Conference paper

Giulia Paggiola, Nolwenn Derrien, Jonathan D. Moseley, Anthony Green, Sabine L. Flitsch, James H. Clark, Con Robert McElroy* and Andrew J. Hunt*

Application of bio-based solvents for biocatalysed synthesis of amides with *Pseudomonas stutzeri* lipase (PSL)

https://doi.org/10.1515/pac-2019-0808

Abstract: Bio-based solvents were investigated for the biocatalysed amidation reactions of various ester-amine combinations by *Pseudomonas stutzeri* lipase (PSL). Reactions were undertaken in a range of green and potentially bio-based solvents including terpinolene, p-cymene, limonene, 2-methyl THF, ɣ-valerolactone, propylene carbonate, dimethyl isosorbide, glycerol triacetate and water. Solvent screenings demonstrated the importance and potential of using non-polar bio-based solvents for favouring aminolysis over hydrolysis; whilst substrate screenings highlighted the unfavourable impact of reactants bearing bulky para- or 4-substituents. Renewable terpene-based solvents (terpinolene, p-cymene, D-limonene) were demonstrated to be suitable bio-based media for PSL amidation reactions. Such solvents could provide a greener and more sustainable alternative to traditional petrochemical derived non-polar solvents. Importantly, once the enzyme (either PSL or CALB) binds with a bulky para-substituted substrate, only small reagents are able to access the active site. This therefore limits the possibility for aminolysis to take place, thereby promoting the hydrolysis. This mechanism of binding supports the widely accepted 'Ping Pong – Bi Bi' mechanism used to describe enzyme kinetics. The work highlights the need to further investigate enzyme activity in relation to para- or 4-substituted substrates. A priority in PSL chemistry remains a methodology to tackle the competing hydrolysis reaction.

Keywords: amides; bio-based; biocatalysis; biocatalysts; enzyme catalysis; fine chemicals; green chemistry; ICGC-8; organic chemistry; organic synthesis; pharmaceuticals; solvents; sustainable chemistry.

Introduction

Selective and efficient amide synthesis is a high priority for the pharmaceutical industry, with at least one in every four pharmaceutically active compounds containing carboxamide functionality [1, 2]. Common synthetic methods include use of $SOCl_2$-activated acids, stoichiometric coupling agents e.g. carbodiimides (DCC,

Article note: A collection of invited papers based on presentations at the 8th IUPAC International Conference on Green Chemistry (ICGC-8), Bangkok, Thailand, 9–14 September 2018.

***Corresponding authors: Con Robert McElroy,** Green Chemistry Centre of Excellence, Department of Chemistry, University of York, Heslington, North Yorkshire YO10 5DD, UK, e-mail: rob.mcelroy@york.ac.uk; and **Andrew J. Hunt,** Materials Chemistry Research Center, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand, e-mail: andrew@kku.ac.th. https://orcid.org/0000-0003-3983-8313
Giulia Paggiola and James H. Clark: Green Chemistry Centre of Excellence, Department of Chemistry, University of York, Heslington, North Yorkshire YO10 5DD, UK
Nolwenn Derrien and Jonathan D. Moseley: CatSci Ltd, CBTC2 Capital Business Park, Wentloog, Cardiff, CF3 2PX, UK
Anthony Green and Sabine L. Flitsch: Manchester Institute of Biotechnology & School of Chemistry, The University of Manchester, Manchester, M1 7DN, UK

EDCI · HCl) and carbodiimidazoles (CDI) or metal-based catalysts, amongst others [3]. These strategies attain lower activation energies, but at the expense of mass efficiency, cost and safety of the synthetic process [4]. The use of enzymes as a safer and more environmentally-friendly alternative has been utilised in combination with greener solvents [5–8]. The production of amides through the use of enzymatic catalysis has been extensively investigated and documented in the literature [9, 10]. Continuous developments in protein expression and purification, are leading to the increased performance of lipases as viable bio-catalysts for industrial amidation chemistry [9, 10].

