr6-catalysed 3’-SL production in the EMR should be subjected to scale-up studies.
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Paper I

Dependency of the hydrogen bonding capacity of the solvent anion on the thermal stability of feruloyl esterases in ionic liquid systems

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Three feruloyl esterases, EC 3.1.1.73, (FAEs), namely FAE A from Aspergillus niger (AnFaeA), FAE C from Aspergillus nidulans (AndFaeC), and the FAE activity in a commercial β-glucanase mixture from Humicola insolens (Ultraflo L) were tested for their ability to catalyse esterification of sinapic acid with glycerol in four ionic liquid (IL) systems. The IL systems were systematically composed of two selected pairs of cations and anions, respectively: [BMIm][PF$_6$], [C$_2$OHMIm][PF$_6$], [BMIm][BF$_4$], and [C$_2$OHMIm][BF$_4$]. AnFaeA had activity in [PF$_6$]-based ILs, whereas the AndFaeC and the FAE in Ultraflo L had no appreciable activities and were generally unstable in the IL systems. FAE stability in the IL systems was apparently highly dependent on enzyme structure, and notably AnFaeA’s similarity to IL-compatible lipases may generally unstable in the IL systems.

Introduction

Ferulic acid esterases (FAEs; EC 3.1.1.73) are accessory plant cell wall-degrading enzymes, which catalyse the hydrolysis of the ester bond between ferulic acid and the monosaccharide to which it is covalently linked; in arabinoxylans the ferulic acid is bound via O-5 bonds to arabinose, whereas linkages of ferulic acid to the O-2 of arabinose and O-6 of galactose have been shown in pectins. FAEs do however also show activity towards simpler esters (e.g. methyl esters) and to a varying degree also towards esters of other hydroxycinnamates such as sinapic acid (SA), caffeic acid (CA), and p-coumaric acid (pCA), and the sub-type FAE nomenclature is based on the specific activity on these latter substrates.

FAEs can also be brought to catalyse the (trans)esterification reaction in solvents that favour synthesis over hydrolysis, i.e. systems with low water content such as organic solvents or ionic liquids (ILs). Recently, modifications of hydroxycinnamates have been performed in order to change their physicochemical and functional properties via such enzyme catalysed esterification reactions. These types of reactions may take place either through addition of aliphatic alcohols to increase lipophilicity or by addition of carbohydrates. The reported FAE catalysed esterification reactions are mainly direct esterifications of hydroxycinnamic acids or transesterification reactions of their esters with primary alcohols, e.g. 1-butanol, glycerol. However, also reactions with a number of monosaccharides, glycosides, and arabino-oligosaccharides have been demonstrated. The reactions have been accomplished in microemulsions of organic solvents with low water levels (<5%), in nearly solvent-free systems, or in a single organic solvent.

Hatzakis and Smounou have shown the ability of an FAE from Humicola insolens to catalyse the transesterification of vinyl acetate with a number of secondary alcohols in a solvent-free system.

During the last decade, the interest in performing enzyme-catalysed (trans)esterification reactions in ionic liquids (ILs) has increased rapidly. This interest has mainly been motivated by a desire to replace volatile organic solvents with non-volatile ILs, which also have the advantage of allowing increased enzyme
enantiom-selectivity and encompass the possibility of solvent tailoring for implementation of new, efficient reaction regimes due to their unconventional solvent properties. To date, most of the enzyme-catalysed reactions in IL systems have been done with lipases, especially lipase B from Candida antarctica (CaLB). Stability of the enzymes in the IL matrix is vital when performing these reactions. Although the enzyme stability issue has been addressed in some cases, only Ulbert et al. and Lou and Zong have tested the lipase stability (a lipase from Candida rugosa and CaLB, respectively) at more than one temperature. Hence, knowledge of enzyme thermal stability in IL systems is scarce. Only a single study has shown the ability of FAE A from Aspergillus niger (AnFaeA) to catalyse the (trans)esterification of sinapic acid and methyl sinapate with glycerol in an IL-water system using \([\text{C}_2\text{OHMIm}][\text{PF}_6]\) and \([\text{C}_5\text{OHMIm}][\text{PF}_6]\) with up to 30\% of water.

It is an obvious premise for successful catalysis that the enzyme is active in the IL. However, it may be hypothesized that the activity and stability of enzymes will be affected by the reaction temperature and that the stability may vary for different enzyme protein structures. Different ILs may moreover affect the enzyme stability differently. The objective of this work was to elucidate the effect of enzyme structure (and origin) and IL nature on the thermal stability and activity of selected FAEs in IL systems. This was done by determining the synthetic activity and thermal stability of three different FAEs in IL-buffer systems using a carefully selected series of four ILs with pairwise similar cations and anions of varying hydrophobicity and polarity, namely \([\text{C}_2\text{OHMIm}][\text{PF}_6]\), \([\text{C}_5\text{OHMIm}][\text{BF}_4]\), \([\text{BMIm}][\text{PF}_6]\), and \([\text{BMIm}][\text{BF}_4]\) (Fig. 1). The choice of \([\text{C}_2\text{OHMIm}][\text{PF}_6]\) was based on the results previously obtained with FAE; the \([\text{BMIm}][\text{PF}_6]\) and \([\text{BMIm}][\text{BF}_4]\) ILs were included because they are currently used widely in enzyme catalysis research – although not with FAEs – whereas \([\text{C}_5\text{OHMIm}][\text{BF}_4]\) was included to complete the series. Furthermore, the study aims to explore the use of the quantum chemistry-based COSMO-RS method for explaining and predicting the effect of given ILs on enzyme stability to provide a first foundation for predicting the optimal IL for a particular FAE-catalysed esterification reaction.

**Materials and methods**

**Materials**

Feruloyl esterase type A from Aspergillus niger and Ultrafl L (the latter is a commercial \(\beta\)-glucanase mixture from Humicola insolens) were provided by Novozymes A/S ( Bagsvaerd, Denmark). Feruloyl esterase type C from Aspergillus nidulans (AnS267.2) was produced by fermentation essentially as described by Stratton et al. The Pichia pastoris clone transformed with the feruloyl esterase gene was obtained from the Fungal Genetics Stock Center as described by Bauer et al. Methyl sinapate (methyl 3-(4-hydroxy-3,5-dimethoxophenyl)prop-2-enolate) was purchased from Apin Chemicals (Abingdon, UK). Anhydrous glycerol was purchased from AppliChem (Darmstadt, Germany). LC-MS grade methanol for HPLC analysis was purchased from Fisher Scientific (Loughborough, UK). Diethyl ether was purchased from Merck (Darmstadt, Germany) and MgSO\(_4\) from Riedel-de Haën (Seelze, Germany). All other chemicals, including sinapic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enolic acid) and the ionic liquids \([\text{BMIm}][\text{BF}_4]\) (1-butyl-3-methylimidazolium tetrafluoroborate) and \([\text{BMIm}][\text{PF}_6]\) (1-butyl-3-methylimidazolium hexafluorophosphate) (purity ≥97\%), were purchased from Sigma–Aldrich (St. Louis, MO, USA).

**Preparation of \([\text{C}_2\text{OHMIm}][\text{PF}_6]\) and \([\text{C}_5\text{OHMIm}][\text{BF}_4]\)**

Ionic liquids 1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate, \([\text{C}_5\text{OHMIm}][\text{PF}_6]\), and 1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate, \([\text{C}_5\text{OHMIm}][\text{BF}_4]\), were prepared according to the method described by Branco et al.

**Feruloyl esterase activity assay on methyl sinapate**

The activity assay was based on the one proposed by Juge et al. In brief, 50 μL enzyme solution was added to 500 μL of a preheated (37 °C) solution of 1 mM methyl sinapate (MSA) in 100 mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer (pH 6.0) to start the reaction. After 10 min of reaction at 37 °C, the reaction was stopped by adding 200 μL glacial acetic acid. Amounts of substrate (MSA) and product (sinapic acid) were analysed by RP-HPLC (see below). Activity (U) is expressed as the amount of enzyme required to release 1 μmol of sinapic acid per minute at 37 °C and pH 6.0.

**Thermal stability**

To mimic the system used for the esterification reaction, the FAEs were incubated in an IL system containing 15\% (v/v) enzyme solution diluted in 100 mM MOPS buffer (pH 6.0). Each reaction was conducted in an Eppendorf tube at 700 rpm and at 30 °C, 40 °C, 50 °C, or 60 °C in a Thermomixer (Eppendorf, Hamburg, Germany) for up to 2 h. After 0, 10, 20, 30, 60, and 120 min of incubation, enzyme samples (50 μL; 11.2 mU) were taken out and tested in the feruloyl esterase activity assay at 50 °C. Residual activity was also measured in the esterification reaction in \([\text{C}_5\text{OHMIm}][\text{PF}_6]\) (see below) after 0, 30, and 60 min of incubation in \([\text{C}_5\text{OHMIm}][\text{PF}_6]\) at 40 °C and 50 °C. Control samples were run without enzyme and no reaction was detected.

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*Fig. 1 Structures of the ionic liquid cations and anions used in this study: 1-butyl-3-methylimidazolium ([BMIm]+), 1-(2-hydroxyethyl)-3-methylimidazolium ([C2OHMIm]+), hexafluorophosphate ([PF6−]), and tetrafluoroborate ([BF4−]).*
Enzyme catalysed esterification of glycerol with sinapic acid

Using the results obtained by Vafiadi et al.\textsuperscript{12} in the esterification of glycerol with sinapic acid using FAE from \textit{A. niger}, the esterification activity of each of the FAEs was tested in a system with 2.5 M glycerol, 0.02 M sinapic acid (solubilised in the IL from its solid form), and 15% (v/v) enzyme solution in 100 mM MOPS buffer (pH 6.0) to give 56 µL. Hence, all reaction mixtures contained 15% (v/v) aqueous buffer, 18% (v/v) glycerol, and 67% (v/v) IL. The total reaction volume was 600 µL, and the reaction took place in an Eppendorf tube kept at 1400 rpm and 40 \textdegree C (30 \textdegree C when using BF\textsubscript{4} -based ILs), in a thermomixer (lower temperature than Vafiadi et al.\textsuperscript{12} to ensure enzyme stability). The reaction was stopped by extracting substrate and product with ethyl acetate (40 µL sample in 1 mL ethyl acetate) for 4 min at 40 \textdegree C and 1400 rpm. After evaporation of the ethyl acetate extract, the remaining solids were re-dissolved in 1 mL of a 1:1 methanol-water solution and analysed by RP-HPLC (see below). Control samples were run without enzyme or without glycerol, and no conversion was detected. Conversion yields were calculated from the molar amount of glycerol sinapate formed compared to the molar amount of sinapic acid originally present.

Analysis of reaction components by RP-HPLC

Quantitative analyses were made by RP-HPLC using a Chemstation 1100 series, Hewlett Packard equipped with a C18 column (150 mm × 4.6 mm, 3 µm; Phenomenex (Torrance, CA, USA)) with DAD detection of the substrate and product with quantification at 320 nm. Pure sinapic acid and methyl sinapate were used as external standards. Elution was conducted at 30 \textdegree C with [methanol]:[10% (v/v) acetic acid in water] [4]:[6] as the mobile phase at a flow rate of 0.5 mL min\textsuperscript{-1} for analysis of the hydrolysis reaction and 0.3 mL min\textsuperscript{-1} for analysis of the esterification reaction. Samples were filtered through a 0.2 µm syringe tip filter prior to analysis.

Generation of ionic liquid σ-profiles by COSMO-RS

The COSMO-file for \texttt{[C\textsubscript{2}OHMIm]+} was made with Gaussian03 (Gaussian Inc. (Wallingford, CT, USA)).\textsuperscript{17} The structure was first optimised with the semi-empirical PM3 method and then refined with B3LYP, first with the basis set 6-31 and then with 6-311++G(d,p). After optimisation the two conformers of \texttt{[C\textsubscript{2}OHMIm]+} were almost identical with regards to σ-profiles, and thus only a single one was used here. σ-profiles were generated with COSMOtherm (COSMOlogic (Leverkusen, Germany))\textsuperscript{18} using the BP-TZVP parameterisation.

Comparison of FAE sequence and structure

Initial sequence similarity searches were performed with protein-protein BLAST using the PSI-BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).\textsuperscript{19} Structure similarity searches for homologous proteins were performed by HHPredd (http://toolkit.tuebingen.mpg.de/hhpred)\textsuperscript{20} using default settings.

Statistics

One-way ANOVA for determination of statistical significance was made in Minitab 16 (Minitab Inc., State College, PA, USA). Statistical significance was established at \( p < 0.05 \).

Results and discussion

Effect of FAE structure on activity in IL-buffer systems

Among the four ILs used, \texttt{[BMIm][PF\textsubscript{6}]}\textsuperscript{-}, \texttt{[C\textsubscript{2}OHMIm][PF\textsubscript{6}]}\textsuperscript{-}, \texttt{[BMIm][BF\textsubscript{4}]}\textsuperscript{-}, and \texttt{[C\textsubscript{2}OHMIm][BF\textsubscript{4}]}\textsuperscript{-} (Fig. 1), the system with \texttt{[BMIm][PF\textsubscript{6}]}\textsuperscript{-} formed two phases due to the water-immiscibility of this IL. Enzyme activity was found in the aqueous phase of this two-phase system only (data not shown). The other three ILs were water-miscible and only one phase was formed in each of these IL reaction systems.

The AndFaeC was inactivated immediately in the \texttt{[C\textsubscript{2}OHMIm]+}-based IL systems, and in less than 10 min in the \texttt{[BMIm]+}-based IL systems. Consequently, only minor esterification activity, i.e. \( \sim 1\% \) conversion, was seen and only in the \texttt{[BMIm][PF\textsubscript{6}]}\textsuperscript{-} system (Table 1). However, in MOPS buffer, pH 6.0, the AndFaeC was completely stable for more than 2 h at 40 \textdegree C (data not shown). This indicated that the AndFaeC was very sensitive to the IL environment. Similarly, the FAE activity in the Ultraflo L preparation was inactivated immediately in the \texttt{[C\textsubscript{2}OHMIm]+}-based IL systems, and in less than 10 min in the \texttt{[BMIm][BF\textsubscript{4}]}\textsuperscript{-} system (Table 1). In \texttt{[BMIm][PF\textsubscript{6}]}\textsuperscript{-}, however, the FAE activity present in the Ultraflo L was stable throughout the 30 min of reaction (data not shown). This stability was possibly due to the enzyme being present in the aqueous phase of this two-phase system, rather than in the IL matrix.

Table 1 Conversion (%) of sinapic acid to glycerol sinapate after 30 min of reaction at 40 \textdegree C for \texttt{[PF\textsubscript{6}]}\textsuperscript{-}-based ILs and 30 \textdegree C for \texttt{[BF\textsubscript{4}]}\textsuperscript{-}-based ILs in an ionic liquid-buffer (15% v/v) system by 56 µL FAE from three different sources: FAE A from \textit{Aspergillus niger} (AnFAeA), FAE C from \textit{Humincola insolens} found as a side activity in the commercial β-glucanase mixture Ultraflo L, and FAE C from \textit{Aspergillus nidulans} (AndFaeC). The number of phases in each IL-buffer system is determined by visual detection. If complete inactivation has taken place during the reaction this is indicated as follows: i0: complete inactivation occurs immediately (less than 30 s); i10: complete inactivation takes place within 10 min.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>No. of phases</th>
<th>AnFAeA</th>
<th>Ultraflo L</th>
<th>AndFaeC</th>
</tr>
</thead>
<tbody>
<tr>
<td>\texttt{[BMIm][PF\textsubscript{6}]}\textsuperscript{-}</td>
<td>2</td>
<td>13 ± 3%\textsuperscript{a}</td>
<td>1.0 ± 0.1%\textsuperscript{a}</td>
<td>1.1 ± 0.1%\textsuperscript{a} (i10)</td>
</tr>
<tr>
<td>\texttt{[C\textsubscript{2}OHMIm][PF\textsubscript{6}]}\textsuperscript{-}</td>
<td>1</td>
<td>21 ± 2%\textsuperscript{a}</td>
<td>0%\textsuperscript{a} (i0)</td>
<td>0%\textsuperscript{a} (i0)</td>
</tr>
<tr>
<td>\texttt{[BMIm][BF\textsubscript{4}]}\textsuperscript{-}</td>
<td>1</td>
<td>0.9 ± 0.1%\textsuperscript{a}</td>
<td>0%\textsuperscript{a} (i10)</td>
<td>0%\textsuperscript{a} (i10)</td>
</tr>
<tr>
<td>\texttt{[C\textsubscript{2}OHMIm][BF\textsubscript{4}]}\textsuperscript{-}</td>
<td>1</td>
<td>0%\textsuperscript{a} (i10)</td>
<td>0%\textsuperscript{a} (i0)</td>
<td>0%\textsuperscript{a} (i0)</td>
</tr>
</tbody>
</table>

Superscript letters a–c indicate significant difference (one-way ANOVA; \( p < 0.05 \)) between reaction outcomes in different ILs for each enzyme, and letters x and y indicate significant difference between enzymes for each IL.
stable, the esterification activity of the FAE activity in Ultraflo L was very low (1% conversion; Table 1).

In contrast, the AnFaeA exhibited significant esterification activity and catalysed 13% conversion in [BMIm][PF6] and 21% conversion in [C2 OHMIm][PF6] (Table 1). The former is remarkable as Vafiadi et al.\textsuperscript{21} reported that no esterification activity was found in [BMIm][PF6], using the same enzyme and a similar system. As pointed out by Park and Kazlauskas,\textsuperscript{11} impurities in the IL and a resulting shift in pH may be the reason for contradictory results. AnFaeA was stable throughout the reaction time in these two [PF6]-based IL-buffer systems, but the enzyme was apparently sensitive to the [BF4]- systems. Consequently, only low and insignificant activity, 1% conversion, was obtained in [BMIm][BF4], where the enzyme had only 36% residual activity after 10 min (Fig. 2a). No esterification activity was observed in [C2 OHMIm][BF4] (Table 1), where the enzyme was completely inactivated within 10 min. This indicated that it is the [BF4]- anion rather than the single-phase system or water-miscible IL to which AnFaeA is sensitive. This anion effect is discussed further below.

The significant difference in esterification activity of AnFaeA observed between [BMIm][PF6] and [C2 OHMIm][PF6] is most likely due to differences in water-miscibility and thus water activity as well as viscosity between the two ILs. Generally, water-miscible ILs exhibit a lower water activity, \( a_w \), at a given water content than water-immiscible ILs do.\textsuperscript{22} Thus, the supposedly lower \( a_w \) of the water-miscible [C2 OHMIm][PF6] system may in part account for the higher esterification activity. However, at this high water content (15% v/v) even the more hydrophilic [BF4]-based systems have an \( a_w \) fairly close to 1.\textsuperscript{23} Similarly, the \( a_w \) of the system containing the more hydrophobic [C2 OHMIm][PF6] will therefore not be dramatically different from the \( a_w \) of the water-immiscible systems which reach maximum \( a_w = 1 \) at much lower water contents.\textsuperscript{24} Even with 18% (v/v) glycerol in the reaction mixture, the effect of the differential viscosity between the ILs (67% (v/v) of the reaction mixture) cannot be ignored, and the markedly higher viscosity of [BMIm][PF6] compared to [C2 OHMIm][PF6] is likely to cause lower reaction rates through mass transfer limitations.\textsuperscript{25} The interface formed in the two-phase system with [BMIm][PF6], the aqueous enzyme solution, and glycerol, may also induce lowered mass transfer and decrease the esterification rate. For an IL-buffer batch system it is therefore advisable to use an IL which is water-miscible and still contains the stabilising [PF6]- anion, e.g. [C2 OHMIm][PF6]. From these results it can therefore be concluded that the AnFaeA exhibited potential for being used for enzyme catalysed esterification reactions in an IL-buffer system, whereas AndFaeC and Ultraflo L did not.

All three FAEs have pH optima in the pH 5–6 range (Table 2). AnFaeA and Ultraflo L have higher temperature optima, 55–60 °C and 60–65 °C, respectively, than AndFaeC (37 °C) (Table 2). AndFaeC was also found to be less thermally stable in MOPS buffer (pH 6.0) than the other two FAEs (data not shown). It was thus to be expected that AnFaeA and Ultraflo L would be more stable, at least in the [BMIm][PF6] system (Table 1).