Pseudomonas stutzeri lipase (PSL) is considered a top candidate for industrial N-acylation catalysis due to its commercial availability, high activity in organic solvents and ability to tolerate high substrate concentrations, as well as for its wide substrate scope, which is known to accept bulky and electronically disfavoured substrates [11]. The work conducted by Van Pelt et al. [12] and Maraite et al. [13] highlighted opportunities for further investigation of this technology, especially in understanding: (a) the factors affecting the observed substrate specificity, (b) the influence of the medium on the activity and selectivity of the catalysis, and (c) the potential for applicability of these biocatalysts in synthetic chemistry [14]. In the context of these three open questions, the present paper aims to deepen the understanding of solvent effects on the activity of PSL in greener and potentially bio-based solvents.

A series of experiments was carried out using high-throughput screening equipment to monitor PSL catalysed reactions in ten solvents and against combinations of four esters and three amines of general synthetic pharmaceutical interest **11**–**17** (Fig. 1) [18]. Studies to date have primarily utilised PSL in water **10** or methyl tert-butyl ether (MTBE) **9**, along with examples of lower activity in other ethers [15, 19]. Although MTBE performs well, it is potentially problematic, currently being investigated (CoRAP) for potential reproductive toxicity [20].

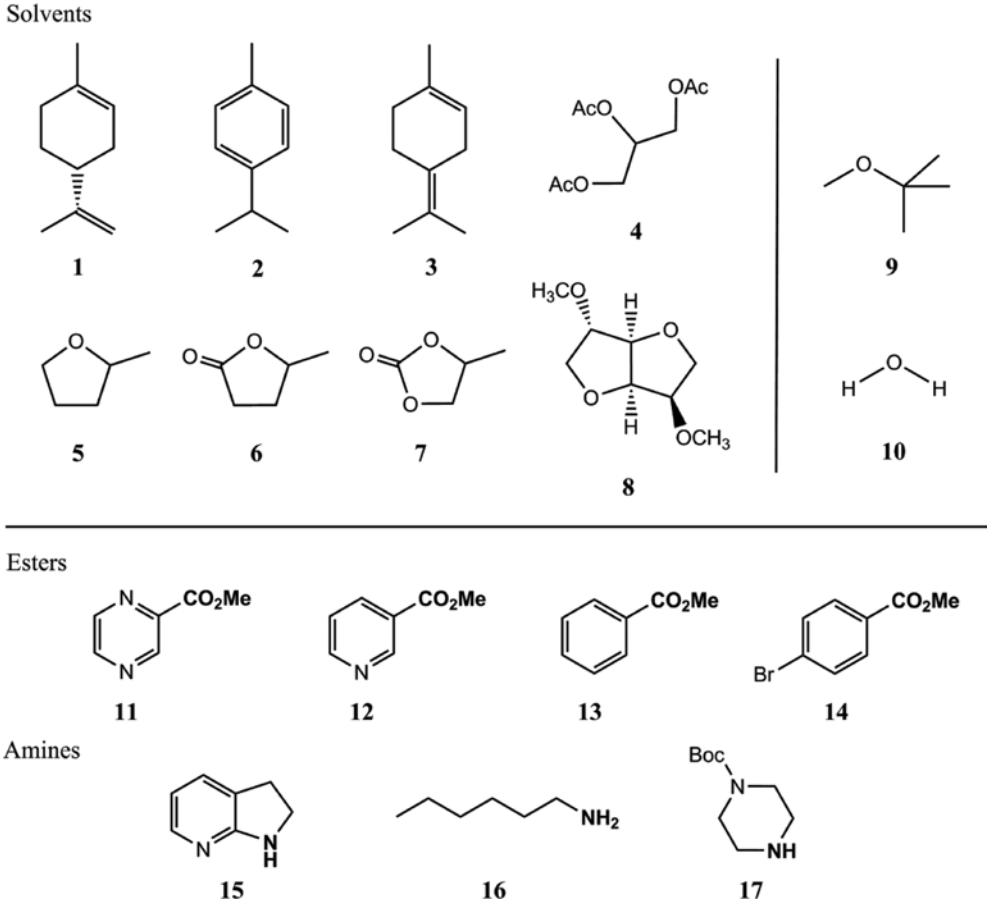

Fig. 1: Solvents (**1**–**10**) and substrates (**11**–**17**) screened in this work. Conditions: 0.1 M ester, 0.15 M amine, 60 mg enzyme catalyst, 100 mg MS 4 Å, run for 24 h at 50 °C in 5 mL of solvent.