Remarkably, the feruloyl esterase activity in Ultraflo L had the lowest affinity towards MSA, the substrate towards which AnFaeA and AndFaeC had the highest affinity. Since the FAEs were dosed according to their hydrolytic activity on MSA, this cannot explain the low esterification activity of Ultraflo L in [BMIm][PF6]. However, if the FAE from \textit{H. insolens} found in Ultraflo L is similar to the one found in Pentowan 500 BG (also a Novozymes blend), which Hatzakis and Smonou\textsuperscript{27} found to have affinity for secondary alcohols, this may in part explain why

![Image](https://example.com/image.png)

**Fig. 2** Thermal stability of FAE A from \textit{A. niger} (AnFaeA) in IL systems: Residual activity of AnFaeA at: (a) 30 °C, (b) 40 °C, (c) 50 °C, and (d) 60 °C when incubated in 100 mM MOPS buffer pH 6.0 (□; open squares), [BMIm][PF6] (●; filled circles), [C2 OHMIm][PF6] (△; open triangles), and [BMIm][BF4] (♦; filled diamonds) for up to 120 min as compared to hydrolytic activity at 0 min of incubation. Thermal stability was also tested in [C2 OHMIm][BF4], but inactivation was immediate (took place in less than 30 s) and for simplicity the data are thus not included in the figure.

<table>
<thead>
<tr>
<th></th>
<th>AnFaeA</th>
<th>Ultraflo L</th>
<th>AndFaeC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of amino acids\textsuperscript{a}</td>
<td>260</td>
<td>273</td>
<td>249</td>
</tr>
<tr>
<td>pH optimum</td>
<td>5</td>
<td>–5–6</td>
<td>6.1</td>
</tr>
<tr>
<td>Temperature optimum</td>
<td>55–60 °C</td>
<td>60–65 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>Substrate affinity</td>
<td>MSA &gt; MFA &gt; MpCA</td>
<td>MCA &gt; MFA &gt; MpCA &gt; MSA</td>
<td>MSA\textsuperscript{a} &gt; MFA</td>
</tr>
</tbody>
</table>

MSA: methyl sinapate; MFA: methyl ferulate; MpCA: methyl p-coumarate; MCA: methyl caffeate.\textsuperscript{*} In the final enzyme, without signal peptide.\textsuperscript{a} MSA tested in this paper; MCA and MpCA not tested.
the activity is lower in the current system. It cannot be excluded that AndFaeC and the feruloyl esterase activity in Ultraflo L might express higher esterification activity at lower water content than AnFaeA, which has been found to have its optimum at the 15% (v/v) used here. However, since AndFaeC and the feruloyl esterase activity in Ultraflo L also showed low stability in the IL systems (Table 1), it seems more relevant to study the structural differences between the three enzymes.

Most importantly, the three FAEs differ in their number of amino acids (aa.), indicating differences in their overall structure. It has already been established that AnFaeA (260 aa.) has sequence and structure similarities to fungal lipases, especially the open form of the lipases from Rhizomucor miehei (37% sequence identity) and Thermomyces lanuginosis (30% sequence identity).28 These particular lipases have previously been found to work fairly well in ionic liquid systems, albeit not as well as the very robust lipase B from Candida antarctica.29 In contrast, AndFaeC (249 aa.) shows a sequence similarity to other (feruloyl) esterases (PSI-BLAST) and when predicting a structure of the enzyme by homology modelling using HHpred, the best match is the ferulic acid esterase domain of the cellulolosal xylanase Z (XynZ) in Clostridium thermocellum (sequence identity 21%; E-value 1.6 \times 10^{-5}). Hermoso et al.28 found that XynZ has poor homology in both sequence and structure with AnFaeA. Although AnFaeA and the FAE domain of XynZ differ in overall structure, their Ser-Asp-His catalytic triads in the active site are identical and they both present a long and narrow substrate-accommodating cavity in contrast to the wide and short ones generally found in lipases.28 Thus, AnFaeA is similar in catalytic mechanism to the FAEs, but similar in structure to some of the fungal lipases – a feature that may explain its higher stability in IL-buffer systems as compared to the other FAEs tested here.

The ferulic acid esterase from H. insolens found in Ultraflo L (273 aa.) showed sequence similarity to feruloyl and acetyl xylan esterases (PSI-BLAST), and structurally it also has some similarity to the FAE domain of XynZ from C. thermocellum, albeit less than AndFaeC (sequence identity 16%; E-value 3.4 \times 10^{-21}). In conclusion, the fact that AnFaeA has a structure more similar to IL-compatible lipases than the other FAEs does seem to be determining for its ability to work well in IL systems.

**Effect of IL nature on enzyme thermal stability**

Only the FAE A from A. niger showed appreciable esterification activity in the IL-buffer systems, and was thus chosen for thermal stability tests at 30–60 °C in [BMIm][PF₆], [C₆OHIm][PF₆], [BMIm][BF₄], and [C₆OHIm][BF₄] containing 15% (v/v) buffer.

Thermal stability has been assessed by determining residual activity in an aqueous medium after incubation in ILs. It has been found that CaLB to some extent (33–73%) refolded upon addition of excess water after having been incubated in denaturing ILs. Later, the same group found denaturation of CaLB to be irreversible in another denaturing IL, and concluded that enzyme inactivation in ILs includes a first reversible step and a second irreversible one.31 The irreversibility of enzyme denaturation in IL systems may (in part) be caused by aggregation of the denatured enzymes.32 Since any reversibility in the denaturation, i.e. re-activation, of AnFaeA, for example upon transfer to an aqueous buffer system for activity measurement, would introduce errors in the thermal stability results, the thermal stability of AnFaeA was also determined as residual esterification activity in the [C₂OHIm][PF₆] system upon incubation in [C₂OHIm][PF₆] for up to 1 h (Fig. 3). The data confirmed that there was no significant difference in thermal stability of AnFaeA when measured as residual hydrolytic activity and when measured as residual esterification activity. It can thus be concluded that the temperature-induced inactivation of AnFaeA in [C₂OHIm][PF₆] is not reversible, and that the thermal stability could be assessed based on hydrolytic activity as well as esterification activity.

At 30 °C and 40 °C, AnFaeA retained full activity for over 2 h in [BMIm][PF₆] and [C₆OHIm][PF₆] and there was no significant difference between the stability in the [PF₆]-based IL systems and stability in the pH 6.0 buffer (Fig. 2a,b). However, at 50 °C AnFaeA was more stable in buffer (kₐ = 0.0012) than in [BMIm][PF₆] (kₐ = 0.0191) and [C₆OHIm][PF₆] (kₐ = 0.0105). For more than 30 min of incubation, AnFaeA showed significantly higher residual activity in [BMIm][PF₆], but after 2 h the activity was significantly higher in [C₆OHIm][PF₆]. After 2 h at 50 °C, the residual activity of AnFaeA was 84% in buffer, 26% in [C₆OHIm][PF₆], and 11% in [BMIm][PF₆] (Fig. 2c). At 60 °C, inactivation was rapid in all media: complete inactivation took place within 10 min in [BMIm][PF₆], within 20 min in [C₆OHIm][PF₆] (kₐ = 0.184), and within 1 h in buffer (kₐ = 0.0646) (Fig. 2d).

In contrast, AnFaeA was highly unstable in the [BF₄]-based IL systems. Even at 30 °C and 40 °C inactivation of AnFaeA was significant in [BMIm][BF₄]: the residual activity was 36% after 10 min and 8% after 2 h at 30 °C (Fig. 2a; kₐ = 0.0174), and 31% after 10 min and 7% after 2 h at 40 °C (Fig. 2b; kₐ = 0.0179). At 50 °C, complete inactivation took place within 10 min (Fig. 2c). In the [C₆OHIm][BF₄] system, AnFaeA was
completely inactivated in less than 30 s (data not shown). The results emphasise that just by changing the anion from \([\text{PF}_6^-]\) to \([\text{BF}_4^-]\) while maintaining the same cations, the effect of the IL on AnFaeA stability changes dramatically. As previously found for lipases,\(^{9a,31}\) the anion has the dominant effect on FAE stability, and the choice of IL is thus of crucial importance when designing an enzyme-IL system for FAE catalysed esterification reactions. Importantly, the effect of \([\text{BF}_4^-]\) anion in particular has been subject to some debate, since some studies have found \([\text{BMIm}][\text{BF}_4]\) to have a significantly negative effect on the stability of CaLB (Novozym 435).\(^{6,24}\) whereas Lau et al.\(^{26}\) reported full activity and no enzyme dissolution using the same CaLB and \([\text{BMIm}][\text{BF}_4]\). Again, different levels of IL purity may explain these contradictory results.\(^{21}\) For FAEs, however, \([\text{BF}_4^-]\) does seem to have a significant, detrimental effect.

In a recent review, Zhao\(^{35}\) listed a number of IL properties that are likely to influence enzyme stability and activity in IL media, namely polarity, hydrogen bond basicity, nucleophilicity, hydrophobicity, viscosity, and in aqueous IL systems also ion kosmotropicity. Even if no solid prediction tool for enzyme-IL compatibility has been developed due to the complex nature of IL-enzyme systems, there seems to be general consensus that low hydrogen bond basicity and nucleophilicity as well as high viscosity and especially high hydrophobicity all favour enzyme stability and activity in ILs.\(^{35}\) Increasing viscosity may however also decrease the reaction rate of the enzyme.\(^{25}\) Similarly, it has been established that the relationship between enzyme activity and hydrophobicity follows a bell-shaped curve: activity increases with hydrophobicity up to a certain point, but then decreases due to a more thermodynamic ground state stabilization of the substrates and thus less tendency to react.\(^{16}\)

No clear relationship between enzyme activity and polarity has been established, although one study indicated that the reaction rate is higher in more polar ILs.\(^{37}\) In general, it is believed that the anion has a larger effect on enzyme stability than the cation does,\(^{11}\) and that large anions with delocalised negative charge on more atoms are generally more stabilising.\(^{38}\) Larger anions have also been suggested to be less destabilising because they are sterically demanding, in that their size would require the enzyme to change conformation – and vice versa.\(^{9a}\) Generally, hydrophobic ILs with \([\text{PF}_6^-]\) and \([\text{TF}_{2}\text{N}^-]\) (bis(trifluoromethanesulfonyl)amide) anions are stabilising while hydrophilic ILs with \([\text{NO}_3^-], [\text{lactate}^-], [\text{TiO}^-]\) (trifluoromethanesulfonate), \([\text{EtSO}_4^-]\), and in some cases also \([\text{BF}_4^-]\) are destabilising to enzymes.\(^{8}\) The thermal stability results obtained in this work (Fig. 2) support this hypothesis and are the first to be presented for FAEs.

In aqueous systems of hydrophilic ILs such as the \([\text{BF}_4^-]\)-based systems used here, where the IL is hydrated and dissociates into individual ions, the importance of having a chaotropic cation and especially a strong kosmotropic anion has been pointed out.\(^{39}\) Therefore, the chaotropic nature of the \([\text{BF}_4^-]\) anion may also play an important role in the FAE inactivation in the IL-buffer system. The same tendency has not been observed for hydrophobic ILs which have low solubility in water and therefore limited ion dissociation,\(^{35}\) explaining why the \([\text{PF}_6^-]\) anion does not cause enzyme inactivation despite its highly chaotropic nature.\(^{40}\)

Cation hydrophobicity has also been found have an effect on enzymes since enzyme enantioselectivity and stability decreases with decreasing alkyl chain length on imidazolium cations from \([\text{OMIm}^+]\) to \([\text{BMIm}^+].^{31}\) This effect may, however, also be confounded with the viscosity effect since a longer alkyl chain length in substituents on the imidazolium cation results in higher viscosity in the range mentioned above (4-8 carbons).\(^{41}\) This cation effect may be restricted to very similar cations like the ones used in the studies mentioned above. At least, it does not extend to the ones used in this work: \([\text{BMIm}][\text{PF}_6]\) has higher viscosity and hydrophobicity than \([\text{C}_4\text{OHIm}][\text{PF}_6]\), but this does not affect the stability of AnFaeA in the two media. There may have been an effect for Ultraflo L (see Table 1), but this may also be explained by the two-phase system formed with \([\text{BMIm}][\text{PF}_6]\), and this difference in water-miscibility is indeed the major cation effect observed in this work.

**Potential of the COSMO-RS method for explaining and predicting FAE stability in ILs**

The quantum chemistry-based method COSMO-RS has been introduced as a fast way of performing semi-quantitative tasks such as solvent screening, e.g. for substrate solubility, especially for complex media like ILs since it has the advantage over the classical group contribution methods (e.g. UNIFAC) that it is not limited to interpolation and partial extrapolation based on available data.\(^{18}\) Therefore, its ability to explain the effects of the four different ILs used in this study on FAE stability has been tested. The COSMO-RS method calculates the polarisation or screening charge density (SCD), \(\sigma\), which can be seen as a local measure of polarity for each molecule. The frequencies of screening charge densities ranging from \(\sigma = -3\ \text{e nm}^2\) to \(\sigma = 3\ \text{e nm}^2\) on the \([\text{BMIm}^-]\) and \([\text{C}_4\text{OHIm}^-]\) cations and \([\text{PF}_6^-]\) and \([\text{BF}_4^-]\) anions – also known as \(\sigma\)-profiles – have been plotted in Fig. 4 along with the \(\sigma\)-profile for FAE’s ‘natural’ solvent, water, for comparison. The hydrogen bonding threshold is \(\pm\sigma_{\text{th}} = \pm0.79\ \text{e nm}^2\), but as hydrogen bonding is weak below \(\pm1\ \text{e nm}^2\) only surface segments with an \(\sigma\)-value beyond \(\pm1\ \text{e nm}^2\) are considered strongly polar and potentially hydrogen bonding.\(^{18}\) From the \(\sigma\)-profiles it is seen that the peak SCD of \([\text{BF}_4^-]\) is found outside the hydrogen bonding limit, whereas the peak SCD of the more hydrophobic \([\text{PF}_6^-]\) is inside this limit (Fig. 4). Thus, the destabilising effect of \([\text{BF}_4^-]\) on the FAEs reported here can be explained by the tendency of \([\text{BF}_4^-]\) to act as a hydrogen bond acceptor and thus interact with the enzyme and disturb its hydrogen bond-based structure. It should also be noted that the SCD peak at \(\sigma > 1\ \text{e nm}^2\) is much larger for \([\text{BF}_4^-]\) than for water, thus explaining why the IL anion has a destabilising effect while water does not. Although the difference in water-miscibility between the two cations is harder to account for by the \(\sigma\)-profiles (the contribution of the hydroxyl group in \([\text{C}_4\text{OHIm}^-]\) can be seen in the range from 0.5 to 1 e nm\(^2\)), the
immiscible [BMIm][PF6] should be used depends on the nature of the IL-buffer system (15% (v/v) water), 85.0% of the [BMIm]+ molecules be preferable. It may be valuable to assess the effect of simple IL systems can thus be used with success at temperatures up to 40 °C. The stability of AnFaeA in ILs, thus, is included for comparison. The vertical lines indicate the hydrogen bonding (HB) limits, i.e. screening charge densities outside these lines indicate the tendency to form hydrogen bonds. σ-profiles are shown for two conformers 0 and 1 of [BMIm]+: COSMOtherm estimates that in this IL-buffer system (15% (v/v) water), 85.0% of the [BMIm]⁺ molecules are in the conformer 0 form, while 10.2% are in the conformer 1 form. The two conformers generated for [C2 OHMIm]⁺ had almost identical σ-profiles; thus, only one is shown.

fact that no major difference in the effect on AnFaeA stability was observed between [BMIm]⁺ and [C2 OHMIm]⁺ is consistent with the two cations having similar σ-profiles in the hydrogen bond donor range (σ < −1 e nm⁻²).

Other studies have also found COSMO-RS to be a useful tool for solvent screening in IL-based biocatalysis. For example, Lue et al. studied enzymatic flavonoid acylation with CaLB in numerous different ILs successfully screened for flavonoid solubility with COSMO-RS and found the method useful for choosing an IL giving the right balance between substrate solubility and enzyme stability.

Conclusions
Of the three FAEs tested here, AnFaeA showed the best potential for being used for esterification in an IL-buffer system, whereas AndFaeC and Ultrafloro L were practically inactive in the systems. AnFaeA has a structure more similar to IL-compatible lipases than the other FAEs, and this seems to be determining for its ability to work well in IL systems. The stability of AnFaeA in the IL-buffer system was found to be anion dependent: AnFaeA was rapidly inactivated in the [BF₄]⁻-based IL systems, but stable in the [PF₆]⁻-based ones, which were only significantly more destabilising than buffer at 50 °C and above. The [PF₆]⁻-based IL systems can thus be used with success at temperatures up to 40 °C. Whether the water-immiscible [C2 OHMIm][PF₆] or the water-immiscible [BMIm][PF₆] should be used depends on the nature of the desired system – in some cases a two-phase system may be preferable. It may be valuable to assess the effect of simple stabilisation methods such as formation of cross-linked enzyme aggregates (CLEAs) on FAE stability, since this has been found to have a positive effect on CaLB stability in ILs.

The COSMO-RS method proved to be a useful tool for explaining the effect of the four ILs studied in this work on AnFaeA stability in terms of hydrogen bonding capacity. Given the importance of hydrogen bonds in maintaining enzyme structure, the COSMO-RS method may thus prove to enable the prediction of useful ILs for enzymatic reactions in terms of stability. Further tests need to be made on other ILs in order to establish the actual potential, but the four ILs used in this study are good representatives of the ILs commonly used in the field. Although other aspects of IL nature such as water miscibility may not be predicted by COSMO-RS, the method can still be used for initial IL screening to give an indication of substrate solubility and enzyme stability in the IL medium.

References
Thermodynamically based solvent design for enzymatic saccharide acylation with hydroxycinnamic acids in non-conventional media

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Enzyme-catalyzed synthesis has been widely studied with lipases (EC 3.1.1.3), but feruloyl esterases (FAEs; EC 3.1.1.73) may provide advantages such as higher substrate affinity and regioselectivity in the synthesis of hydroxycinnamylate saccharide esters. These compounds are interesting because of their amphiphilicity and antioxidative potential. Synthetic reactions using mono- or disaccharides as one of the substrates may moreover direct new routes for biomass upgrading in the biorefinery. The paper reviews the available data for enzymatic hydroxycinnamylate saccharide ester synthesis in organic solvent systems as well as other enzymatic hydroxycinnamylate acylations in ionic liquid systems. The choice of solvent system is highly decisive for enzyme stability, selectivity, and reaction yields in these synthesis reactions. To increase the understanding of the reaction environment and to facilitate solvent screening as a crucial part of the reaction design, the review explores the use of activity coefficient models for describing these systems and – more importantly – the use of group contribution model UNIFAC and quantum chemistry based COSMO-RS for thermodynamic predictions and preliminary solvent screening. Surfactant-free microemulsions of a hydrocarbon, a polar alcohol, and water are interesting solvent systems because they accommodate different substrate and product solubilities and maintain enzyme stability. Ionic liquids may provide advantages as solvents in terms of increased substrate and product solubility, higher reactivity and selectivity, as well as tunable physicochemical properties, but their design should be carefully considered in relation to enzyme stability. The treatise shows that thermodynamic modeling tools for solvent design provide a new toolbox to design enzyme-catalyzed synthetic reactions from biomass sources.

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Introduction

Interest in production of biofuels and biomaterials from renewable cellulosic biomass has increased dramatically in the recent years. The available work has made it clear that in addition to improving the efficiency of the core conversion technologies for biofuel production, it is important to add economic value and sustainability to the biofuel process via concomitant production of higher value products from the biomass. This biorefinery manufacturing concept also encompasses the utilization of other biomass components than glucose to produce, for example, building block compounds, fine chemicals, and various advanced functional compounds [1].

Novel enzyme-catalyzed synthesis reactions show promise to become an important part of such biorefinery processes. Enzyme-catalyzed reactions are more efficient in terms of selectivity and have significant potential as being more sustainable as compared to chemical catalysis and classic chemical synthesis reactions [2,3]. However, as opposed to more conventional enzyme-catalyzed hydrolysis reactions generally taking place in water, the solvent system, and in turn proper solvent design, is crucially important for accomplishing enzyme-catalyzed synthesis reactions successfully. Targeted solvent design is not a well-explored field for enzyme-catalyzed reactions, but holds great potential for improving the enzyme stability, reaction yields, and reaction selectivity in such synthetic enzyme reactions. To aid the development of this potential, this review will explore the use of thermodynamics as a tool for solvent design via predictive solvent engineering with particular focus on enzyme-catalyzed synthesis of hydroxycinnamate saccharide esters from biomass components. The treatise will also present a comprehensive overview of the available data concerning enzymatic hydroxycinnamate saccharide synthesis reactions and hydroxycinnamate (trans)esterification reactions with particular focus on the solvent systems, the substrates, the enzymes, and the yields obtained.

Enzymatic saccharide ester synthesis reactions

Many successful esterifications of saccharides using lipases and proteases have been reported [2], whereas feruloyl esterases (FAEs) have been used only in a few cases. Lipases (EC 3.1.1.3) are known to be active at low water activity conditions [4,5], and most enzymatic synthesis reactions studied so far indeed include the use of lipases, especially lipase B from Candida antarctica (CaLB), which is an unusually stable enzyme and which is moreover commercially available in an immobilized form. Feruloyl esterases (FAEs) (EC 3.1.1.73) are classified as carboxylic ester hydrolases (EC 3.1.1.3) and catalyze hydrolytic reactions in their natural solvent, water (reaction (2) in Fig. 1). The anhydrous nature of organic solvents and ionic liquids is therefore determining the ability of these enzymes to catalyze synthetic reactions. Synthetic reactions include both direct esterification (reaction (1)) and transesterification (reaction (3)) (Fig. 1). FAEs are related to lipases, but probably more typical in terms of stability in non-conventional media than lipases. The FAE type A from Aspergillus niger (AnFAeA) has been found to be structurally similar to lipases from Rhizomucor miehei (Rml) and Thermomyces lanuginosus (TIL) [6]. These lipases both work well in non-conventional media [7], and AnFAeA and other FAEs have indeed been demonstrated to be able to catalyze synthetic (trans)esterification reactions in non-conventional media (Tables 1a and 2b).

Saccharide esters are receiving increasing attention because of their promising properties such as surface activity [8], antitumor activity [9] and plant growth-inhibiting activity [10,11]. Saccharide esters are amphiphilic, non-toxic, and biodegradable, and are increasingly used as low-calorie sweeteners or biosurfactants in food, pharmaceutical and cosmetic industries [2]. Several biocatalyzed esterifications of mono-, di- and polysaccharides have been carried out in organic solvents [2] and to a lesser extent in ionic liquids (ILs) [12,13]. As discussed in more detail later, conversion yields vary from a few percent to more than 90%. The hydroxycinnamate saccharide esters that are receiving particular attention in this review are interesting in their own right because of their antioxidant properties [14], but have mainly been chosen as a subject for this review because both substrates, that is, the hydroxycinnamic acids (Fig. 2) and monosaccharides may be present biomass processing streams. This particular reaction system also represents an example of enzyme-catalyzed change in solubility.
properties of both reactants. We also believe that any reactions using monosaccharides as one of the substrates may direct new avenues for saccharide upgrading for biorefinery purposes.

**Thermodynamic tools for reaction design for enzymatic synthesis reactions**

Solvents are important in synthetic enzymatic reactions as they influence reaction rate, selectivity, and reaction yield, and moreover affect enzyme stability. The use of thermodynamic models for *in silico* solvent screening can save a lot of time in the laboratory and generate knowledge about the reaction system at an early stage. There are many thermodynamic models which could be potentially useful, roughly divided into equations of state and activity coefficient models. However, most of them, such as the local-composition models NRTL [15] and UNIQUAC [16] are particularly suitable for correlation purposes (for binary systems).

**TABLE 1A**

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Saccharide</th>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl ferulate</td>
<td>L-Arabinose</td>
<td>StFaeC</td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>39% (160)</td>
<td>97</td>
</tr>
<tr>
<td>Ethyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/buffer (3.2%)</td>
<td>45% (120)</td>
<td>98</td>
</tr>
<tr>
<td>n-Propyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>6.3% (—)</td>
<td>32</td>
</tr>
<tr>
<td>n-Butyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>3.8% (—)</td>
<td>32</td>
</tr>
<tr>
<td><em>iso</em>-Propyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>3.4% (—)</td>
<td>32</td>
</tr>
<tr>
<td>2-Butyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>3.3% (—)</td>
<td>32</td>
</tr>
<tr>
<td><em>iso</em>-Butyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/water (3.2%)</td>
<td>2.7% (—)</td>
<td>32</td>
</tr>
<tr>
<td>Methyl ferulate</td>
<td></td>
<td></td>
<td><em>n</em>-Hexane/t-butanol/buffer (3.2%)</td>
<td>4.2% (—)</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 1**

Enzyme-catalyzed direct esterification (1), hydrolysis (2), and transesterification (3) of an alcohol with hydroxycinnamic acids. $R_1$ and $R_2$: H, OH, or OCH$_3$ (Fig. 2); $R_3$: any primary (or in some cases secondary) alcohol, cf. section “Regioselectivity”; $R_4$: alkyl, mainly CH$_3$, or vinyl.
### TABLE 1A (Continued)

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Saccharide</th>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl ferulate Ethyl ferulate</td>
<td>L-Arabinose</td>
<td>TsFaeC</td>
<td>n-Hexane/t-butanol/water (3.2%)</td>
<td>21.2% (96)</td>
<td>[90]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Arabinose</td>
<td>Depol 740 H. insolens</td>
<td>n-Hexane/1-butanol or 2-butanone/buffer (3%)</td>
<td>17.7% (144)</td>
<td>[37]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Arabinose</td>
<td>Flavourzyme A. oryzae</td>
<td>n-Hexane/1-butanol/buffer (3%)</td>
<td>32.9% (144)</td>
<td>[37]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Arabinose</td>
<td>Multifect P3000 B. amyloliquefaciens</td>
<td>n-Hexane/1-butanol or 2-butanone/buffer (3%)</td>
<td>36.7% (144)</td>
<td>[37]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Arabinose</td>
<td>Depol 670 T. reesei</td>
<td>n-Hexane/1-butanol or 2-butanone/buffer (3%)</td>
<td>9.6% (144)</td>
<td>[37]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Arabinose</td>
<td>Ceremix Bacillus spp.</td>
<td>n-Hexane/1-butanol/buffer (3%)</td>
<td>32.5% (144)</td>
<td>[37]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>D-Galactose</td>
<td>RP-1 B. subtilis</td>
<td>n-Hexane/1-butanol or 2-butanone/buffer (3%)</td>
<td>19.8% (144)</td>
<td>[37]</td>
</tr>
</tbody>
</table>

n.q.: not quantified, but there is activity; StFaeC: FAE C from Sporotrichum thermophile; TsFaeC: FAE C from Talaromyces stipitatus. Systems with <1% conversion are not included. Reactions with substrates that (1) lack the double bond (hydroxyphenolpropionic acids), or (2) do not have hydroxyl-groups on the aromatic ring are not included as these are not considered hydroxycinnamic acids.

### TABLE 1B

Enzymatic hydroxycinnamate (HC) saccharide syntheses in pure organic solvents or nearly solvent-free systems reported in the literature.

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Saccharide</th>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>Arabinose</td>
<td>Pectinase PL A. niger</td>
<td>Nearly solvent-free (buffer and DMSO added)</td>
<td>n.q. (0.5)</td>
<td>[100]</td>
</tr>
<tr>
<td>Fructose</td>
<td></td>
<td></td>
<td></td>
<td>n.q. (0.5)</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td></td>
<td></td>
<td>n.q. (0.5)</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td>n.q. (0.5)</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td>n.q. (0.5)</td>
<td></td>
</tr>
<tr>
<td>Vinyl ferulate</td>
<td>L-Arabinose</td>
<td>Lipolase 100T T. lanuginosus</td>
<td>Acetonitrile</td>
<td>83% (62)</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methyl isobutyl ketone</td>
<td>90% (65)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>25–60% (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetonitrile</td>
<td>64% (64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methyl isobutyl ketone</td>
<td>49% (65)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>25–60% (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-Butanol</td>
<td>25–60% (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Esulin</td>
<td>53% (69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methyl isobutyl ketone</td>
<td>41% (65)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>&lt;25% (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-Butanol</td>
<td>25–60% (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n-Pentyl d-galactose</td>
<td>63% (67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methyl isobutyl ketone</td>
<td>88% (65)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>60–95% (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-Butanol</td>
<td>25–60% (63)</td>
<td></td>
</tr>
<tr>
<td>TFE ferulate</td>
<td>Methyl glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinyl ferulate</td>
<td>Methyl glucose</td>
<td>Pentopan 500 BG T. lanuginosus</td>
<td>Chloroform</td>
<td>25–60% (72)</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetonitrile</td>
<td>60–95% (64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetonitrile</td>
<td>25–60% (88)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chloroform</td>
<td>25–60% (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toluene</td>
<td>&lt;25% (72)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-Butanol</td>
<td>&lt;25% (72)</td>
<td></td>
</tr>
</tbody>
</table>
and in turn for predictions for multicomponent systems. This is because interaction parameters are needed which should be obtained from binary data. Thus, such models are typically not suitable for solvent screening purposes. Predictive models which can be used for such purposes include group contribution activity coefficient models like UNIFAC [17–19] and the more recently developed theoretically based COSMO-RS approach [20]. As pointed out by Halling [21], non-conventional multicomponent systems for enzymatic synthesis are not at equilibrium, let alone ideal. Nevertheless, thermodynamic analysis should be able to assist and contribute to the understanding of the reaction systems, because many of the processes probably do come close to equilibrium – a requirement for precise application of standard thermodynamics – and many predictions will in turn be correct within an acceptable accuracy [21].

Many thermodynamic methods are not suitable for predicting complex ionic liquid (IL) solvent systems, for example, for calculating substrate and product solubility. For such IL systems, one suitable choice for solvent screening is the quantum chemistry-based COSMO-RS method. It is relatively easy to use and it may provide qualitatively reliable predictions without the need for experimental data for parameter fitting [22]. For example, with the purpose of identifying the optimal IL structure for maximal flavonoid solubility, the solubility of a particular flavonoid has been predicted in a wide range of ILs using COSMO-RS, and the approach also assisted in concluding that the flavonoid solubility in ILs was strongly anion-dependent [23]. On this basis, it is probable that COSMO-RS will also be a useful tool for predicting solubility in ILs of hydroxycinnamic acids, hydroxycinnamate derivatives, and their saccharide conjugates. The COSMO-RS solvent screening can also be extended to include organic solvents with reasonable success [24]. Recently, it has moreover been shown that the effect of IL anion nature on the stability of AnFaeA (which can catalyze hydroxycinnamate saccharide ester synthesis) can also be predicted to a large extent or at least explained by COSMO-RS-generated σ-profiles [25]. It has thus turned out that in ILs not only reactant and product solubility, but also enzyme stability is highly anion-dependent [25–27].

COSMO-RS is thus a tool that in many cases may be useful for qualitative solvent screening, but from the quantitative point of view, the results may not always be satisfactory. Unfortunately, in these cases the results cannot be further improved, as there are no adjustable parameters in the model. Nevertheless, the significant advantage of COSMO-RS is that it can assist in pre-selecting IL solvents [28] and point to suitable solvent candidates, thus aiding the design and optimization of the actual experimental work. If just a few data points are available, as is often the case in these new systems, “local” UNIFAC models can also be used with more success than ordinary group contribution models, which sometimes fail when it comes to complex mixtures. In such “local” UNIFAC models a sensitivity analysis is first performed to identify the most sensitive pair of group interaction parameters and then these parameters are regressed to the few available experimental data points. The finally obtained “local” UNIFAC model is still

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Saccharide</th>
<th>Enzyme</th>
<th>Solvent system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFE ferulate</td>
<td>Methyl glucose</td>
<td>Chloroform</td>
<td>25–60% (72)</td>
<td>[33]</td>
<td></td>
</tr>
<tr>
<td>Vinyl ferulate</td>
<td>L-Arabinose</td>
<td>Acetonitrile</td>
<td>25–60% (70)</td>
<td>&lt;25% (88)</td>
<td></td>
</tr>
<tr>
<td>Vinyl p-coumarate</td>
<td>Arbutin</td>
<td>Nearly solvent-free (THF added)</td>
<td>36% (96)</td>
<td>[38]</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>Arbutin</td>
<td>t-Butanol</td>
<td>62% (168)</td>
<td>[34]</td>
<td></td>
</tr>
<tr>
<td>Vinyl ferulate</td>
<td>Isoquercitrin</td>
<td>Acetonitrile</td>
<td>50% (&lt;)</td>
<td>[101]</td>
<td></td>
</tr>
<tr>
<td>Vinyl p-coumarate</td>
<td>Isoquercitrin</td>
<td>Acetone</td>
<td>&gt;40% (72)</td>
<td>[102]</td>
<td></td>
</tr>
</tbody>
</table>

TTE: 2,2,2-trifluoroethyl; n.q.: not quantified, but there is activity; AnLA: lipase A from Aspergillus niger; PeL: lipase from Penicillium expansum; CaL: lipase B from Candida antarctica; PCL: lipase from Pseudomonas cepacia (now known as Burkholderia cepacia lipase, BlC); DMSO: dimethyl sulfoxide; THF: tetrahydrofuran. Systems with <1% conversion not included. Reactions with substrates that (1) lack the double bond (hydroxyphenolpropionic acids), or (2) do not have hydroxyl-groups on the aromatic ring are not included as these are not considered hydroxycinnamic acids.

![FIGURE 2](image)

The structures of the common, naturally occuring hydroxycinnamic acids shown in their protonated form: (a) ferulic acid, (b) sinapic acid, (c) caffeic acid, and (d) p-coumaric acid. With pKₐ values in the 4.4–4.6 range [104], the hydroxycinnamic acids are most probably neither fully protonated nor fully dissociated in most reactions, but the names of the protonated forms are used in this paper to designate this hydroxycinnamic acid-hydroxycinnamate equilibrium.
predictive in the sense that it can be used for other similar systems with the same groups. “Local” UNIFAC models have been used successfully for screening of classic solvents for, for example, pharmaceuticals [29], and could provide an alternative to COSMO-RS.

Enzymatic synthesis of hydroxycinnamate saccharide esters in organic solvent systems

Tables 1a and 1b summarize the yields reported in the literature as obtained in enzyme-catalyzed (trans)esterifications of hydroxycinnamic acids or their esters and mono-, di-, or oligosaccharides or saccharide derivatives. Both direct esterifications and transesterification reactions have been reported. All reactions published so far have taken place in systems based on organic solvents, that is, surfactant-free microemulsions (Table 1a), pure organic solvents (Table 1b), or nearly solvent-free systems (Table 1b).

Substrates

In almost all of the reported enzymatic hydroxycinnamate saccharide synthesis reactions that have been accomplished in organic solvent systems (Tables 1a and 1b), ferulic acid or its ester has been used as the hydroxycinnamate substrate, irrespective of whether the reaction has been a direct esterification or a transesterification reaction. The reason may be that ferulic acid is the most abundant type of hydroxycinnamic acid present in currently processed biomass. In unprocessed materials ferulic acid is mainly found in a bound, esterified form [30,31], but hydrothermal treatment of biomass will liberate a significant portion of the ferulic acid into solution. Interestingly, several FAEs show higher affinity for sinapic acid in the esterification reaction [25]. Various monosaccharides, including both hexoses and pentoses, especially arabinose, and monosaccharide derivatives such as β-PO-arabinose, methyl glucose, penta-galactose, esculin, arbutin, and arabinobio-ligosaccharides have been evaluated as saccharide substrates for these acylation reactions (Tables 1a and 1b).

Enzymes

Multiple types of enzymes have been used for enzymatic hydroxycinnamate saccharide synthesis reactions. Hence, in addition to FAEs, lipases, as well as commercial blends containing FAEs (Pectinase PL, Pentopan 500 BG, Depol 740, Depol 670), or lipases (Lipolase 100T), proteases (Flavourzyme, Multifect P3000, Cere-mix), and even a 5'-phosphodiesterase (RP-1) have been employed for these reactions (Tables 1a and 1b). The quite narrow specificity of the different hydrolyses seems to broaden significantly when catalyzing the reverse reaction, for example, as seen for StFaEc [32] (Table 1a) and the Lipolase 100T and Pentopan 500 BG mixtures [33] (Table 1b). The data show that the enzymes used apparently exhibit a broad specificity towards the saccharide acceptor with no chiral selectivity in these reactions (Tables 1a and 1b). As discussed later, apart from the recent results reported by Ishihara et al. [34], lipase action seems to require vinyl or 2,2,2-trifluoroethyl (TFE) activation of the reactant hydroxycinnamic acids (Table 1b).

Solvent systems

Mainly two kinds of organic solvent systems have been used for enzymatic hydroxycinnamate saccharide ester synthesis: pure organic solvents (Table 1b) and surfactant-free microemulsions of organic solvents and water or aqueous buffer (Table 1a), respectively. In pure organic solvents, Mastihubova et al. [33] obtained higher yields in the least hydrophobic solvents acetonitrile and methyl isobutyl ketone because of a higher solubility of the saccharide acceptor in these solvents. Surfactant-free microemulsions are thermodynamically stable ternary mixtures of a hydrocarbon, an alcohol, and water or aqueous buffer. Aqueous microdroplets are stabilized in dispersion by alcohol molecules adsorbed to their surface, and several enzymes have been found to retain stability in these systems presumably because the enzymes become entrapped in the aqueous droplets and are thus protected from the organic solvent contact [35,36]. When using a surfactant-free microemulsion it is possible to separate the phases and recover the product in the organic phase and the enzyme in the aqueous phase [35]. In reactions with saccharides, the aqueous phase in the microemulsions further accommodates the low saccharide solubility in non-aqueous media [37]. The yields are generally lower in the microemulsion systems (Table 1a) than in the pure organic solvent systems (Table 1b), but this may also be due that mainly FAEs and proteases have been used in the microemulsion systems, while lipases have been more prevalently employed in the pure organic solvent systems; these two effects cannot be separated. Reaction times for these reactions are generally quite long, ranging from 30 min up to seven days (Table 1b).

Conversion yields

The conversion yields range from less than 1% (not included in the tables) to 90% or above (Tables 1a and 1b). The highest yields, up to 90% in 65 hours, have been obtained with Lipolase 100T catalyzing the reaction of l-arabinose with vinyl-activated ferulate in acetonitrile and methyl isobutyl ketone [33] (Table 1b). Also CaLB has given high product yields in t-butanol with ferulic acid and arbutin during extended reaction (62% in 168 hours) [34] (Table 1b). The FAE in Depol 670 has also been reported to produce good yields with ferulic acid and galactose as reactants in a microemulsion (61.5% in 144 hours [37]; Table 1a) and so has the FAE in Pentopan 500 BG in catalyzing the reaction between esculin and vinyl ferulate in acetonitrile (60–95% in 64 hours [33]; Table 1b). Generally, most of the reported reactions have had conversion yields of 20–50% regardless of the solvent system. Regarding solvent-free systems, only one quantified yield has been reported. The yield was at the level of the other reaction systems, namely 36% conversion in 96 hours [38] (Table 1b). The conversion yields of 21–45% obtained with FAEs in transesterifications with methyl ferulate and arabinose (Table 1a) may be improved by applying reduced pressure to remove the produced methanol in situ thus preventing the unwanted reverse reaction. Vacuum in situ removal of methanol or ethanol in transesterifications with methyl ferulate and ethyl ferulate, respectively, has been successfully implemented in both organic solvent systems [39], solvent-free systems [40] and IL systems [41] resulting in increases in conversion yields from 50% to 83%, 78% to 96%, and 61% to 91%, respectively. These data indicate that much can be gained by careful reactor design.

Enzymatic synthesis of hydroxycinnamate esters in ionic liquid systems

Enzymatic syntheses of hydroxycinnamate saccharide esters have only been carried out in organic solvent systems and solvent-free
systems (Tables 1a and 1b), but several authors have investigated other esterifications with hydroxycinnamic acids in IL systems using lipases (Table 2a) and FAEs (Table 2b) as well as esterifications of saccharides. So far, no enzymatic (trans)esterifications of hydroxycinnamic acid (esters) and saccharides have been reported. The saccharide (trans)esterifications accomplished in ILs have so far mainly employed lipases, especially CaLB, reaching conversion yields ranging from 80% to 98% [12,13,42,43]. The interest in ILs is mainly motivated by a desire to replace volatile organic solvents with non-volatile ILs. Advantages of using ILs often include their increased selectivity and ability to solubilize both polar and non-polar substrates and products as well as the ability to adjust other physicochemical properties such as density, viscosity, and solvating power through tailoring of cation and anion type to meet the needs of specific systems. Working with saccharides, the major advantage of using ILs as compared to organic solvent systems is the increased substrate solubility. Furthermore, higher yields in shorter reaction times as well as increased regioselectivity may also be achieved in enzymatic acylation of saccharides in ILs compared to organic solvents [44].