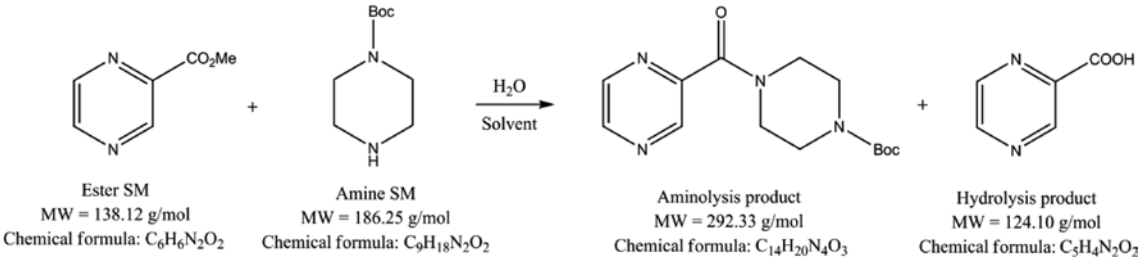

Fig. 2: Typical reaction scheme for amide formation.

Herein, eight bio-based solvents were evaluated as sustainable options for green non-aqueous biocatalysis to form an amide (Fig. 2) and benchmarked against neat MTBE **9** and water **10** for comparison with previous literature. Solvent screenings included the use of D-limonene **1**, p-cymene **2**, terpinolene **3**, glycerol triacetate **4**, 2-methyltetrahydrofuran **5**, γ-valerolactone **6**, propylene carbonate **7** and dimethyl isosorbide **8**, Fig. 1. This work not only investigates the use of non-polar bio-based solvents for aminolysis but, substrate screenings also highlight the impact of reactants on the reaction.

Materials and methods

Materials

Table 1 provides a list of reagents used in the study with their associated CAS number, source and also COSHH information. The colour highlighted in the COSHH sections provides the readers with an indication of how environmentally sustainable is likely to be: green is safe to use, amber is a warning and if possible greener alternatives should be used and finally red indicates that the chemical is not sustainable and therefore not recommended for use.

Methods

Sixty milligrams of *Pseudomonas Stutzeri* lipase (dried free enzyme preparation) were weighed into tubes using Flexi-weight (design ND112). Then ~100 mg mol sieves (powder) were added to all tubes before adding

Table 1: Reagents and solvents with their associated CAS number, source and COSHH information.

Reagent/Solvent	CAS	Source/Quantity	COSHH
PSL dry enzyme	N/R	AG(MAN) 60 mg	Unknown (Amber)
Methyl 2-pyrazinecarboxylate (E2)	[6164-79-0]	Aldrich 50 mg	H315-H319-H335
1-Boc-piperazine	[57260-71-6]	Aldrich 1.2 eq 81 mg	H315-H319-H335
Pyrazinecarboxylic acid	[98-97-5]	Aldrich	Amber
MTBE	[1634-04-4]	Fisher 5.0 mL	H315 (Amber)
Terpinolene	[586-62-9]	Aldrich 5.0 mL	None (Green)
p-cymene	[99-87-6]	Aldrich 5.0 mL	H226-H315-H319-H335
Limonene	[5989-27-5]	Aldrich 5.0 mL	H226-H304-H315-H317-H410
2-MethylTHF	[96-47-9]	Aldrich 5.0 mL	H225 (Green)
γ-Valerolactone	[108-29-2]	Aldrich 5.0 mL	None (Green)
Propylene carbonate	[108-32-7]	Aldrich 5.0 mL	H319 (Green)
Dimethyl isosorbide	[5306-85-4]	Aldrich 5.0 mL	None (Green)
Glycerol triacetate	[102-76-1]	Aldrich 5.0 mL	Green
Water	[7732-18-5]	Aldrich 5.0 mL	Green

Table 2: HPLC conditions, where A = 0.03 % TFA in H_2O, B = 0.03 % TFA in MeCN.