**Conversion yields**

An evaluation of lipase-catalyzed hydroxycinnamate (trans)esterifications in ILs shows that CaLB-catalyzed reactions have been reported to give particularly high conversion yields, above 95%, in the transesterifications with methyl caffeate [41], whereas RmL-catalyzed conversions have produced slightly higher yields than CaLB in direct esterification reactions [45] (Table 2a). Generally,

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Alcohol</th>
<th>Ionic liquid system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>Methanol</td>
<td>[C4MIm][PF6]</td>
<td>41.7% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>[C4MIm][PF6]</td>
<td>40.7% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Propanol</td>
<td>[C4MIm][PF6]</td>
<td>39.7% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>1-Butanol</td>
<td>[C4MIm][PF6]</td>
<td>32.9% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>1-Hexanol</td>
<td>[C4MIm][PF6]</td>
<td>30.3% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>1-Octanol</td>
<td>[C4MIm][PF6]</td>
<td>17.5% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Geraniol</td>
<td>[C4MIm][PF6]</td>
<td>6.7% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>1-Octanol</td>
<td>[C4MIm][PF6]</td>
<td>27.4% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td></td>
<td>[C4MIm][PF6]</td>
<td>8.4% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td></td>
<td>[C4MIm][PF6]</td>
<td>0.4% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1-Butanol</td>
<td>[C4MIm][BF4]</td>
<td>2.3% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][BF4]</td>
<td>3.4% (72)</td>
<td>[45]</td>
</tr>
<tr>
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<td></td>
<td>[C4MIm][BF4]</td>
<td>7.6% (72)</td>
<td>[45]</td>
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<td>[C4MIm][BF4]</td>
<td>23.4% (72)</td>
<td>[45]</td>
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<td></td>
<td>[C4MIm][BF4]</td>
<td>55.6% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][BF4] (90%)/[C4MIm][PF6] (10%)</td>
<td>3.9% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][BF4] (50%)/[C4MIm][PF6] (50%)</td>
<td>10.2% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][BF4] (10%)/[C4MIm][PF6] (90%)</td>
<td>31.5% (72)</td>
<td>[45]</td>
</tr>
<tr>
<td>Ethanol</td>
<td>[C4MIm][BF4]</td>
<td>2.0% (72)</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[C4MIm][BF4]</td>
<td>3.4% (72)</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[C4MIm][BF4]</td>
<td>5.1% (72)</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[C4MIm][BF4]</td>
<td>51.0% (72)</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td>Methyl caffeate</td>
<td>cis-9-Octadecen-1-ol</td>
<td>[C4MIm][PF6] (25%)/iso-octane (75%)</td>
<td>~100%* (288)</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][PF6] (25%)/iso-octane (75%)</td>
<td>~100%* (288)</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>3-Cyclohexyl-1-propanol</td>
<td>[C4MIm][NTf2] (reduced pressure)</td>
<td>97.7% (24)</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][NTf2]</td>
<td>93.8% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>2-Cyclohexylethanol</td>
<td>[C4MIm][PF6]</td>
<td>97.6% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][BF4]</td>
<td>45.8% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[C4MIm][CF3SO3]</td>
<td>7.6% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[PMPra][NTf2]</td>
<td>1.6% (96)</td>
<td>[41]</td>
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<td></td>
<td></td>
<td>[MPPro][NTf2]</td>
<td>71.2% (96)</td>
<td>[41]</td>
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<tr>
<td></td>
<td></td>
<td>[MPPip][NTf2]</td>
<td>68.2% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[TMPA][NTf2]</td>
<td>72.2% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69.0% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>4-Phenyl-1-butanol</td>
<td>[C4MIm][NTf2]</td>
<td>96.7% (96)</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>5-Phenyl-1-pentanol</td>
<td>[C4MIm][NTf2]</td>
<td>84.0% (96)</td>
<td>[41]</td>
</tr>
</tbody>
</table>

---

*Experimental results apparently exceeded the theoretical maximum.
TABLE 2B
Enzymatic hydroxycinnamate (HC) (trans)esterifications in ionic liquid systems with FAEs as reported in the literature.

<table>
<thead>
<tr>
<th>HC (ester)</th>
<th>Alcohol</th>
<th>Enzyme</th>
<th>Ionic liquid system</th>
<th>Yield (hours)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinapic acid</td>
<td>Glycerol</td>
<td>AnFaeA</td>
<td>[C2OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>13% (0.5)</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[C3OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>1% (0.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[C2OHMIm][BF4] (85%)/MOPS buffer (15%)</td>
<td>72.5% (24)</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[C2OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>9% (24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[C2OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>7% (24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[C2OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>2.5% (24)</td>
<td></td>
</tr>
<tr>
<td>Methyl sinapate</td>
<td>Glycerol</td>
<td>AndFaeC</td>
<td>[C2OHMIm][PF6] (85%)/MOPS buffer (15%)</td>
<td>1% (0.5)</td>
<td>[25]</td>
</tr>
</tbody>
</table>

Conversion yields have been higher in the hydrophobic [PF6] and [NTf2]-based IL systems where the lipases have turned out to be more stable [41,45] (Table 2a). Vafaiidi et al. [46] also achieved a high conversion yield (72.5% in 24 hours) in the optimized direct esterification of glycerol with sinapic acid in [C2OHMIm][PF6] containing 15% buffer using AnFaeA (Table 2b). Results with FAEs in IL systems are scarce, but at least AnFaeA holds potential for future reactions also including saccharides because of its appreciable stability in [PF6]-based IL systems (the anion effect on enzyme stability in ILs is discussed in the section “Enzyme stability in non-conventional media”).

A comparison of the results obtained in IL systems (Tables 2a and 2b) to equivalent data obtained in solvent-free systems or organic solvent systems shows that the yields are generally higher and the reaction times shorter in the IL systems. Katsoura et al. [45] thus obtained a conversion yield of 17.5% in 72 h in the esterification of ferulic acid with 1-octanol in [C2OHMIm][PF6] using CaLB (Table 2a), while Stamatis et al. [47] obtained only 11% conversion during 288 hours in the corresponding solvent-free system and Compton et al. [39] reported a conversion yield of 14% after reaction for 312 hours in t-butanol. With RmL, the comparable yield was 30% after 288 hours in the solvent-free system [47] and 35% after 72 hours in [C2OHMIm][PF6] [45] (Table 2a). Similarly, the yield of ethyl ferulate with CaLB was 20% after 312 hours in t-butanol [39] and 41% in 72 hours in [C2OHMIm][PF6] [45] (Table 2a). In the CaLB-catalyzed esterification of cis-9-octadecen-1-ol with ferulic acid, Chen et al. [48] obtained almost 100% conversion in 288 hours in a 25/75 mix of [C2OHMIm][PF6] or [C6MIm][PF6] and iso-octane (Table 2a), while Guyot et al. [49] obtained only 14% in 360 hours in a solvent-free system. With FAE from A. niger, the yields reported have been more similar across different solvents. Hence, Tsuchiyama et al. [50] obtained 70% conversion in 24 hours in the esterification of glycerol with sinapic acid in a nearly solvent-free system (i.e. with some dimethyl sulfoxide (DMSO) added), while Vafaiidi et al. [46] obtained 72.5% conversion in 24 hours in a 85/15 mix of [C2OHMIm][PF6] and buffer (Table 2b). Similarly, in the acylation of glucose protected at O-6 with vinyl acetate, for example, CrL catalysis produced higher yields at shorter reaction times, and exhibited a significantly improved regioselectivity in [C2OHMIm][PF6] and [C2OHMIm][PF6] as compared to similar reactions in tetrahydrofuran (THF) and chloroform, respectively [44]. Thus, the use of ILs may improve reaction efficiency in enzymatic synthesis reactions of this kind.

**Organic solvents vs. ionic liquids**

The major driving force in enzyme catalysis is the energy of binding between the enzyme and its substrate [51]. For binding to occur, the substrate must undergo desolvation, and the more energetically favorable this desolvation is the greater is the net binding energy. High solubility of a compound is caused by strong interactions between the compound and its solvent. Such substrate stabilization causes lower reaction rates because the energetic advantage of binding to the enzyme is decreased. The weaker interaction between a fairly hydrophilic substrate in a hydrophobic solvent increases the activity of the substrate and thus its ability to bind to the enzyme and react. By contrast, a more hydrophobic product (e.g. an acylated saccharide) exhibits stronger interactions with the hydrophobic solvents and is thus stabilized in the solvent. This product stabilization makes the reverse reaction (hydrolysis) unfavorable. Katsoura et al. [45] found that the ratio between ethyl ferulate (product) solubility and ferulic acid (substrate) solubility Xproduct/Xsubstrate (where X is solubility) in five ionic liquids and two organic solvents was directly correlated to the conversion yield independently of the lipase used, thus underlining the solvent effect. It is however possible that the reaction, although supposed to be a reversible, thermodynamically controlled reaction, was kinetically controlled because yields were often low when the initial rate was low [45].

Low substrate solubility has been shown to cause problems with regioselectivity in glucose acylations catalyzed by lipases. It has for example been reported that in acetone or THF where the desired 6-O-acetylated glucose is much more soluble than the glucose substrate, the increased solubility of the product caused CaLB, which usually favors primary hydroxyl groups, to catalyze another acylation yielding a 3,6-O-diacyl derivative [52]. This undesired loss of regioselectivity was not observed when substrate and product solubilities were more similar, for example, when alky glucosides rather than just glucose were used as substrates or when using hydrophilic [BF4]^- based ILs, where glucose solubility was found to be up to 100 times higher than in acetone of THF [52]. Similarly, regioselectivity was low in the hydrophobic [C3MIm][PF6], where glucose solubility was fairly low whereas much higher regioselectivity of CaLB was reported for transesterification of polyhydroxylated phenolics and flavonoid glucosides with vinyl butyrate in the hydrophilic [C3MIm][BF4] than in [C3MIm][PF6] or acetone because of higher glucose solubility in the [C3MIm][BF4] [53]. Thus, saccharide solubility is important for regioselectivity, at least when working with lipases known for...
their broad specificity. For FAEs, no problems with regioselectivity in saccharide esterifications have been reported so far; most FAEs are specific for primary alcohol groups, with a single exception (as discussed further in the section ‘Regioselectivity’).

ILs seem to be more suited for saccharide reactions than organic solvents when the reaction is not possible in a purely aqueous medium [52]. Indeed, saccharides are only soluble in appreciable amounts in a few organic solvents such as pyridine, DMF, DMSO and t-butanol, possibly because of the strong Lewis acid–Lewis base interactions between saccharides and these solvents [54]. Thus, these solvents have been extensively used for saccharide and carbohydrate ester synthesis with lipases and a few proteases, but they are harmful to less robust enzymes [3,55–58]. Because of their highly polar nature, the solubility of saccharides is also low in hydrophobic ILs. More hydrophilic ILs can dissolve larger amounts of carbohydrates such as cellulose, but these ILs may at the same time have a detrimental effect on enzyme stability unless carefully designed to avoid enzyme inactivation [13]. Nevertheless, several successful esterifications of glucose and methylglucose have been reported in IL systems, all with high regioselectivity using immobilized CaLB [43,52,59]. Recently, Lee et al. [60] introduced a new method for increasing the saccharide concentration in ILs by mixing the IL with an aqueous glucose, fructose, or sucrose solution before removing the water by vacuum evaporation, thus making a supersaturated saccharide solution in the IL. Ha et al. [42] studied the effect of IL hydrophobicity on this supersaturated system using mixtures of the hydrophobic [C4MIm][NTf2] or [C4MIm][NTf2] and the more hydrophilic [C4MIm][TFO] and found that the solubility of both glucose and lauric acid as well as enzyme activity increased with increasing hydrophilicity, while the stability of immobilized lipase B from Candida antarctica decreased with increasing hydrophilicity. Consequently, a 50/50 mixture of [C4MIm][TFO] and [C4MIm][NTf2] was chosen for further reactions [42]. Zhao et al. [13] designed new ILs which could solubilize cellulose (Avicel) in appreciable amounts (10–15 wt%) without completely inactivating CaLB. Their strategy was to decrease the concentration of the enzyme–harming anion by using very large anions. The best compromise between cellulose solubility and initial reaction rate was obtained in triethyl [2-(2-methoxyethoxy)ethoxy]-ethyl-ammonium acetate ((MeOEt)2EtOAc). Such a compromise between substrate (and product) solubility and enzyme stability is central when working with biocatalysis in non-aqueous media.

While the interest in replacing organic solvents with ILs has been driven by the factors mentioned above, ILs still have some disadvantages compared to organic solvents. These disadvantages concern recovery of non-volatile products and cost. Coating of an immobilized enzyme with IL coupled with application of this coated enzyme under solvent-free conditions, that is, using the liquid substrate as the ‘solvent’ rather than having the IL as the main solvent, has been proposed as a method to facilitate product removal and to reduce IL costs [61]. Indeed, transesterification of citronellol and direct esterification of methyl glucose with a range of fatty acids using IL-coated immobilized CaLB (Novozym 435) have been reported [43,62]. Because hydroxycinnamic acids do not readily melt at ‘usual’ reaction temperatures, a similar approach cannot be used in the synthesis of hydroxycinnamate esters. Recently, van Doorslaer et al. [63] demonstrated solvent-resistant nanofiltration to be a useful method for separation of non-volatile products from IL reaction mixtures, the number of filtration steps required to obtain >95% rejection of the IL ranging from one to three depending on the molecular weight of the IL.

**Enzyme stability in non-conventional media**

Due to the quite long reaction times required for enzymatic synthesis reactions, including those in non-conventional media (summarized in Tables 1a, 1b, 2a, 2b) it is exceedingly important that the enzymes are stable in these reaction media. Enzyme stability is often better in organic solvents than in aqueous media [64]. Enzymes are generally only suspended – not dissolved – in organic solvents, and do not tolerate strongly interacting solvents, except water. The increased stability is however only observed in weakly interacting organic solvents and not in very hydrophilic ones such as DMSO and formamide which dissolve and hence inactivate enzymes [65]. Indeed, Faulds et al. [66] recently showed that the use of DMSO, acetone, and 1,4-dioxane as co-solvents in aqueous systems caused inactivation of four different FAEs, except at low concentrations. In organic solvents, increased enzyme stability often occurs at the price of a reduced reaction rate because of reactant stabilization, that is, increased reactant solubility, transition state destabilization, reduced flexibility, and/or suboptimal pH conditions, if care is not taken to prevent such a reduction [51]. The desire to improve low reaction rates has been a driving force in the introduction of ILs as alternative non-aqueous media: their highly polar nature may remedy transition state destabilization and the loss of flexibility because of stripping of water molecules. In general, catalytic efficiency is increased in ILs compared to organic solvents as discussed further in ‘Enzymatic synthesis of hydroxycinnamate esters in ionic liquid systems’.

Water-immiscible ILs generally do not inactivate enzymes, and it has been reported for both lipases and FAEs that only when the IL – and especially its anion – exhibits too strong hydrogen bonding capacity does the IL harm the enzyme [25,27]. AnFaeA has been found to show appreciable stability in [PF6][IL]–based IL systems for more than two hours at temperatures up to 40 °C, but the FAE from Ultrafló L and especially AndFaeC are unstable in these systems [25] (Table 2b). The difference in stability among these enzymes, and notably the stability of AnFaeA, were explained by the similarity in structure of AnFaeA to IL-compatible lipases such as RmL and TIL [25]. The FAEs were rapidly inactivated in the [BF4][IL]–based systems and only 1% conversion was achieved with AnFaeA in [BMIm][BF4] [25] (Table 2b).

Enzyme stability and activity in ILs are governed by several factors recently reviewed by Zhao [67]. These factors are hydrogen bond basicity, nucleophilicity, hydrophobicity, viscosity, and in aqueous IL systems also ion kosmotropicity. No universal prediction tool for enzyme-IL compatibility has been developed because of the complex nature of IL-enzyme systems, but there seems to be general consensus that enzyme stability in ILs is favored by low hydrogen bond basicity and nucleophilicity as well as high viscosity and especially an adequately high hydrophobicity [67]. In aqueous systems of hydrophilic ILs, where the IL is hydrated and dissociates into individual ions, the importance of having a chaotropic cation and especially a strong kosmotropic anion has been pointed out [68]. Generally, the anion has a larger effect on enzyme stability than the cation does [25,26], possibly because the cation is
significantly larger than the anion and has its positive charge delocalized over most of the molecule whereas the anion is more compact and has a more localized negative ion charge [69]. Similarly, it has also been suggested that large anions with the negative charge delocalized on more atoms are more stabilising [70], and that larger anions are less destabilising because they are sterically demanding, that is, their size will require many hydrogen bonds in the protein matrix to be broken to form a few new ones, making such an interaction less favorable [27]. Hydrophobicity, nucleophilicity, and hydrogen bond basicity should not be confused with each other, but the general picture is that the more hydrophilic IL anions are also the ones with the higher nucleophilicity and hydrogen bond basicity, rendering them more probably to interact with the enzyme thus causing it to change conformation – and vice versa. Generally, hydrophobic ILs with [PF₆]⁻ and [Tf₂N]⁻ anions are stabilizing while hydrophilic ILs with [NO₃]⁻, [lactate]⁻, [EtSO₄]⁻, and in some cases also [BF₄]⁻ are destabilizing to enzymes [25,27,45,67]. In biphasic aqueous-IL systems, enzymes have little or no contact with the IL phase and enzyme-IL incompatibilities are therefore not to be expected [71]. However, some enzyme inactivation must be expected to occur at the interface between the two phases. Indeed, for AnFaEA the thermal stability was lower in a system of the water-immiscible [C₄MIm][PF₆] and aqueous buffer than in pure aqueous buffer and was comparable to the thermal stability observed in the system comprising the water-immiscible [C₂OHMim][PF₆] and aqueous buffer [25]. The choice of solvent may often be a compromise between maintaining enzyme stability and increasing substrate and/or product solubility.

Thermodynamically based design of enzymatic hydroxycinnaminate saccharide esterifications

When working with enzymatic synthesis of hydroxycinnamate esters in non-conventional media – or enzymatic synthesis in general – solid–liquid equilibria (SLE), that is, substrate and product solubility in the liquid phase, and liquid–liquid equilibria (LLE), that is, equilibrium between a mixture of solvents are the most interesting thermodynamic properties to model and/or predict. Vapor–liquid equilibria (VLE) are important in systems of ILs and supercritical CO₂. Thermodynamic models may also be used for modeling the relationship between water activity and water content as well as for prediction of enzyme stability in non-conventional media. Thermodynamic data and modeling on hydroxycinnamic acid-containing systems are scarce, but this section reviews the work which has already been done and provides suggestions for further work in this area.

Solid–liquid equilibria (SLE): prediction of hydroxycinnamic acid solubility

Data and thermodynamic modeling on systems containing hydroxycinnamic acids are scarce. In a series of recent studies, Voutas and co-workers measured the solubility of three hydroxycinnamate methyl esters, namely methyl ferulate (MFA), methyl sinapate (MSA) and methyl p-coumarate (MpCA), in several organic solvents [72], imidazolium-based [PF₆]⁻ and [BF₄]⁻ ILs [73] and their mixtures [74] and modeled the systems with the activity coefficient models NRTL and UNIQUAC. Except for the system with MSA and t-butanol, both models correlated the data successfully in the single solvent systems with UNIQUAC being only slightly better than NRTL (absolute average deviation (AAD) generally below 10%). Using the binary interaction parameters between solute and solvent molecules obtained in these single solvent studies, Panteli and Voutas [74] evaluated the ability of NRTL and UNIQUAC to correlate solubility data in mixtures of ILs and organic solvents (SLE). The binary interaction parameters between the two solvents were either set to zero, estimated by fitting literature LLE data, or one of the two interaction parameters was estimated by fitting to a single data point on the solubility curve while the other was set to zero. As expected, the latter approach gave the best results. This is expected because it will almost always give a better result when a model is developed to fit a certain set of data as opposed to predicting data beyond the experimental data set. For NRTL, estimating the binary interaction parameters by fitting literature LLE data had only little or in some cases negative effect on the model performance. This is an indication that the model does not capture the true LLE behavior of these systems, and that more advanced models may be required to describe them. Indeed, UNIQUAC which is a slightly more advanced activity coefficient model benefitted more from estimating the binary interaction parameters by fitting literature LLE data. This approach significantly improved the performance of UNIQUAC in all of the systems with MSA, while the improvement was less pronounced or in some cases even absent for the MFA and MpCA systems. Generally, NRTL gave better results than UNIQUAC when using binary interaction parameters set to zero, while this overall tendency was reversed when fitting to literature LLE data. In general, both models performed best for the MFA systems, where in most cases the absolute deviation was less than 10% [74]. Currently, the available data do not offer any explanation as to why the MFA systems were more easily modeled.

To test the predictive potential of COSMO-RS in hydroxycinnamic acid systems, the model was tested on the same systems of organic solvents and ILs for its ability to predict solubilities of MFA and MSA (Fig. 3, details on the calculations are given in the figure legend).

The COSMO-RS predictions in fact fit the data rather poorly, but these results (Fig. 3) are included because of the lack of similar results in this field, and because the discussion of the COSMO-RS predictions permits the highlighting of some important points: hence, although the COSMO-RS predictions substantially overestimate the solubility of MFA and MSA in the pure organic solvents, the COSMO-RS predictions are qualitatively correct except for t-butanol (Fig. 3). In the UNIQUAC and NRTL models of MSA solubility, the solvent t-butanol also caused problems [72]. For the pure IL systems, COSMO-RS significantly underestimated the solubility, but it again performs qualitatively correctly for MFA. For MSA, the predictions are also qualitatively correct when looking at [PF₆]⁻-based systems and [BF₄]⁻-based systems separately (Fig. 3). For the mixtures of organic solvents and ILs, the solubilities are significantly overestimated for MFA, and qualitatively correct only when looking at t-butanol systems and ethyl acetate systems separately. The predictions for the mixtures are slightly more accurate for MSA, but again an underestimation is observed (Fig. 3). The deviations in MSA solubility between these mixtures are too small to give qualitatively correct predictions with COSMO-RS, but the general picture that [C₄MIm]⁺ gives higher solubility of MSA than [C₄MIm]⁺ in the [BF₄]⁻ mixtures
Experimental data for solubility of methyl ferulate (MFA; left) and methyl sinapate (MSA; right) in organic solvents [72], ILs [73], and their mixtures with $x_{\text{Org./m}} = 0.5$ [74] at 30°C compared to the solubilities calculated for this work by COSMO-RS version 2.1 revision 01.06 using $\Delta_{\text{H}}^{\text{m}}$ and $T_{\text{m}}$ data from [73]. The COSMO-files for MFA, MSA, and [C$_2$OHMIm]$^+$ were made with Gaussian03 [105]. The structures were first optimized with the semi-empirical PM3 method and then refined with B3LYP, MSA and MFA first with the basis set 6–31G and then with 6–31G(d), while [C$_2$OHMIm]$^+$ was refined first with the basis set 6–31 and then with 6–311++G(d,p).

is conveyed (Fig. 3). Thus, with average percentage deviations ranging from 19% to more than 200% in the investigated systems, COSMO-RS does not provide quantitatively accurate predictions. However, the predictions of hydroxycinnamate methyl ester solubility are generally qualitatively correct and therefore the model is a useful tool for preliminary solvent screening for systems of organic solvents and/or ILs containing hydroxycinnamic acids, although it clearly cannot yet replace experimental work for more accurate solubility data. It should be emphasized that the results of local composition models such as NRTL and UNIQUAC obtained by Panteli and co-workers [72–74] and those of COSMO-RS obtained here are not entirely comparable. NRTL and UNIQUAC are models suitable for correlations, while the COSMO-RS results are predictions. Unlike the activity coefficient models, COSMO-RS does not require experimental data for parameter fitting and can therefore be extended to a wide range of completely new systems. It should be noted that compatibility of both substrates and enzyme with the solvent(s) must be accommodated and may significantly constrain the possible solvents for a given reaction because a compromise between substrate solubility and enzyme stability is often required. Both aspects can be explored through the use of thermodynamic tools [12,25]. To develop the use of thermodynamic tools for SLE predictions in hydroxycinnamic acid-containing systems and improve the performance of these tools, it is clear that future investigations must be expanded to include more, relevant systems.

**Prediction of liquid–liquid equilibria (LLE) in ionic liquid systems**

Many non-conventional enzymatic systems contain at least two phases: the relatively non-polar continuous bulk phase which functions as a reservoir or transport medium for (some of) the substrates and products and the relatively polar phase containing the enzyme. The reaction takes place in the polar phase or in some cases at the interface [4]. The main interest in the present context is in systems that accommodate saccharide solubility and enzyme stability, namely IL-containing systems and surfactant-free micro-emulsions. When working with ILs and a co-solvent with which the IL is not completely miscible at the given working temperature and pressure, for example, water or iso-octane [48], it is useful to know the liquid-liquid equilibria behavior of the system, that is, how much of the IL is dissolved in the co-solvent and vice versa and the amount of each of the two phases in a two-phase system.

LLE for many IL/organic solvent or IL/water systems have been modeled with the activity coefficient models NRTL [75,76] and UNIQUAC [77], the group contribution model UNIFAC [78], and COSMO-RS [79–81], but only a few systems have been modeled with more than one model to compare the applicability of the models to the various mixtures. Some systems for which more models have been applied and the modeling results given as absolute average deviation in the molar fraction of IL, $x_{\text{IL}}$, in % (AAD%) are shown in Table 3.

It can be seen that UNIQUAC correlated the data fairly successfully [77; Table 3]. Alevizou et al. [78] extended the UNIFAC model to mixtures containing [C$_3$C$_3$Im][PF$_6$] ILs by determining interaction parameters between the imidazolium and hexafluorophosphate groups and the main existing UNIFAC groups, CH$_3$, OH, and H$_2$O. The predictive capability was good, although the alkyl chain length increased in [C$_3$MIm][PF$_6$], the performance of UNIFAC decreased, both in the 1-alcohol mixtures where UNIFAC over-estimated the upper critical solution temperature (UCST) and with water (Table 3). A similar trend was observed for COSMO-RS for the 1-alcohol mixtures (Table 3). The AAD% levels were generally much higher for COSMO-RS, but again it must be stressed that COSMO-RS provides predictions without the need for parameter fitting. In the systems of [C$_3$MIm][PF$_6$] and ethanol, 1-propanol, or 1-butanol, the COSMO-RS predictions appear to be qualitatively correct, but
the prediction is quantitatively poor with large overestimations of UCST [80]. In the systems of [C₄MIm][PF₆] and 1-butanol, the difference between calculated and experimental UCST is small and the predictions are qualitatively correct, but xₘ is overestimated, giving rise to the high deviations [81] (Table 3). The COSMO-RS predictions in the water/[C₄MIm][PF₆] systems show quite low AAD% (Table 3) and they are qualitatively correct; the model slightly underestimates xₘ for water/[C₄MIm][PF₆] in the water-rich phase side and overestimates xₘ for all three systems in the IL-rich phase side [79]. Clearly, more IL/water and IL/organic solvent systems need to be modeled to develop the full potential of these tools for LLE predictions.

Surfactant-free microemulsions are ternary mixtures of a hydrocarbon, a polar alcohol and water or aqueous buffer. Zoumpangioti et al. [82] used the ternary LLE phase diagram to choose monophasic mixtures for their study on lipases in microemulsions. Indeed, data for these ternary mixtures, which are often composed of hexane, 1- or t-butanol, and water (Table 1a), are readily available, for example, from the NIST database (http://srdata.nist.gov). Islam et al. [83] correlated data for hexane/1-butanol/water with activity coefficient models UNIFAC and NRTL and found the three-parameter NRTL superior because the two-parameter UNIQUC could not adequately capture the behavior of the immiscible hexane–water pair. Magnusson et al. [84] provided UNIFAC parameters and ternary phase diagrams for water/1-propanol/hexane, water/2-propanol/hexane and many other related ternary systems. It is thus plausible that surfactant-free microemulsions can be adequately modeled with these classic thermodynamic tools.

**Water activity vs. water content**

Although the water content is generally quite low in enzyme-catalyzed synthetic reactions to avoid unwanted hydrolysis, the water content greatly affects the kinetics [21]. When choosing an enzyme for synthesis in non-conventional media, it is important to consider that the so-called water activity profiles, that is, enzyme activity vs. water activity in the reaction mixture, differ from enzyme to enzyme [4]. Water activity is a much more useful measure of how much water is required for a given enzyme to work in non-conventional media than water content or concentration, because the water concentration required for optimal catalysis may differ from solvent to solvent while the required water activity is the same and dependent on the enzyme only [4,21,85]. Furthermore, it has been reported that the water activity profiles for four different lipases were independent of the reaction used for determining lipase activity [86]. Because the relationship between water content and water activity differs from solvent to solvent, each reaction system must be pre-equilibrated to a given optimal (enzyme dependent) water activity before reaction. To ease this work, Voutsas et al. [87] presented a thermodynamic method for predicting water activity in a wide range of organic solvents on the basis of the UNIFAC group contribution model. The authors concluded that UNIFAC is not recommended for (very) hydrophobic solvents, but it predicts the water activity satisfactorily for solvents with substantial solubility of water. Both for the water activity and the effect of reactants on water activity, the modified UNIFAC [18] performed better than the other UNIFAC variants tested by Voutsas et al. [87]. With UNIFAC parameters already existing for most organic solvents and the current work on expanding UNIFAC to ILS [78] it is probable that this will be a useful tool for predicting water activity in various (not too hydrophobic) solvents in the future. More work should however be done, expanding the investigations to more solvents.

**Prediction of enzyme stability in non-conventional media**

As reviewed in ‘Enzyme stability in non-conventional media’, enzyme stability in non-conventional media depends on several factors and is obviously an essential prerequisite for enzyme catalysis. However, the efforts to predict enzyme stability rather than using time-consuming empirical methods are almost non-existent. Recently, it was shown that COSMO-RS-generated σ-profiles could be used for explaining why AnFaeA was stable in [PF₆]⁻–based IL systems, but rapidly inactivated in [BF₄]⁻–based IL systems in terms of hydrogen bonding capacity given as screening charge density, σ, which is a local measure of polarity for each molecule [25]. Thus, the COSMO-RS method holds potential for

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**TABLE 3**

Summary of LLE modeling results for IL-containing systems modeled with more than one thermodynamic model (COSMO-RS, UNIFAC, or UNIQUC) given as absolute average deviation in the molar fraction of IL, xₘ, in % (AAD%).

<table>
<thead>
<tr>
<th>System</th>
<th>COSMO-RS</th>
<th>UNIFAC</th>
<th>UNIQUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAD% in xₘ</td>
<td>AAD% in xₘ</td>
<td>AAD% in xₘ</td>
</tr>
<tr>
<td>Ethanol/[C₄MIm][PF₆]</td>
<td>100</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>1-Propanol/[C₄MIm][PF₆]</td>
<td>56</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>1-Butanol/[C₄MIm][PF₆]</td>
<td>50</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>1-Butanol/[C₅MIm][PF₆]</td>
<td>66</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>1-Butanol/[C₆MIm][PF₆]</td>
<td>90</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>1-Butanol/[C₇MIm][PF₆]</td>
<td>98</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>1-Butanol/[C₈MIm][PF₆]</td>
<td>100</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Water/[C₄MIm][PF₆]</td>
<td>19</td>
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<td></td>
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<tr>
<td>Water/[C₅MIm][PF₆]</td>
<td>12</td>
<td>5</td>
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<tr>
<td>Water/[C₆MIm][PF₆]</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*Marsh et al. [80].

*Wu et al. [81].

*Freire et al. [79].

*Alevizou et al. [78].

*Sahandzhieva et al. [77].
being employed in preliminary solvent screening not only for substrate/product solubility, but also for enzyme stability. Whether the predictive power of this tool can be expanded to cover more ILs and organic solvents is however yet to be explored.

**Lipase or feruloyl esterase?**

Lipases (EC 3.1.1.3) and FAEs (EC 3.1.1.73), respectively, are the enzyme types that have mainly been used to catalyze hydroxycinnamate (trans)esterifications (Tables 1a and 1b). Lipases are known to be more stable than FAEs in non-conventional media, display high activity at low water content [4], and give high yields in many of the reactions used here (Tables 1b and 2a). FAEs are in contrast generally more selective, that is, FAEs exhibit higher substrate specificity than lipases.

**Affinity for hydroxycinnamic acids**

CaLB seems to accept numerous phenolic acid substrates [88], and does not exhibit the same strict specificity for the length of the aliphatic chain between the aromatic ring and the carboxyl group as FAEs do [89–91], and successful reactions with CaLB and hydroxycinnamic acids have been reported, especially in IL systems, for example, achieving reaction yields above 95% in a 96-hour reaction [41] (Table 2a). Nevertheless, lipases may not be the optimal choice of catalyst for synthetic, enzyme-catalyzed reactions with hydroxycinnamic acids. Indeed, several reports have shown that the presence of the α,β-unsaturation in the aliphatic chain between the aromatic ring and the carboxylic acid found in cinnamate derivatives dramatically lowers or even eliminates the activity of lipases from Candida antarctica (CaLB), Rhizomucor miehei (RmL) and Candida rugosa (CrL), esterase from Fusarium oxysporum (FoE), and cutinase from Fusarium solani (FsC), which all readily catalyze the esterification of the saturated variants, for example, p-hydroxyphenylpropionic acid [45,47,82,88]. The lowered lipase activity may be explained by the active site structure. Hence, it is known that the active acyl binding site structure in CaLB consists of a hydrophilic ‘bottom’ region, where the catalysis takes place, and a hydrophobic ‘top’ region, the so-called hydrophobic crevice, where the aromatic ring should fit in [88]. The α,β-unsaturation in the aliphatic chain of cinnamates reduces the flexibility in the aliphatic chain, in turn producing a poorer substrate fit and consequently lower CaLB activity [88]. The loss of lipase activity observed for hydroxycinnamic acids is however not the only matter of steric effects caused by the unsaturated aliphatic chain. Indeed, the results by Guyot et al. [49], Katsoura et al. [45], and Yang et al. [38] indicate that the simultaneous presence of a hydroxyl group in the para-position and the double bond in the aliphatic chain typical for hydroxycinnamic acids give rise to an even greater loss of lipase activity because of resonance effects in the conjugated system, that is, between the hydroxyl substituent and the reacting carboxylic acid. The presence of the p-hydroxyl group without the double bond did not have a similarly dramatic effect [88].

**Regioselectivity**

Otto et al. [88] reported that the high regioselectivity observed for CaLB was caused by steric repulsion in the alcohol binding site preventing other orientations of the glucose molecule. The binding site could also accommodate glucose substituted at C1 with hydrophobic moieties [88]. Similarly, Yang et al. [38] reported that PeL was highly selective for O-6 on arbutin. However, CaLB has been found to catalyze esterification on secondary hydroxyl groups on several occasions. For instance, CaLB readily catalyzes the acylation of the flavonoid glucoside rutin, which has no primary hydroxyl groups on the saccharide moiety, but is then very specific for O-3 [92]. Furthermore, when substrate solubility is low, CaLB loses regioselectivity and catalyzes diacetylation [52,53]. For FAEs it has generally been found that (trans)esterification with a hydroxycinnamic acid only takes place at primary hydroxyl groups on the acceptor [33,92–94]. It is therefore surprising that Couto et al. [37] could use 1-butanol as a microemulsion solvent in the enzymatic formation of feruloylated monosaccharides because this primary alcohol must be expected to compete with the monosaccharides in the esterification reaction. Indeed, the same type of hexane/1-butanol/water microemulsion has been used for producing butyl hydroxycinnamates by enzymatic transesterification [94]. However, Couto et al. [37] analyzed their products by LC-MS and did not mention any formation of a butyl ferulate byproduct. Only a FAE from Humicola insolens (reported to be present in the commercial blend Pentapan 500 BG [95]) has been reported to be specific towards secondary alcohols with high enantiopurity for the R-configuration on the alcohol-bearing stereocenter [95]. For AnFaEa, Vafadhi et al. [94] have reported 78% conversion in 120 hours in the transesterification of MSA with 1-butanol, while the conversion was only 9% with 2-butanol in 120 hours. This result indicates that AnFaEa can catalyze (trans)esterification with secondary hydroxyl groups (at least when no primary hydroxyl groups are present), but much prefers the primary ones. No cases of diesterification have been reported for FAEs on the polyhydroxylated saccharides.

**Vinyl activation required?**

Except for one study [34], all the reported lipase-catalyzed hydroxycinnamate saccharide ester syntheses have used hydroxycinnamate vinyl or 2,2,2-trifluoroethyl (TFE) esters as acyl donors, and these TFE esters had to be chemically synthesized before reaction (Table 1b). In transesterification of the triglyceride triolein with CaLB, greater reaction efficiency was obtained with vinyl ferulate compared to ethyl ferulate as substrate, regardless of the reaction conditions [96]. In case the objective is to upgrade bio compounds, this need for a chemical synthesis step before the enzymatic reaction may be an obstacle. Ways to circumvent this requirement do exist, as recently shown by Ishihara et al. [34], but the requirement for special synthesized acyl donors is a clear disadvantage for the use of lipases in these reactions. Many of the FAE-catalyzed reactions are also transesterifications, notably with methyl ferulate, but ferulic acid has been used as well [37] (Table 1a). Furthermore, in the synthesis of glycerol sinapate in ILs, AnFaEa was found to give higher yields in the direct esterification than in the transesterification [46].

**Conclusions**

Synthesis of hydroxycinnamate esters can be catalyzed by lipases and feruloyl esterases. The available data for such enzymatic saccharide acylation reactions with hydroxycinnamates show that:

- Use of ILs may improve enzymatic catalysis efficiency in terms of higher yields and shorter reaction times compared to organic solvent and solvent-free systems as seen in synthesis of hydroxycinnaminate alcohol esters.
• Use of supersaturated saccharide-IL solutions may improve catalytic efficiency.

When designing an enzymatic saccharide acylation reaction the following points should be considered to maximize reaction rate, selectivity, enzyme stability, and reaction yields:

• Saccharide solubility and enzyme stability should be accommodated through careful selection of (a mixture of) ILs or use of surfactant-free microemulsions. A compromise between solubility and stability may be required.

• A high ratio \( X_{\text{product}} / X_{\text{substrate}} \) (where \( X \) is solubility) increases reactivity, but too low \( X_{\text{substrate}} \) can have adverse effects on regioselectivity.

• Choice of enzyme class should be considered: for systems containing hydroxycinnamic acids FAEs may to be the superior choice, while the more robust lipases may be advantageous when working with other aromatic acids.

• Enzymes successfully applied in microemulsions and organic solvent systems should be tested in IL systems.

• Choice of hydroxycinnamic acid donor could be changed from the widely used ferulic acid to sinapic acid or other hydroxycinnamic acids on the basis of the enzyme’s affinity.

• Continuous removal of MeOH by vacuum may improve reaction yields in transesterification reactions with hydroxycinnamates methyl esters.

Application of thermodynamic tools to these novel complex reaction systems is limited by the lack of data, but predictive thermodynamic tools do exist. The use of thermodynamic tools can ease the solvent design process and enhance the understanding of the reaction system as follows:

• COSMO-RS can be used for predicting substrate and/or product solubility in a preliminary solvent screening, being aware of its quantitative inaccuracies.

• COSMO-RS can be used for predicting enzyme stability in ILs as part of a preliminary solvent screening.

• Activity coefficient models, UNIFAC and/or COSMO-RS can be used to assess the LLE of the solvent system depending on the data and parameter availability for a given system.

• UNIFAC may be used for predicting water activity in various solvents, except very hydrophobic ones.

Acknowledgements

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Improvement of trans-sialylation versus hydrolysis activity of an engineered sialidase from *Trypanosoma rangeli* by use of co-solvents

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Abstract

Biocatalytic trans-sialylation is relevant for designing biomimetic oligosaccharides such as human milk oligosaccharides. $t$-Butanol and ionic liquids EAN (ethylammonium nitrate), [MMIm][MeSO₄] (1,3-dimethylimidazolium methyl sulfate), and [C₂OHMIm][PF₆] (1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate) were examined as co-solvents to improve the synthesis vs. hydrolysis ratio in the trans-sialylation of lactose catalysed by an engineered sialidase from Trypanosoma rangeli, Tr6. Use of 25% (v/v) $t$-butanol as a co-solvent significantly increased the 3’-sialyllactose product concentration by 40% from 1.04 ± 0.09 mM to 1.47 ± 0.01 mM. The enzymatic synthesis vs. hydrolysis ratio correspondingly increased 1.2 times. 1% (v/v) [C₂OHMIm][PF₆] and 1-2.5% (v/v) EAN also improved the synthesis vs. hydrolysis ratio, up to 2.5 times, but simultaneously decreased the 3’-sialyllactose yields, probably due to enzyme inactivation caused by the ionic liquid. [MMIm][MeSO₄] had a detrimental effect on trans-sialylation yield and on the ratio between synthesis and hydrolysis.

Keywords

3’-sialyllactose, casein glycomacropeptide (CGMP), co-solvent, ionic liquids, $t$-butanol, trans-sialidase, Trypanosoma rangeli
Introduction

A sialidase from *Trypanosoma rangeli* has previously been genetically modified to provide a significantly increased *trans*-sialidase activity (Paris et al. 2005). Recently, it was shown that this mutant enzyme, Tr6, could be expressed in *Pichia pastoris* after codon-optimization, and used for producing 3’-sialyllactose in gram quantities using cheap dairy industry side stream components as substrates for the *trans*-sialidase reaction, namely casein glycomacropeptide (CGMP) and lactose (Michalak et al. 2013). However, while the introduction of the six amino acid substitutions that make Tr6 different from the wild type *T. rangeli* sialidase dramatically increased the *trans*-sialidase activity, it also increased the $k_{cat}/K_m$ of the hydrolysis of 3’-sialyllactose 1.8-fold (Paris et al. 2005). Obviously, both high substrate hydrolysis (primary hydrolysis) and high product hydrolysis (secondary hydrolysis) rates are undesirable when it is intended to promote the *trans*-sialylation reaction for synthesis of sialylated glycosides (Figure 1).