Time	% A
0	99
3.0	60
4.2	5
4.51	99

the substrate 50 mg and the amine. Solvent was then added by Eppendorf and the reactions stirred at 50 °C for 42 h. Reactions were run on the Mettler-Toledo Mini-Mapper in a standard double-sealed reaction block at 50 °C with stirring with a simple magnetic flea. The reactions were automatically sampled on the Mini-Mapper at 3, 6, 12, 24 and 42 h.

Analysis

The UV absorbance was converted to mmol/mL using the formula from the linear regression from the calibration curve (see Supporting Information). A standard of the product was prepared by treatment with MeI. A calibration was done with the acid, SM and product at the following concentration. The acid was more difficult to calibrate due to its poor solubility. The conversion was calculated as Product/(Product + Acid + SM) and the hydrolysis calculated as Acid/(Acid + Product + SM). The solvents peaks did not interfere with the samples. Samples were taken on mini-mapper at, 3, 6, 12 and 24 h, by taking 100 μL into 600 μL of diluent. Vials were filled with 4:1 MeCN/water with 0.2 mg/mL of internal standard (4,4′-t-butyl-biphenyl) pre-dissolved in it. Needle wash was 3.0 mL DMAc. Samples were spun down on the centrifuge before being run on the LCMS using BEH Shied RP18 column 2.1*100 mm with CatSci Generic 2.1 method (Acquity), which was conducted at a flow of 0.8 mL/min, for 5 min at 30 °C under the conditions outlined in Table 2. Prior to undertaking the study significant work was conducted to test reaction protocols to ensure reproducibility, a number of preliminary scouting studies were initially conducted, and automatic replicates were compared in each screening block (not reported in this work). Due to limited quantities of the enzyme and some solvents, extensive reproducibility tests could not be conducted. Two duplicates in MTBE provided a reproducibility control for each batch and allowed a comparison against existing literature. Standard deviation among these was applied as error bars to all solvents. In the case of three bio-based solvents across the polarity range (p-cymene, limonene and 2-methyl THF) repeat reactions were also conducted to demonstrate the validity of the results. The HPLC method was also validated by conducting repeated analysis on all samples to ensure that the results are both reliable and accurate.

Control reactions

Test reactions without enzyme and with pre-denatured enzyme did not proceed, confirming that the enzymatic activity is purely due to the proteins tri-dimensional conformation. Test reactions with equivalent acid as starting material did not proceed to the amide. Control experiments were also conducted in duplicate to ensure thoroughness of the controls.

Results and discussion

In Fig. 3 the conversion plots of four reactions (3a–d) are outlined, presenting the reactions in three bio-based solvents (p-cymene **2**, D-limonene **1**, 2-methyltetrahydrofuran **5**) and against benchmarks in MTBE **9** and