In another *trans*-glycosylation reaction catalysed by glycoside hydrolases, namely the β-galactosidase-catalysed synthesis of *N*-acetyl-D-lactosamine (LacNAc) from *N*-acetyl-D-glucosamine (GlcNAc) and a β-galactosyl donor such as lactose or *p*-nitrophenyl-β-galactopyranoside, non-aqueous co-solvents have been used for improving *trans*-galactosylation yields. These co-solvents include organic solvents (Brienda et al. 2010), glycerol-based bio-solvents (Sandoval et al. 2013), and ionic liquids (ILs) (Kaftzik et al. 2002; Sandoval et al. 2012). It is hypothesized that the co-solvents improve *trans*-galactosylation yields by improving the selectivity of the enzyme for the desired glycosyl acceptor compared to water - an effect that exceeds what may be explained by the concomitant change in water activity (Lang et al. 2006).
The objective of the present study was to examine whether the co-solvent approach to improve glycosidase-catalysed synthesis can be used for improving trans-sialylation, using Tr6 and the production of 3'-sialyllactose as an example (3'-sialyllactose being a human milk oligosaccharide model case compound). The overarching objective is to improve the ratio between biocatalytic synthesis and hydrolysis, i.e. the [3'-sialyllactose]/[sialic acid] ratio, without compromising 3'-sialyllactose yields. Bridiau et al. (2010) found that t-butanol had the least detrimental effect on β-galactosidase stability of the six organic co-solvents tested; in fact, t-butanol stabilized the enzyme at concentrations around 10% (v/v). Therefore, t-butanol is included as an organic co-solvent in the current study. Furthermore, three different ILs are tested as co-solvents, including EAN and [MMIm][MeSO₄], which have had positive effects on tyrosinase (Goldfeder et al. 2013) and β-galactosidase specificity (Kaftzik et al. 2002; Lang et al. 2006), respectively, and [C₂OHMIm][PF₆], which had the least detrimental effect on feruloyl esterase stability in the synthesis of glycerol sinapate (Zeuner et al. 2011).

Insert Figure 1 here.

Materials & Methods

Chemicals

Casein glycomacropeptide (CGMP) in the form of the commercially available product Lacprodan® CGMP-20, containing a total of 5.7% (w/w), equivalent to 0.2 mmol/g dry matter, of covalently linked sialic acid, was a gift from Arla Foods amba (Viby, Denmark).

Before use, low molecular weight impurities in the CGMP solution were removed by filtration on a 5 kDa membrane (Sartorius AG, Goettingen, Germany) as a technical
precaution relating to HPAEC analysis. Ethylammonium nitrate (EAN) was purchased from Iolitec GmbH (Heilbronn, Germany). The 3’-sialyllactose standard was purchased from Carbosynth (Compton, United Kingdom). Ionic liquid 1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate ([C2OHMIm][PF6]) was prepared according to the method described by Branco et al. (2002). β-D-lactose, N-acetylneuraminic acid (sialic acid) standard, 1,3-dimethylimidazolium methyl sulfate ([MMIm][MeSO4]), t-butanol (2-methyl-2-propanol), and all other chemicals were purchased from Sigma-Aldrich.

**Production of Tr6 and trans-sialidase activity assay**

His-tagged (6 x His) Tr6 was genetically modified and expressed in *Pichia pastoris* as described previously (Michalak et al. 2013). The enzyme was purified by Ni²⁺ affinity column chromatography using a 5 mL HisTrap™ HP column (GE Healthcare, Uppsala, Sweden). After purification, salts were removed and the enzyme concentrated in 5 kDa Hydrosart® Vivaspin spin columns (Sartorius AG, Goettingen, Germany), before adding 10% (v/v) glycerol to the final solution for enzyme stabilization. The enzyme was kept at -80°C until further use.

Tr6 was assayed for trans-sialidase activity using β-D-lactose as acceptor and CGMP as donor of sialic acid at the optimal conditions established previously (Michalak et al. 2013): 117 mM lactose, 4.6 mM sialic acid bound in CGMP, 25°C, and pH 5.5 (10 mM citrate buffer). Co-solvent concentrations ranged from 0.1% (v/v) to 25% (v/v). The reaction was monitored for up to 100 min and terminated by thermal inactivation of the enzyme at 90°C for 10 min. The enzyme dosage was 1.2% E/S (w/w), where the substrate (S) equals the total sialic acid covalently bound to CGMP. After reaction, the samples were filtered in 5 kDa
polyethersulfone spin columns (Vivaspin, Sartorius AG, Goettingen, Germany) for removal of CGMP (incl. partially de-sialylated CGMP) prior to HPAEC analysis. All experiments were made in duplicates.

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Separation and quantification of 3’-sialyllactose and sialic acid were achieved by HPAEC-PAD analysis using a CarboPac™ PA100 (4 mm x 250 mm) analytical column equipped with a CarboPac™ PA100 (4 mm x 50 mm) guard column on a Dionex ICS-3000 system (all from Dionex Corp., Sunnyvale, CA). The eluent system included deionized water (A), 0.5 M NaOH (B), and 0.5 M NaOAc with 0.02% (w/v) NaN₃ (C). Using a flow rate of 1 mL/min, elution started with isocratic elution at 89:10:1 (% A:B:C, by vol.) from 0 to 3 min followed by a linear gradient from 89:10:1 (% A:B:C, by vol.) to 60:10:30 (% A:B:C) from 3 to 19 min. Strongly retained anions were then removed by isocratic elution at 5:10:85 (% A:B:C, by vol.) for 4 min. Finally, the column was re-equilibrated at 89:10:1 (% A:B:C, by vol.) for 5 min. For samples containing organic or IL co-solvent, co-solvent-containing standard samples were included to quantify the effect of the co-solvents on sample preparation and analysis.

Statistics

One-way ANOVA for determination of statistical significance was made in Minitab 16 (Minitab Inc., State College, PA, USA). Statistical significance was established at \( p < 0.05 \).
Results & Discussion

Insert Figure 2 here.

Due to product hydrolysis, Tr6-catalysed 3’-sialyllactose production reached a maximum after 40-60 min after which the 3’-sialyllactose concentration declined (Figure 2). Addition of 10-25% (v/v) t-butanol increased the 3’-sialyllactose concentration and lowered the degree of product hydrolysis (Figure 2). At t-butanol concentrations of 20-25% (v/v) the 1.3-1.4-fold increase in 3’-sialyllactose concentration was statistically significant (Table 1). Significant increases in the ratios between synthesis and hydrolysis, i.e. the [3’-sialyllactose]/[sialic acid] ratios, were found at 15-25% (v/v) t-butanol after 40 min (where 3’-sialyllactose concentrations were maximal) and at 10-25% (v/v) t-butanol after 100 min of reaction (Table 1). The relative increase in the synthesis/hydrolysis ratio after 40 min reaction with 15-25% (v/v) t-butanol was mainly a result of the increased 3’-sialyllactose concentration since sialic acid levels were not significantly reduced by the co-solvent indicating a higher total enzyme activity (Figure 2), whereas the higher [3’-sialyllactose]/[sialic acid] ratio observed after 100 min at 10-25% (v/v) t-butanol may be explained by a lowered product hydrolysis (Figure 2). Concentrations of t-butanol below 10% (v/v) had no effect on 3’-sialyllactose or sialic acid concentrations (data not shown). Bridiau et al. (2010) reported that the activity of β-galactosidase from Bacillus circulans (BcβGal) increased at t-butanol concentrations of 5-10 % (v/v); the results obtained for the Tr6-catalysed 3’-sialyllactose production thus indicate that relatively high co-solvent levels of t-butanol are required for achieving a significant improvement in the [3’-sialyllactose]/[sialic acid] ratio.

Insert Table 1 here.

The ionic liquid [MMIm][MeSO₄] successfully improved trans-galactosylation yields of BcβGal by suppressing both substrate and product hydrolysis when used at 30%
(v/v) (Kaftzik et al. 2002). For Tr6 however, in the present study, 0.1-1% (v/v) [MMIm][MeSO₄] had a detrimental effect on the synthesis vs. hydrolysis ratio as well as on the overall 3’-sialyllactose concentration (Table 2). For [C₂OMIm][PF₆] and EAN, increasing the co-solvent concentration to 1-2.5% (v/v) had a positive effect on the [3’-sialyllactose]/[sialic acid] ratio at reaction times of 60-100 min (Table 2), mainly because the 3’-sialyllactose concentration increased throughout the reaction time and sialic acid levels were lowered compared to the reaction in pure aqueous medium (data not shown). However, this relative increase in the synthesis versus hydrolysis came at the expense of trans-sialylation yields: at 1% (v/v) [C₂OHMIm][PF₆] the 3’-sialyllactose concentration was only 57% of that obtained without co-solvent, and for EAN the 3’-sialyllactose concentration decreased to 36 and 9% of the concentration without co-solvent when increasing the co-solvent concentration to 1 and 2.5% (v/v), respectively (Table 2). Consequently, increasing the IL co-solvent concentration further was irrelevant.

Insert Table 2 here.

The detrimental effect of the ILs on trans-sialylation yields are most likely due to destabilization of the Tr6 enzyme by the ILs. Enzyme stability generally decreases with increasing co-solvent concentration and careful selection of IL co-solvents is required in order to avoid enzyme inactivation caused by the tendency of especially the IL anion to form hydrogen bonds that disrupt the protein structure (Kaftzik et al. 2002; Lang et al. 2006; Bridiau et al. 2010; Zhao 2010). In general, thermostable enzymes more often remain active in non-conventional media including ILs (Ferdjani et al. 2011). Therefore, hyperthermostable glycosidases have usually been preferred in systems with IL co-solvents (Lang et al. 2006; Sandoval et al. 2012).
Previously, no detrimental effect of the IL [CPMA][MeSO₄] (cosalskyl pentaethoxy methylammonium methyl sulfate) on enzyme stability was observed when a hyper-thermostable β-galactosidase from *Thermus thermophilus* (TTP0042) was employed for the LacNAc synthesis, (Sandoval et al. 2012). Nevertheless, the effect of the IL co-solvent on β-galactosidase selectivity correlated to changes in the enzyme’s secondary and tertiary structure and increased enzyme flexibility (Sandoval et al. 2012). IL co-solvents might exert a similar effect on Tr₆, but due to the lower enzyme stability the co-solvent-induced conformational changes apparently resulted in enzyme inactivation already at low IL concentrations.

The 1.4-fold improvement in 3’-sialyllactose concentration obtained at 25% v/v t-butanol (Table 1) was smaller than the approx. 7-fold increase in LacNAc yield obtained with BcβGal at 20-30% (v/v) t-butanol (Bridiau et al. 2010). This difference may in part be explained by the fact that the co-solvent approach was tested at conditions that were already optimized for trans-sialylation, i.e. an acceptor:donor ratio of 25. Indeed, the LacNAc yield obtained with BcβGal at 20-30% (v/v) t-butanol at an acceptor:donor ratio of 1 was comparable to the yield obtained without co-solvent at an acceptor:donor ratio of 7-8, but smaller than that obtained with an acceptor:donor ratio of 10 (Bridiau et al. 2010).

Furthermore, an acceptor:donor ratio of 9.6 in the BcβGal-catalysed synthesis of LacNAc produced a 1.9-fold improvement in LacNAc yield with 30% (v/v) [MMIm][MeSO₄], which is more comparable to the results obtained here (Kaftzik et al. 2002).

The 1.2-fold improvement in [3’-sialyllactose]/[sialic acid] ratio obtained with 25% (v/v) t-butanol at the optimal reaction time (40 min, Table 1) is comparable to the improvement in synthesis vs. hydrolysis ratio that was obtained previously by Lang et al. (2006) with the hyperthermostable β-glycosidase from *Pyrococcus furiosus* (CelB) using 45%
(v/v) [MMIm][MeSO₄] as co-solvent for the trans-galactosylation of a number of hydroxylated acceptors. Comparing the synthesis vs. hydrolysis ratios in the BcβGal-catalysed synthesis of LacNAc from GlcNAc and lactose at the reaction times which gave optimal yields, the ratio was improved 2.4 times by addition of [MMIm][MeSO₄] (Kaftzik et al. 2002). However, a 20-fold increase was observed when the ratios after 90 min of reaction were compared, because the IL co-solvent suppressed the secondary product hydrolysis more efficiently than what was obtained in the Tr6-catalysed production of 3'-sialyllactose (Kaftzik et al. 2002; Figure 2; Table 2). In more recent studies of TTP0047-catalysed LacNAc synthesis, lack of regioselectivity was a more important issue than hydrolysis and the [LacNAc]/[galactose] ratio was thus improved 1.4 times only, even if the LacNAc yield was more than doubled by addition of 30% (v/v) [OMIm][PF₆] (Sandoval et al. 2012). Lack of regioselectivity is not an issue with Tr6 for two reasons: (1) donor self-condensation does not take place because the transferred sialyl group cannot act as an acceptor (Vandekerckhove et al. 1992), and (2) Tr6 transfers sialyl groups with high regiospecificity, catalysing the formation of α(2, 3)-bonds only (Michalak et al. 2013).

_t_-Butanol forms an azeotrope with water and cannot be fully removed by simple distillation (Xu and Wand 2006). However, the recovery of 3'-sialyllactose from the aqueous reaction mixture already requires an anion exchange chromatography step, which will also remove the co-solvent (Michalak et al. 2013).

**Conclusions**

Although the effect is not dramatic, 20-25% (v/v) _t_-butanol co-solvent may be used to improve the Tr6-catalysed production of 3’-sialyllactose since it improves reaction yields as
well as the synthesis vs. hydrolysis ratio. Optimization of the process and careful co-solvent selection may improve the co-solvent effect. Indeed, the need for an acceptor:donor ratio of 25 as used at the optimal conditions for Tr6-catalysed \textit{trans}-sialylation (Michalak et al. 2013) may be obviated by the use of a co-solvent, e.g. \textit{t}-butanol, as previously shown for BcβGal in the synthesis of LacNAc (Bridiau et al. 2010). This may be relevant when working with more expensive acceptor substrates, e.g. lacto-\textit{N}-tetroases and lacto-\textit{N}-fucopentaoses, which also function as acceptor substrates for Tr6 (Michalak et al. 2013). IL co-solvents also had a positive effect on the synthesis vs. hydrolysis ratio, but a negative effect on \textit{trans}-sialylation yields, probably due to destabilization of the enzyme.

\textbf{Acknowledgements}

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References


**Tables**

**Table 1.** Relative values of the [3'-sialyllactose]/[sialic acid] ratio with 10-25% (v/v) *t*-butanol co-solvent compared to the the [3'-sialyllactose]/[sialic acid] ratio obtained during the reaction in buffer after 10-100 min of reaction. Bold typeface indicates significant increase in [3'-sialyllactose]/[sialic acid] ratio within each reaction time (*p* < 0.05). The maximum 3'-sialyllactose concentration (3'-'SL conc.) obtained at each *t*-butanol concentration is indicated relative to the maximum 3'-sialyllactose concentration obtained without co-solvent (mM/mM); bold typeface indicates significant increase in 3'-sialyllactose concentration.

<table>
<thead>
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</tr>
<tr>
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<td><strong>1.6 ± 0.17</strong></td>
<td><strong>1.5 ± 0.15</strong></td>
<td><strong>1.5 ± 0.15</strong></td>
</tr>
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</table>

| Relative 3'-SL conc. | 1.2 ± 0.11 | 1.1 ± 0.09 | **1.3 ± 0.10** | **1.4 ± 0.11** |
Table 2. Relative values of the [3’-sialyllactose]/[sialic acid] ratio with 0.1-2.5% (v/v) ionic liquid co-solvents compared to the the [3’-sialyllactose]/[sialic acid] ratio obtained during the reaction in buffer after 10-100 min of reaction. Bold typeface indicates significant increase in [3’-sialyllactose]/[sialic acid] ratio ($p < 0.05$). The maximum 3’-sialyllactose concentration (3’-SL conc.) obtained at each solvent condition is indicated relative to the maximum 3’-sialyllactose concentration obtained without co-solvent (mM/mM).

| Time   | [MMIm][MeSO₄] | | | [C₂OHMIm][PF₆] | | | EAN | | |
|--------|---------------|---|---|-----------------|---|---|---|
|        | 0.1% | 1% | 0.1% | 1% | 0.1% | 1% | 2.5% | 1% | 2.5% |
| 10 min | 1.0 ± 0.06 | 0.5 ± 0.05 | 1.0 ± 0.04 | 1.0 ± 0.12 | 0.9 ± 0.05 | 0.4 ± 0.01 | 0.4 ± 0.01 | |
| 20 min | 0.9 ± 0.12 | 0.4 ± 0.07 | 0.9 ± 0.12 | 0.9 ± 0.12 | 0.9 ± 0.11 | 0.6 ± 0.09 | 0.5 ± 0.09 | 1.0 ± 0.07 | 0.6 ± 0.06 |
| 40 min | 0.6 ± 0.05 | 0.3 ± 0.04 | 0.9 ± 0.12 | 1.0 ± 0.07 | 0.8 ± 0.07 | 1.0 ± 0.07 | 1.0 ± 0.07 | 0.6 ± 0.06 | |
| 60 min | 0.8 ± 0.09 | 0.4 ± 0.04 | 1.1 ± 0.11 | 1.4 ± 0.12 | 1.1 ± 0.20 | 1.5 ± 0.21 | 1.6 ± 0.20 | |
| 100 min| 0.7 ± 0.04 | 0.3 ± 0.02 | 1.0 ± 0.05 | 1.7 ± 0.09 | 1.7 ± 0.15 | 2.2 ± 0.11 | 2.5 ± 0.14 |

Relative 3’-SL conc. 0.92 ± 0.04 0.51 ± 0.06 0.99 ± 0.02 0.57 ± 0.01 0.97 ± 0.05 0.36 ± 0.00 0.09 ± 0.00
Figure legends

**Figure 1.** General reaction scheme for the reactions catalysed by sialidases with trans-sialidase activity such as Tr6: The sialyl donor (e.g. CGMP) binds to the enzyme and forms a covalent sialyl-enzyme intermediate (Damager et al. 2008). This intermediate undergoes nucleophilic attack by a β-galactosyl acceptor (e.g. β-lactose) resulting in synthesis of a sialylated glycoside (e.g. 3′-sialyllactose) or by water resulting in hydrolysis and formation of free sialic acid. The sialylated glycoside product formed by the trans-sialidase activity is also a substrate for the sialidase activity (secondary hydrolysis).

**Figure 2.** Tr6-catalysed formation of 3′-sialyllactose (3′-SL; solid symbols, solid lines) and sialic acid (SA; open symbols, dashed lines) in the presence of 0-25% (v/v) t-butanol. Reaction conditions: 117 mM lactose, 4.6 mM sialic acid bound in CGMP, 25°C, and pH 5.5. The values are adjusted for t-butanol evaporation, etc. during reaction, sample preparation, and analysis.
Figures

Figure 1.
Figure 2.
Optimizing the biocatalytic productivity of an engineered sialidase from *Trypanosoma rangeli* for 3’-sialyllactose production

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Abstract

An engineered sialidase, Tr6, from Trypanosoma rangeli was used for biosynthetic production of 3'-sialyllactose, a human milk oligosaccharide case compound, from casein glycomacropeptide (CGMP) and lactose, components abundantly present in industrial dairy side streams. Four different enzyme re-use methods were compared to optimize the biocatalytic productivity, i.e. 3'-sialyllactose formation per amount of Tr6 employed: i) His-tag immobilization on magnetic Cu$^{2+}$-imino diacetic acid-functionalized nanoparticles (MNPs), ii) membrane immobilization, iii) calcium alginate encapsulation of cross-linked Tr6, iv) Tr6 catalysis in a membrane reactor. Tr6 immobilized on MNPs gave a biocatalytic productivity of 84 mg 3'-sialyllactose per mg Tr6 after seven consecutive reaction runs. Calcium-alginate and membrane immobilization were inefficient. Using free Tr6 in a 10 kDa membrane reactor produced a 9-fold biocatalytic productivity increase compared to using free Tr6 in a batch reactor giving 306 mg 3'-sialyllactose per mg Tr6 after seven consecutive reaction runs. The 3'-sialyllactose yield on α-2,3-bound sialic acid in CGMP was 74%. Using circular dichroism, a temperature denaturation midpoint of Tr6, T$_{m}$, of 57.2°C was determined. The thermal stability of free Tr6 was similarly high and the Tr6 was stable at the reaction temperature (25°C) for at least 24 hours.

Keywords: Trans-sialidase; Trypanosoma rangeli; 3'-sialyllactose; enzyme immobilization; enzymatic membrane reactor; thermal stability.
1. Introduction

Recently, it was found that an engineered sialidase from *Trypanosoma rangeli*, Tr6, containing 6 designated amino acid mutations (I37L, M96V, A98P, S120Y, G249Y, Q284P) [1] could be expressed at high yield in *Pichia pastoris* and used for production of 3’-sialyllactose in gram quantities using casein glycomacropeptide (CGMP) and lactose as substrates for the trans-sialidase enzyme reaction (Figure 1) [2]. Sialylated compounds constitute approx. 16% of the total oligosaccharides in human milk [3]. 3’-sialyllactose, a human milk oligosaccharide (HMO) model case compound, has been shown to possess several beneficial features including the ability to inhibit the binding of cholera toxin *in vitro* [4] and to induce growth of various *Bifidobacterium* strains [5]. Furthermore, sialylated compounds in human milk, of which sialylated HMOs constitute the majority, are presumably involved in brain development in infants [6, 7]. Despite being highly abundant in human milk, HMOs are generally scarce in nature, especially in bovine milk from which infant formula is produced [8]. It is therefore of major interest to produce 3’-sialyllactose and other sialylated compounds from cheap, abundant side stream components, including e.g. lactose abundant in whey, and CGMP, which is the soluble glycosylated casein residue produced as a cleavage product after chymosin action on κ-casein during cheese manufacture.

Tr6 is a sialidase which has been genetically engineered to increase its *trans*-sialidase activity. However, its hydrolytic activity has not been completely removed [2] and it is therefore important to control reaction conditions, not least reaction time, to avoid extensive substrate and product hydrolysis. The reaction conditions for production of 3’-sialyllactose were optimized previously [2], but in order to increase the biocatalytic productivity of Tr6 with the aim of reducing enzyme cost and optimizing the enzymatic reaction for large scale use, a first option is to re-use the enzyme in a number of consecutive time-controlled reactions. This paper investigates methods for increasing the
biocatalytic productivity of Tr6 by enzyme re-use, i.e. via immobilization and use of an enzymatic membrane reactor approach, respectively.

Enzymatic membrane reactors (EMRs), allowing continuous or semi-continuous product removal whilst retaining the enzyme in the reactor, have proven successful for improving biocatalytic productivity in reactions where product inhibition severely reduces product yields [9, 10]. In a similar manner, a reduction in product yield due to product hydrolysis may be avoided by continuously removing the product from the EMR while confining the enzyme to the reactor thus enabling biocatalysis for extended reaction times, and in turn improve the biocatalytic productivity.

The enzyme can be free in the reactor or immobilized on the membrane. The latter often results in enzyme stabilization [11, 12]. No previous reports of immobilization of a sialidase from T. rangeli or its mutants exist, but other enzymes displaying trans-sialidase activity have been immobilized in order to improve their industrial potential. These include the Trypanosoma cruzi trans-sialidase which was immobilized on concanavalin A-sepharose [13-15] and a sialidase from Vibrio cholerae which was immobilized on VA-epoxy [16]. For the trans-sialidase from T. cruzi immobilization caused an activity loss of 25%, but increased enzyme stability [13, 15]. Immobilization of a sialidase from V. cholerae resulted in a decrease in trans-sialidase regioselectivity from a molar ratio of approx. 1:6 between 3'- and 6'-isomers for the free enzyme to approx. 1:3 for the immobilized enzyme, but the total trans-sialylation yield increased from 9.5% to 20% [16].

In the current study, three different types of enzyme immobilization are investigated, namely the widely used calcium alginate encapsulation of cross-linked Tr6, immobilization of Tr6 on Cu$^{2+}$-iminodiacetic acid (IDA)-functionalized carbon-coated magnetic nanoparticles (MNPs) via the His-tag, and membrane immobilization. In addition, the thermal stability of Tr6 as well as its stability at the reaction temperature for extended reaction times are determined, since stability is a requirement for high biocatalytic productivity.
2. Materials and methods

2.1 Chemicals

3’-sialyllactose standard was purchased from Carbosynth (Compton, United Kingdom). Casein glycomacropeptide (CGMP) in the form of the commercially available product Lacprodan® CGMP-20, containing a total of 5.7% (w/w), equivalent to 0.2 mmol/g dry matter, of covalently linked sialic acid was a gift from Arla Foods amba (Viby, Denmark). Before use, low molecular weight impurities in the CGMP solution were removed by filtration on a 5 kDa membrane (Sartorius AG, Goettingen, Germany) as a technical precaution relating to HPAEC analysis.

Glutaraldehyde, β-D-lactose, N-acetylneuraminic acid (sialic acid) standard, and all other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Production of the Tr6 enzyme

The 6xHis-tagged Tr6 enzyme was genetically modified and subsequently expressed in Pichia pastoris as described previously [2]. Tr6 was purified from the ultrafiltered cell-free extract from P. pastoris by Ni²⁺ affinity column chromatography using a 5 mL HisTrap™ HP column (GE Healthcare, Uppsala, Sweden). After purification, salts were removed and the enzyme concentrated in 5 kDa Hydrosart® Vivaspin spin columns (Sartorius AG, Goettingen, Germany), before adding 10% glycerol to the final enzyme solution for enzyme stabilization. The enzyme was kept at -80°C until further use. The purification yield was determined by measuring volumes and trans-sialidase activities of crude and purified Tr6, respectively. Protein concentration was determined by measuring absorbance at 280 nm and using a molecular weight of 72.7 kDa and an extinction coefficient for Tr6 of 114000 M⁻¹ cm⁻¹ estimated by ProtParam [17] based on the amino acid sequence.
2.3 Standard trans-sialidase activity assay

Tr6 was assayed for trans-sialidase activity using lactose as acceptor and CGMP as donor of sialic acid at the optimal conditions established previously [2], namely 117 mM lactose, 4.6 mM sialic acid bound in CGMP, 25°C, and pH 5.5 (10 mM citrate buffer). The reaction time was 20 minutes unless stated otherwise. The reaction was stopped by inactivation at 90°C for 10 minutes. The enzyme loading was 1.4 % E/S (w/w), where the substrate [S] is total sialic acid covalently bound to CGMP. After reaction, the samples were filtered on a 5 kDa polyethersulfone membrane (Vivaspin, Sartorius AG, Goettingen, Germany) to remove CGMP. Concentrations of 3’-sialyllactose and sialic acid were determined by HPAEC-PAD as detailed below.

2.4 High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Separation and quantification of the reaction products 3’-sialyllactose and sialic acid were carried out by HPAEC-PAD analysis using a CarboPac™ PA100 (4 mm x 250 mm) analytical column equipped with a CarboPac™ PA100 (4 mm x 50 mm) guard column (Dionex Corp., Sunnyvale, CA) on a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA) at a flow rate of 1 mL/min. The eluent system comprised deionized water (A), 0.5 M NaOH (B), and 0.5 M NaOAc with 0.02% (w/v) NaN₃ (C). The elution started with isocratic elution at 89:10:1 (% A:B:C) from 0 to 3 minutes followed by a linear gradient from 89:10:1 (% A:B:C) to 60:10:30 (% A:B:C) from 3 to 19 minutes. Afterwards, strongly retained anions were removed from the column by isocratic elution at 5:10:85 (% A:B:C) for 4 minutes. Finally, the column was re-equilibrated for 5 minutes at 89:10:1 (% A:B:C). This allowed for baseline separation of lactose, sialic acid, and 3’-sialyllactose.
2.5 Calcium alginate encapsulation of cross-linked Tr6

Tr6 was cross-linked with glutaraldehyde for 4 h at 25°C at 0.2% (v/v) glutaraldehyde at an enzyme concentration of 2 mg/mL in a 40 mM EPPS buffer (pH 7.0). A control sample without glutaraldehyde was included and subsequently assayed for activity along with a sample of the cross-linked enzyme. The cross-linked enzyme was then mixed with sodium alginate in 10 mM citrate buffer (pH 5.5) to give a final sodium alginate concentration of 3.75% (w/v) and a final enzyme concentration of 0.4 mg/mL. Upon overnight incubation at 5°C, the enzyme-sodium alginate solutions were dropped from a needle-equipped syringe from approx. 20 cm height into a solution of 200 mM CaCl$_2$ in 10 mM citrate buffer (pH 5.5). The formed gel beads were left to cure for 2 hours at room temperature applying gentle magnetic stirring. Control calcium alginate beads without enzyme were prepared similarly. The gel beads were stored in a solution of 20 mM CaCl$_2$ in 10 mM citrate buffer (pH 5.5) at 5°C until use. Activity analyses were carried out in quadruplicates according to the trans-sialidase activity assay (Section 2.3) in a 10 mL reaction volume with a theoretical maximum enzyme loading of 1.4% E/S (w/w). Free enzyme control samples were included as positive controls (in duplicate). The curing buffer was also assayed for activity.

2.6 Immobilization of Tr6 on carbon-coated magnetic nanoparticles (MNPs)

200 mg of iminodiacetic acid (IDA)-functionalized carbon-coated magnetic nanoparticles (TurboBeads® Complexon, TurboBeads Llc., Zürich, Switzerland) were loaded with Cu$^{2+}$ in a 200 mM CuSO$_4$·5H$_2$O solution for 18 h at room temperature. The MNPs were then washed three times in MilliQ water to remove unbound Cu$^{2+}$. Subsequently, 1.44 mg Tr6 in 10 mM citrate buffer (pH 5.5) was added and incubated with the MNPs for 2 h at room temperature. The Tr6-MNPs were then washed three times in MilliQ water and the washing water collected for activity analysis. Immobilization efficiency was determined by measuring *trans*-sialidase activity in the
immobilization supernatant as well as in the washing water. The Tr6-MNPs were split into two
aliquots, each theoretically containing 0.72 mg Tr6 (giving an enzyme dosage of 1.4 % E/S) and
used for duplicate 3’-sialyllactose production in 250 mL baffled Erlenmeyer flasks (35 mL working
volume). Free enzyme and negative control samples (MNPs without enzyme) were included in
duplicates. The flasks were incubated on a shaking platform (150 rpm) at 25°C for up to 2.5 hours,
with samples being removed for heat inactivation after 0, 10, 20, 40, 60, 100, and 150 minutes. The
Tr6-MNPs were tested for reusability in seven consecutive runs of 60 minutes (the optimal time, i.e.
highest level of 3’-sialyllactose produced), replacing the entire substrate volume between each run.

2.7 Enzymatic membrane reactor with free and immobilized Tr6
Free Tr6 was used for production of 3’-sialyllactose in a 50 mL commercial stirred cell (Amicon
8050, Millipore, Billerica, MA) equipped with a 10 kDa regenerated cellulose membrane
(Millipore, Billerica, MA) and operated as described previously [11]. The new membrane was first
soaked in a 5% NaCl solution for 30 minutes; filtration was then initiated with deionized water for
another 30 minutes according to the manufacturers’ instructions. Afterwards, the water permeability
of the membrane was measured with buffer (10 mM citrate, pH 5.5) at 2 bar for 20 minutes. For the
enzymatic reaction, 0.10 mg purified Tr6 was added to a working volume of 6 mL (117 mM lactose
and 4.6 mM sialic acid bound in CGMP in 10 mM citrate buffer, pH 5.5), giving an enzyme loading
of 1.4% E/S (w/w). Pressure and agitation were applied to the extent necessary to obtain 5 mL
permeate in 1 hour. When 5 mL permeate was obtained (one cycle), the filtration was suspended
and 5 mL of fresh substrate was added to the reactor for the next cycle. In order to ensure that the
reaction time was the same (namely 60 min) for each of the seven consecutive cycles, pressure was
adjusted from 0 to 2.4 bar and agitation was increased from 100 to 300 rpm (pressure < 1 bar: 100
rpm; 1-1.5 bar: 150 rpm; 1.5-2 bar: 200 rpm; > 2 bar: 300 rpm). Permeate samples were heated at
90°C for 10 minutes to terminate any enzyme activity. Duplicate experiments were made. The membrane permeability after seven cycles was measured and the permeability loss was negligible because the CGMP fouling layer was removed by gentle washing.

Immobilization of Tr6 on the 10 kDa regenerated cellulose membrane was tested in the same reactor setup. For immobilization, 0.10 mg Tr6 (resulting in a theoretical maximum enzyme loading of 1.4% E/S (w/w)) in 42.5 mL of 10 mM citrate buffer (pH 5.5) in the later reaction was filtered through the membrane in a dead-end filtration at a pressure of 1-3 bar without agitation. The permeate was recirculated into the cell manually in order to make this fouling-fostering enzyme immobilization last for 1 h. After filtration, the membrane was washed by permeate and in the last step 7.5 mL fresh buffer. Both permeate and washing residues were collected in order to calculate the immobilized enzyme amount by mass balance. The permeability of membranes before and after filtration was measured; there was negligible permeability loss because the enzyme amount used for immobilization was low. The EMR was then operated as described above (using a substrate solution of 117 mM lactose and 4.6 mM sialic acid bound in CGMP), but with a reaction time of 20 minutes in each of the seven consecutive cycles. All experiments in the EMR were performed at 23°C.

2.8 Stability of free Tr6
Thermal stability of free Tr6 was tested by incubating the enzyme at 45°C, 55°C, 60°C, and 65°C at relevant time intervals for up to 100 minutes. After incubation, the \textit{trans}-sialidase activity was tested in the standard \textit{trans}-sialidase activity assay (see above). Furthermore, the stability of free Tr6 at reaction temperature (25°C) was tested at extended incubation times for up to 24 hours following the same procedure as for the thermal stability determination.
2.9 Circular dichroism

All CD measurements were carried out in an AVIV 410 circular dichroism spectropolarimeter (Lakewood, NJ, USA) equipped with a temperature control unit. The enzyme was assayed in 10 mM citrate buffer (pH 5.5). Far-UV CD spectra were recorded at 4°C, 25°C and 95°C with Tr6 concentrations of 29 μM in a 1 mm cell. Control baselines were measured with buffer in the absence of Tr6. Secondary structure contents were estimated from the spectra using the K2D2 server [18]. Thermal transitions were measured from 4°C to 85°C with a Tr6 concentration of 2.9 μM in a sealed 10 mm cell while stirring. After reaching the desired temperature, Tr6 was incubated for 2 minutes and data was collected for 1 minute. Thermal denaturation was followed at 232 nm where the change in ellipticity as a function of temperature [\(\Theta\)] was most pronounced.

Unfolding curves were fitted by Eq. (1):

\[
[\Theta]_T = \alpha([\Theta]_F - [\Theta]_D) + [\Theta]_D
\]  

where \(\alpha\) is defined as the fraction of folded Tr6 at any temperature. \([\Theta]_F\) and \([\Theta]_D\) describe the ellipticity of the fully folded and denatured form and were normalized to 1 and 0, respectively. The temperature at the midpoint of denaturation (\(T_m\)) was determined with \(\alpha = 0.5\).

2.10 Statistics

One-way ANOVA for determination of statistical significance was made in Minitab 16 (Minitab Inc., State College, PA, USA). Statistical significance was established at \(p < 0.05\).
3. Results

3.1 Immobilization of Tr6

3.1.1 Calcium alginate encapsulation of cross-linked Tr6

After cross-linking with 0.2% (v/v) glutaraldehyde to 2 mg/mL purified Tr6 the retained trans-sialidase activity was only 33 ± 2%, indicating that cross-linking was harmful to the enzyme. Encapsulation of the cross-linked Tr6 in calcium alginate beads deprived Tr6 of almost all its activity: in the first 20-minute run after calcium alginate encapsulation, only 0.1% of the original trans-sialidase activity was obtained.

3.1.2 Immobilization of Tr6 on carbon-coated MNPs

Purified Tr6 was immobilized on Cu$^{2+}$-IDA-functionalized carbon-coated MNPs with an immobilization efficiency of 94 ± 0.7%. The production of 3'-sialyllactose with Tr6-MNPs in a 35 mL working volume was followed for up to 2.5 hours and compared to the production of 3'-sialyllactose obtained with free Tr6 (Figure 2). The initial trans-sialidase rate for the free Tr6 (0.070 mM/min.) was 2.4 times higher than for the Tr6-MNPs (0.029 mM/min.), giving higher 3'-sialyllactose yields at short reaction times with free Tr6. However, free Tr6 also hydrolyzed the 3'-sialyllactose product at a higher rate than the immobilized Tr6 did. Thus, after 40 minutes of reaction the level of 3'-sialyllactose reached a maximum (1.30 ± 0.10 mM) in the free Tr6 reaction before decreasing due to product hydrolysis (Figure 2). With the immobilized Tr6, though, the 3'-sialyllactose levels kept increasing until 60 minutes, thus reaching a higher level (1.85 ± 0.17 mM) of 3'-sialyllactose before decreasing due product hydrolysis after 60 minutes (Figure 2). Consequently, the level of free sialic acid was much lower with MNP-immobilized Tr6 than with free Tr6 (Figure 2).
Subsequently, Tr6-MNPs were tested for reusability over seven consecutive 60-minute runs in the 35 mL working volume (Figure 3). At the end of each run, the Tr6-MNPs were separated from the reaction mixture by an external magnet, and the entire substrate volume was replaced. In the second reaction run, the 3’-sialyllactose level reached only 46% of the level in the first 60-minute reaction run (Figure 3). The \textit{trans}-sialidase activity of the Tr6-MNPs kept decreasing over the 7 hours of reaction, reaching a 3’-sialyllactose level in the seventh run, which was only 7% of the level reached in the first reaction run (Figure 3). The level of free sialic acid was generally low and followed the same decreasing trend as the 3’-sialyllactose level (Figure 3).

3.2 Tr6 in EMR

3.2.1 Free Tr6 in EMR

Free Tr6 was used in EMR equipped with a 10 kDa regenerated cellulose membrane in seven consecutive 60-minute reaction cycles in order to produce 3’-sialyllactose, which could then pass the membrane in order to avoid product hydrolysis. After each cycle, fresh substrate was added to the reactor. The permeate concentration of 3’-sialyllactose was 1.33 ± 0.02 mM in the first 60-cycle, but then increased to approx. 1.8 mM in the third cycle and remained constant at this level throughout the seven cycles (Figure 4). In contrast, the sialic acid yield kept increasing throughout the seven 60-minute cycles, starting at 0.65 ± 0.1 mM in the first cycle and ending at 2.03 ± 0.06 mM in the seventh cycle (Figure 4). The level of 3’-sialyllactose in the retentate obtained after the seventh cycle was very low (0.05 ± 0.005 mM), whereas the concentration of free sialic acid was 3.7 ± 0.07 mM (Figure 4). The average pressure applied in each cycle in order to obtain uniform reaction times and permeate volumes increased from 0.46 bar in the first cycle to 1.72 bar in the seventh cycle due to the increasing osmotic pressure or fouling resistance by the accumulated CGMP in the retentate (Figure 4).
3.2.2 Membrane immobilization of Tr6

The immobilization efficiency on the 10 kDa regenerated cellulose membrane operated in normal mode (i.e. skin layer up) during the fouling-induced immobilization was 35 ± 1%. The production of 3’-sialyllactose and free sialic acid followed the same trend over seven consecutive reaction cycles as observed with free Tr6 in the EMR (Figure 4), albeit with lower yields due to the low immobilization efficiency (data not shown).

3.3 Stability of free Tr6

Thermal stability of free Tr6 was tested by incubating the enzyme at 45°C, 55°C, 60°C, and 65°C at relevant time intervals up to 100 minutes. At 45°C, the thermal inactivation constant, $k_D$, for free Tr6 was approx. 0, indicating that the enzyme is stable at this temperature for at least 100 minutes (Figure 5). From 55°C $k_D$ increased from 0.02, over 0.13 at 60°C, to 0.81 at 65°C, where the thermal inactivation was fast: only 2% of the initial trans-sialidase activity was retained after 5 minutes of incubation at 65°C (Figure 5). From the Arrhenius plot an activation energy for thermal inactivation, $E_a$, Tr6 of 346 kJ/mol was estimated.

Furthermore, Tr6 was incubated in 10 mm citrate buffer (pH 5.5) at 25°C for up to 24 hours. No significant loss in trans-sialidase activity was observed, indicating that Tr6 was completely stable at the reaction temperature for at least 24 hours (data not shown).

Figure 6 shows the CD spectra and temperature transition of Tr6 in 10 mM citrate buffer (pH 5.5). From the spectral data, a secondary structure content of 4% α-helix and 44% β-sheet was estimated using the K2D2 server [18]. The midpoint denaturation temperature, $T_m$, of Tr6 was determined to be 57.2°C.
4. Discussion

4.1 Immobilization of Tr6

4.1.1 Calcium alginate encapsulation of cross-linked Tr6

Whether the loss of 67\% activity during glutaraldehyde cross-linking for calcium alginate immobilization is due to extensive aggregation blocking the active site or a result of mass transfer limitations for the enzyme aggregates to react with the with the relatively large CGMP donor substrate is unknown. Using the same enzyme:glutaraldehyde ratio and concentrations for a reference enzyme (feruloyl esterase type C from *Emericella nidulans*), no activity was lost during cross-linking (data not shown). Encapsulation of cross-linked enzyme in a calcium alginate matrix is a method commonly used for enzyme immobilization. In the case of Tr6 used for producing 3'-sialyllactose from lactose and CGMP, however, this method turned out not to be a viable immobilization method since only 0.1\% of the original *trans*-sialidase activity was recovered upon encapsulation of glutaraldehyde cross-linked Tr6 in calcium alginate.

4.1.2 Immobilization of Tr6 on carbon-coated MNPs

Superparamagnetic nanoparticles are emerging as carriers for enzyme immobilization, using a variety of immobilization strategies [19]. In the current study, the His-tag used for immobilized metal affinity chromatography (IMAC) protein purification was exploited for enzyme immobilization on carbon-coated MNPs functionalized with IDA residues. The same strategy has previously been used for immobilizing *Bacillus stearothermophilus* L1 lipase on silica-coated MNPs [20], *Solanum tuberosum* epoxide hydrolase on poly(glycidyl methacrylate) (PGMA)-coated MNPs [21], and both T4 DNA ligase and T7 RNA polymerase on magnetomicelles [22] using either Cu$^{2+}$-IDA or Ni$^{2+}$-nitrilotriacetic acid (NTA) as the chelating complex. His-tag facilitated immobilization provides high immobilization specificity given that only one His-tagged protein is
present [21, 22]. Previously, it was reported that higher lipase activity and markedly better 
reusability were obtained using Cu\textsuperscript{2+}-IDA-functionalized silica-coated MNPs for immobilization 
than with Cu\textsuperscript{2+}-IDA-functionalized silica gel or silica-coated MNPs without the Cu\textsuperscript{2+}-IDA-
functionalization [20].

Comparing the performance of free Tr6 and Tr6-MNPs for the production of 3’-sialyllactose 
from lactose and CGMP for up to 2.5 hours showed that MNP-immobilized Tr6 produced higher 
levels of 3’-sialyllactose than free Tr6 due to a less pronounced product hydrolysis (Figure 2). The 
ratio between the transfer reaction rate and the hydrolysis rate was significantly higher for the Tr6-
MNPs than for the free enzyme, giving rise to a more desirable product composition and higher 3’-
sialyllactose yields. Enzyme immobilization naturally introduces mass transfer limitations. Since 
the product concentration in the reaction mixture is lower than the substrate concentration, the 
immobilized Tr6 is in principle more likely to encounter unreacted substrate than a hydrolysable 
product molecule. The optimal reaction time was lower for free Tr6 than for MNP-immobilized 
Tr6, but because the 3’-sialyllactose yield was higher for the Tr6-MNPs, there was no statistically 
significant difference between the biocatalytic productivity rates obtained for the two enzymes at 
the optimal reaction times: the biocatalytic productivity rate was thus 0.84 mg 3’-sialyllactose/(mg 
Tr6 ∙ minute) for free Tr6 and 0.79 mg 3’-sialyllactose/(mg Tr6 ∙ minute) for Tr6-MNPs. In 
addition, since Tr6 is an expensive enzyme the objective was to increase biocatalytic productivity 
rather than shortening reaction time. Thus, MNP-immobilized Tr6 was more efficient in producing 
3’-sialyllactose than free Tr6 without even being reused. The biocatalytic productivity obtained in 
one 60-minute reaction run was 33.5 mg 3’-sialyllactose/mg Tr6 for free Tr6 and 47.6 mg 3’-
sialyllactose/mg Tr6 for the Tr6-MNPs; see Table 1.

In theory, only negligible amounts of enzyme should be released from the MNPs due to a 
strong interaction between the His-tag and the Cu\textsuperscript{2+}-IDA complex. For the interaction between His
and Ni$^{2+}$-NTA, the dissociation constant is $10^{-13}$ M at pH 8 [23], corresponding to only 0.02% of the enzyme being released after reaching equilibrium in each cycle [21]. The Cu$^{2+}$-IDA interaction is also strong: Cu$^{2+}$ binds IDA with an association constant of $10^{11}$ M$^{-1}$ [24]. Indeed, high enzyme-MNP recyclability has been reported previously using this immobilization strategy: Wang et al. (2011) [21] retained 80% epoxide hydrolase productivity after 8 cycles, while Kim et al. (2009) [20] reported more than 70% of initial lipase activity after 6 cycles. Unfortunately, the reusability of Tr6 is not similarly high: After 7 cycles, only 7% of the initial trans-sialidase activity was retained (Figure 3). To test whether this was due to loss of enzyme from the carrier or enzyme inactivation, a test was made where the Tr6-MNPs were washed between each 60-minute reaction run and the washing water subsequently analyzed for activity. 3-7% of the initial trans-sialidase activity was lost in each wash, the amount of activity lost decreasing over time. This does not explain the entire loss of activity, but it is likely that some enzyme is also lost in the reaction mixture. IDA is a much stronger chelator of Cu$^{2+}$ than citrate [25], but the use of citrate in the reaction may account for some of this loss. Thus, using a different buffer may improve the reusability of the Tr6-MNPs. Even so, the yield of 3’-sialyllactose obtained with Tr6 immobilized on MNPs is higher than the yield obtained with free Tr6 already in the first reaction run (Figure 2). Thus, immobilizing Tr6 increases the biocatalytic productivity, even if a more stable immobilization would be desirable.

Using the His-tag for immobilization offers the possibility of integrating the immobilization with the IMAC purification, and was successfully used by Wang et al. (2011) [21] who immobilized epoxide hydrolase from a cell-free extract from P. pastoris on PGMA-coated MNPs. Therefore, His-tag facilitated immobilization on carbon-coated MNPs of crude Tr6, i.e. the ultrafiltered cell-free extract from P. pastoris, and IMAC-purified Tr6, respectively, was compared in the current study. The trans-sialidase activity obtained when immobilizing crude Tr6 was about half of that obtained when immobilizing the purified Tr6 (data not shown). The decay in
immobilized activity observed over time (no. of runs) with crude Tr6 was similar to that obtained
with purified Tr6 (Figure 3). The purification yield was 84%, meaning that 1.2 times more enzyme
must be used when immobilizing purified enzyme, but since the immobilized trans-sialidase
activity was twice as high with purified Tr6, it follows that for the enzyme reaction system used in
the current study it is not advisable to integrate the purification step with the immobilization step.
The decrease in retained trans-sialidase activity obtained when using crude Tr6 can in part be
explained by lower immobilization efficiency (approx. 70% for crude Tr6 vs. approx. 100% for
purified Tr6), indicating that other components in the cell-free extract may block the
immobilization, even if His-tag-facilitated immobilization is known to be highly specific [21, 22].
A decrease in retained activity was also reported in the one-pot purification and immobilization of
His-tagged fuculose-1-phosphate aldolase from *E. coli* on high density Co$^{2+}$-IDA-functionalized
sepharose support compared to the immobilization of the purified enzyme [26].

The main limitation of using MNPs for enzyme immobilization in industrial scale is the fact
that magnetic field strength decreases with increasing distance, thus reducing the separation
efficiency. Therefore, it is recommended to use a long reactor with a small diameter, e.g. a
serpentine tube in which the reaction mix containing Tr6-MNPs is circulated. Then, upon reaching
the desired reaction time, an external magnet is placed along the side of the tube drawing the Tr6-
MNPs to the side of the reactor. The reaction mix containing the sialylated product can then be
removed from the reactor by opening it since the Tr6-MNPs are retained inside by the external
magnetic field. Alternatively, another IDA-functionalized resin can be used in a different reactor
design. In conclusion, immobilization of Tr6, be it on Cu$^{2+}$-IDA-functionalized MNPs or another
carrier, presents a means of avoiding product hydrolysis through control of reaction time while at
the same time providing for enzyme re-use thus increasing the biocatalytic productivity. However,
higher recyclability is desirable. Another way to achieve the same advantages is to use an EMR.
4.2 Tr6 in EMR

High yields of 3'-sialyllactose were obtained when using free Tr6 in the EMR. Furthermore, the 3'-sialyllactose yield remained stable over 7 hours, which agrees with the enzyme being stable for extended reaction times at 25°C. Furthermore, the level of 3'-sialyllactose in the retentate was desirably low. Fresh substrate was added after each 60-minute cycle, but since CGMP was retained in the reactor while lactose could pass the membrane the acceptor:donor ratio decreased over time thus favoring substrate hydrolysis. This was also evident from the level of free sialic acid in the permeate, which increased linearly over the seven 60-minute cycles (Figure 4). Consequently, filtration resistance by CGMP was severe as indicated by the increase in applied pressure (Figure 9). Indeed, CGMP build-up inside the EMR may be the most significant problem to overcome in large-scale production of 3'-sialyllactose. Fortunately, the CGMP layer was easily removed from the membrane, which regained full permeability after simple washing. It is likely that CGMP fouling can be decreased by using another membrane set-up, e.g. cross-flow filtration, rather than dead-end filtration. Recovering Tr6 from the reaction mixture will require a new IMAC purification, and it is therefore advisable to ensure that the reactor can be operated for extended reaction times, taking the stability of Tr6 into consideration. The EMR thus seems a viable setup to use for producing 3'-sialyllactose from CGMP and lactose. Using more expensive acceptors than lactose, e.g. lacto-N-tetraoses and lacto-N-fucopentaoses, acceptor recycling is important. Therefore, we are currently working on a filtration-based reactor setup, where the acceptor can be separated from the product and recycled.

4.2.1 Membrane immobilization

Recently, the potential of fouling-induced enzyme immobilization in membrane reactors using alcohol dehydrogenase (ADH) and glutamate dehydrogenase (GDH) as models for a multi-enzyme
system was examined [11]. The data showed that immobilization of the enzymes in reverse mode, i.e. placing the membrane with the support layer up, maximized the enzyme loading. Such reverse mode immobilization of Tr6 was also tested, but CGMP fouling was severe during the reaction and the permeate flux consequently so low that reasonable amounts of permeate could not be obtained within the desired reaction time (data not shown). It was thus concluded that reverse mode was unfavorable in this system. Unfortunately, using fouling-induced membrane immobilization, irreversible fouling mainly occurs in a reverse membrane set-up mode where the enzyme enters the support layer, whereas in normal mode the enzymes form a cake layer on the membrane, which is easily removed by washing [11]. In addition, the enzyme more readily diffuses back into the bulk solution in normal mode and will thus be lost upon emptying the membrane reactor. It thus follows that using membrane immobilization mainly gives the advantage over using free enzyme in EMR that then enzyme can be stabilized on the membrane. However, Tr6 is so stable that it does not require immobilization to work for extended reaction times (cf. Section 3.3). Therefore, using Tr6 free in the EMR is to be favored over membrane immobilization, since the latter causes undesirable enzyme loss due to low enzyme loading (35 %) and requires an extra process step (immobilization).

4.3 Tr6 stability

Of the methods investigated in order to increase biocatalytic productivity of Tr6, the use of free Tr6 in the EMR proved to be favorable. When optimizing the biocatalytic productivity of an enzymatic reaction, enzyme stability is just as important as activity. Therefore, the stability profile of free Tr6 was elucidated in the current study, indicating that the enzyme could easily be used for extended reaction times at reaction temperature (25°C). Furthermore, thermal stability of Tr6 was studied both kinetically and thermodynamically, both indicating that Tr6 is stable at temperatures higher than 25°C (Figures 5 and 6b). The results suggest that the reaction temperature may be elevated to
increase reaction rates. This is in agreement with the results obtained by Michalak et al. (2013) [2] in the reaction condition optimization, where the optimal temperature of 25°C was in fact the highest temperature included in the optimization study.

Based on the CD data, the K2D2 server estimates a secondary structure content comprising 4% α-helix and 44% β-sheet, which agrees with the crystal structure of a mutant sialidase from *T. rangeli*, a close homologue of Tr6 (PDB: 1WCS) [1]. CD data has not previously been published for Tr6 or related sialidases.

4.4 Biocatalytic productivity and molar yield on sialic acid donor

The biocatalytic productivities of Tr6 obtained when using free Tr6 in EMR and MNP-immobilized Tr6 clearly show the potential of these methods for optimizing the production of 3’-sialyllactose from lactose and CGMP compared to using free Tr6 in batch reactor (Table 1). Immobilizing Tr6 on Cu$^{2+}$-IDA-functionalized MNPs increased the biocatalytic productivity from 33.5 (free Tr6 in batch reactor) to 84.1 when used for seven consecutive runs, corresponding to a 2.5-fold increase. Due to the loss of Tr6 from the carrier, the increase in biocatalytic productivity from 4 consecutive reaction runs (73.3) was however not statistically significant (Table 1). Confining free Tr6 in the EMR equipped with a 10 kDa membrane did however increase biocatalytic productivity dramatically, since the Tr6 was stable at the reaction temperature and kept producing 3’-sialyllactose at high yield in at least seven consecutive 60-minute reaction runs, giving a biocatalytic productivity of 305.6 (Table 1). Thus, using the EMR a more than 9-fold increase in biocatalytic productivity of Tr6 could be obtained.

The molar yield on the limiting substrate, sialic acid bound in CGMP, was 37% after seven consecutive runs in the EMR (Table 1). The mutations introduced in the wild-type sialidase from *T. rangeli* to yield Tr6 were based on the amino acid sequence of a trans-sialidase from *T. cruzi* [1, 2].
which catalyzes the transfer of α-2,3-bound sialic acid but not of α-2,6-bound sialic acid [27]. It can thus be speculated that Tr6 is also specific for α-2,3-bound sialic acid only. Hence, taking the theoretical distribution of sialic acid linkages in CGMP into account (50% is α-2,3-bound and 50% is α-2,6-bound [28]), the yields on GCMP given in Table 1 may be doubled, i.e. a yield on α-2,3-bound sialic acid in CGMP of 74% was achieved with free Tr6 in the EMR.

5. Conclusions

Three different immobilization methods were tested in order to optimize the biocatalytic productivity of Tr6 for production of 3'-sialyllactose from CGMP and lactose. Encapsulation of cross-linked Tr6 in calcium alginate suffered from immediate loss of enzyme activity, whereas membrane immobilization of Tr6 suffered from low immobilization efficiency (35%). Immobilization of Tr6 on carbon-coated MNPs via the His-tag gave high immobilization efficiency (94%) and an improved ratio between trans-sialidase activity and unwanted sialidase activity, giving higher 3'-sialyllactose yields than free Tr6 already in the first reaction run. However, recyclability of the Tr6-MNPs was low due to loss of enzyme from the carrier. Using free Tr6 in EMR with a 10 kDa membrane provides a method for enzyme re-use while continuously removing 3'-sialyllactose from the reactor. In the EMR free Tr6 was stable for extended reaction times and a biocatalytic productivity of 306 mg 3'-sialyllactose per mg Tr6 was obtained after seven consecutive 60-minute reaction runs in the EMR, corresponding to a 9-fold increase in biocatalytic productivity compared to that obtained with free Tr6 in batch reactor (the reference scenario). The yield on α-2,3-bound sialic acid in CGMP (the limiting substrate) was 74%. The biocatalytic productivity of Tr6 was thus successfully optimized for larger scale production of 3'-sialyllactose from components abundantly present in dairy side streams.
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References


**Tables**

**Table 1.** Biocatalytic productivity (mg 3’-sialyllactose/mg enzyme) and molar yield on sialic acid in CGMP obtained with free Tr6 and MNP-immobilized Tr6 (Tr6-MNPs) in a 35 mL batch reactor working volume and of free Tr6 in enzymatic membrane reactor (EMR) with a 6 mL working volume. The enzyme loading was 1.4% E/S (w/w) based on sialic acid bound in CGMP for free Tr6 and approx. 1.3% E/S (w/w) for Tr6-MNPs (immobilization efficiency: 94%). The reaction time was 40 minutes for the free Tr6 in batch reactor and 60 minutes in each run for MNP-immobilized Tr6 (optimal reaction times, cf. Figure 2) as well as for the free Tr6 in EMR (for comparison). Adjusted for purification yield (84%). Superscript letters indicate whether the numbers are significantly different ($p < 0.05$).

<table>
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<th>Biocatalytic productivity [mg 3’-sialyllactose/mg Tr6]</th>
<th>Molar yield on sialic acid donor [mol 3’-sialyllactose/mol sialic acid in CGMP] x 100%</th>
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<tr>
<td>Free Tr6</td>
<td>33.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28%&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tr6-MNPs (1 run)</td>
<td>47.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40%&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Tr6-MNPs (4 runs)</td>
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<td>15%&lt;sup&gt;g&lt;/sup&gt;</td>
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<tr>
<td>Tr6-MNPs (7 runs)</td>
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<td>10%&lt;sup&gt;h&lt;/sup&gt;</td>
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<tr>
<td>Free Tr6 in EMR (1 run)</td>
<td>34.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29%&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>Free Tr6 in EMR (4 runs)</td>
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<td>35%&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free Tr6 in EMR (7 runs)</td>
<td>305.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>37%&lt;sup&gt;g&lt;/sup&gt;</td>
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Figure legends

Figure 1. Stylized scheme of the reactions catalyzed by Tr6 using CGMP and β-lactose as substrates. The structures of the sialic acid-containing side chains in CGMP were determined by Saito and Itoh (1992) [28]. Sialic acid can be α-2,3-bound (light grey) or α-2,6-bound (dark grey) in CGMP, the two structures being present in a 50:50 ratio [28]. Using lactose as acceptor, Tr6 catalyzes the transfer of sialic acid from CGMP to form 3'-sialyllactose (trans-sialidase activity). Using water as acceptor, Tr6 can also catalyze the hydrolysis of both sialic acid bound in CGMP and of the desired product, 3'-sialyllactose (sialidase activity).

Figure 2. Time study on free Tr6 and Tr6-MNPs. Production of 3'-sialyllactose (solid symbols) and free sialic acid (open symbols) with Tr6 immobilized on MNPs (circles) and free Tr6 (triangles) at 117 mM lactose, 4.6 mM sialic acid covalently bound to CGMP, 25°C, and pH 5.5 for up to 2.5 hours in a 35 mL working volume (250 mL baffled shake flasks, 150 rpm). Negative control (buffer instead of enzyme) values subtracted. Standard deviation is shown. Lines are meant as a guide to the eye.

Figure 3. Reusability of Tr6-MNPs. Production of 3'-sialyllactose (dark grey) and free sialic acid (light grey) with Tr6 immobilized on MNPs at 117 mM lactose, 4.6 mM sialic acid covalently bound to CGMP, 25°C, and pH 5.5 in seven consecutive 60-minute reactions (runs) in a 35 mL working volume (250 mL baffled shake flasks, 150 rpm). At the end of each run, Tr6-MNPs were separated from the reaction mixture by an external magnet, and the entire substrate volume was replaced. Negative control (MNPs without Tr6) values subtracted. Standard deviation is shown. The percentages indicate the level of 3'-sialyllactose produced comparing to the first 60-minute reaction run.

Figure 4. Free Tr6 in EMR. Permeate concentrations of 3'-sialyllactose (dark grey) and free sialic acid (light grey) produced with free Tr6 during seven consecutive 60-minute cycles in an enzymatic membrane reactor (EMR) equipped with a 10 kDa regenerated cellulose membrane and containing 117 mM lactose and 4.6 mM sialic acid bound in CGMP at 25°C and pH 5.5. Fresh substrate was added after each 60-minute cycle.
New permeate was collected during each reaction run, i.e. the yields are not cumulative. The retentate was analyzed for 3'-sialyllactose and free sialic acid after all seven cycles had been completed. Average pressure applied during each cycle to keep the reaction time and permeate volume identical in each cycle is shown (triangles; right-hand axis). Standard deviation is indicated. Lowercase letters indicate significant difference between permeate 3'-sialyllactose levels ($p < 0.05$).

**Figure 5.** Thermal stability of free Tr6. Thermal inactivation constants, $k_D$, are determined from the linear regression slopes at 45°C (squares), 55°C (triangles; $R^2 = 0.965$), 60°C (circles; $R^2 = 0.984$), and 65°C (diamonds; $R^2 = 0.935$) in the ln($v$) vs. incubation time plot, where $v$ is given as µM 3'-sialyllactose per minute. Standard deviation is indicated.

**Figure 6.** Circular dichroism measurements on Tr6. (a) CD spectra of Tr6 in 10 mM citrate buffer (pH 5.5) at 4°C, 25°C, and 95°C. (b) Thermal transition of Tr6 in 10 mM citrate buffer (pH 5.5) at 232 nm. The data are normalized to show the fraction of folded Tr6 at a given temperature. The line is the fit used for determining $T_m$, fitted to the data with the Levenberg-Marquardt algorithm. $T_m$ is 57.2°C.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.