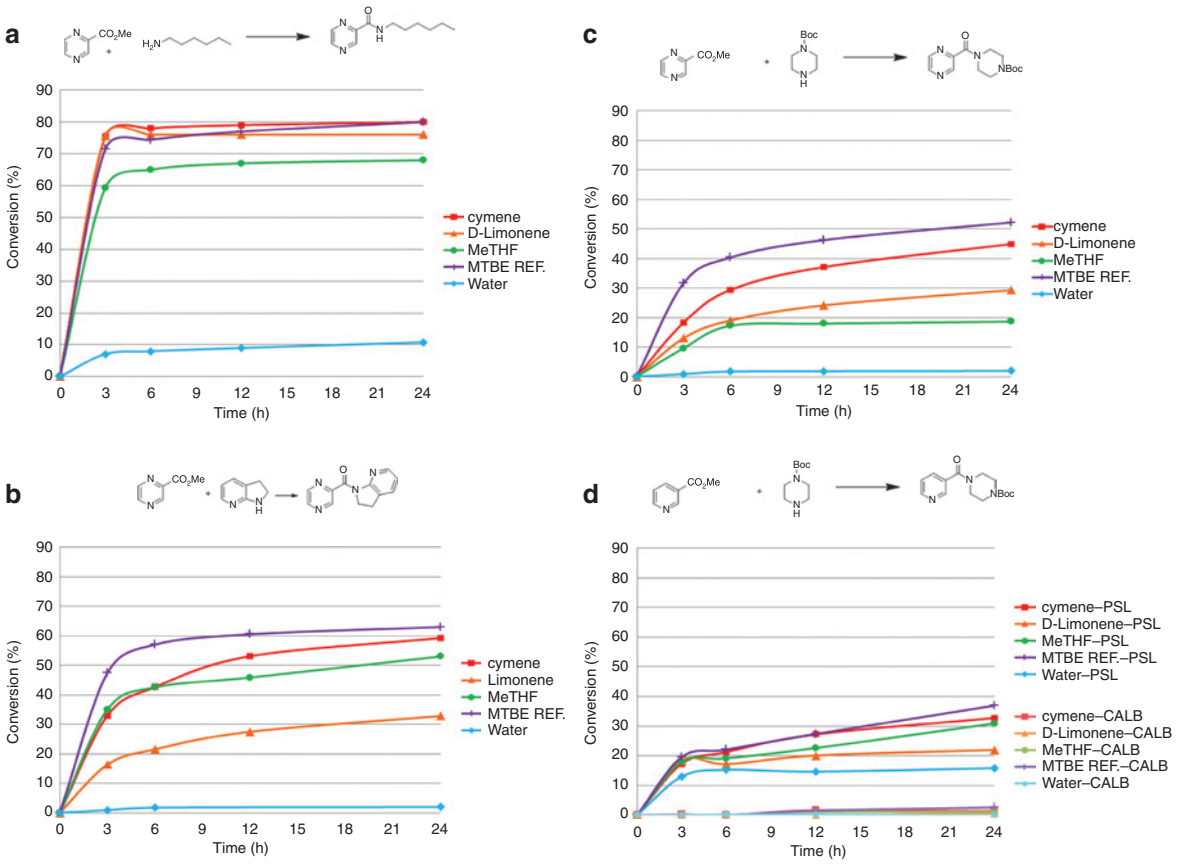

Fig. 3: Conversion plots of enzyme catalysed reactions in different solvents. Plots (a)–(c) compare reactions of methyl 2-pyrazinecarboxylate with (a) n-hexylamine, (b) 7-azaindole, (c) 1-Boc-piperazine. Plot (d) compares the activities of PSL and CALB in the reaction of methyl 2-pyrazinecarboxylate with 1-Boc-piperazine in various solvents.

water **10**. Profiles of other bio-solvents are available in the Supporting Information (Figs. S9, S13, S15, S22). The first three reaction figures share a common ester substrate, pyrazine carboxylate **11**. In each plot, the substrate was reacted with the three different amines (**15** in 3b, **16** in 3a, **17** in 3c), yielding significant differences in terms of conversion, reaction profiles, distribution, and final equilibrium.

The highest yields were achieved with substrate pair consisting of pyrazine carboxylate with the linear aliphatic amine (in Fig. 3a), in the range of 70–80 %. These values were achieved in less than 3 h in all organic solvents, with bio-based D-limonene and p-cymene matching the profile in MTBE. The accessibility and low steric hindrance of hexylamine **16** are believed to play a predominant role in the reaction, thus outweighing most solvent effects.

Solvent effects became more prominent once less favourable substrate combinations were used. Reactions in Fig. 3b and c present two secondary amines commonly used as building blocks in medicinal chemistry, 2,3-dihydro-7-azaindole **15** and N-Boc-piperazine **17**: the first bearing fused aromatic ring which electronically deactivates the reactive amine and adds steric hindrance; the second presenting a t-butyloxycarbonyl (Boc-) hindering group diametrically opposed to the amine binding site. A comparison of the early reaction profiles suggests that the azaindole **15** reacts more quickly with the ester despite of the deactivation effect and geminal hindrance. Van Pelt et al. had previously reported high PSL activity against deactivated amines [8], and other studies also compared the specificity of PSL with other widely used lipases in particular *Candida Antarctica* lipase type B (CALB) and benzoin-like substrates [8–11]. However, previous work had not investigated cyclic amines and bulky 'para'-substituents, such as N-Boc-piperazine **17**. This work suggests that 4-substituted amines are less favoured as substrates for PSL catalysis compared to 2-substituted amines and primary amines. p-cymene was demonstrated as a reliable alternative to MTBE.

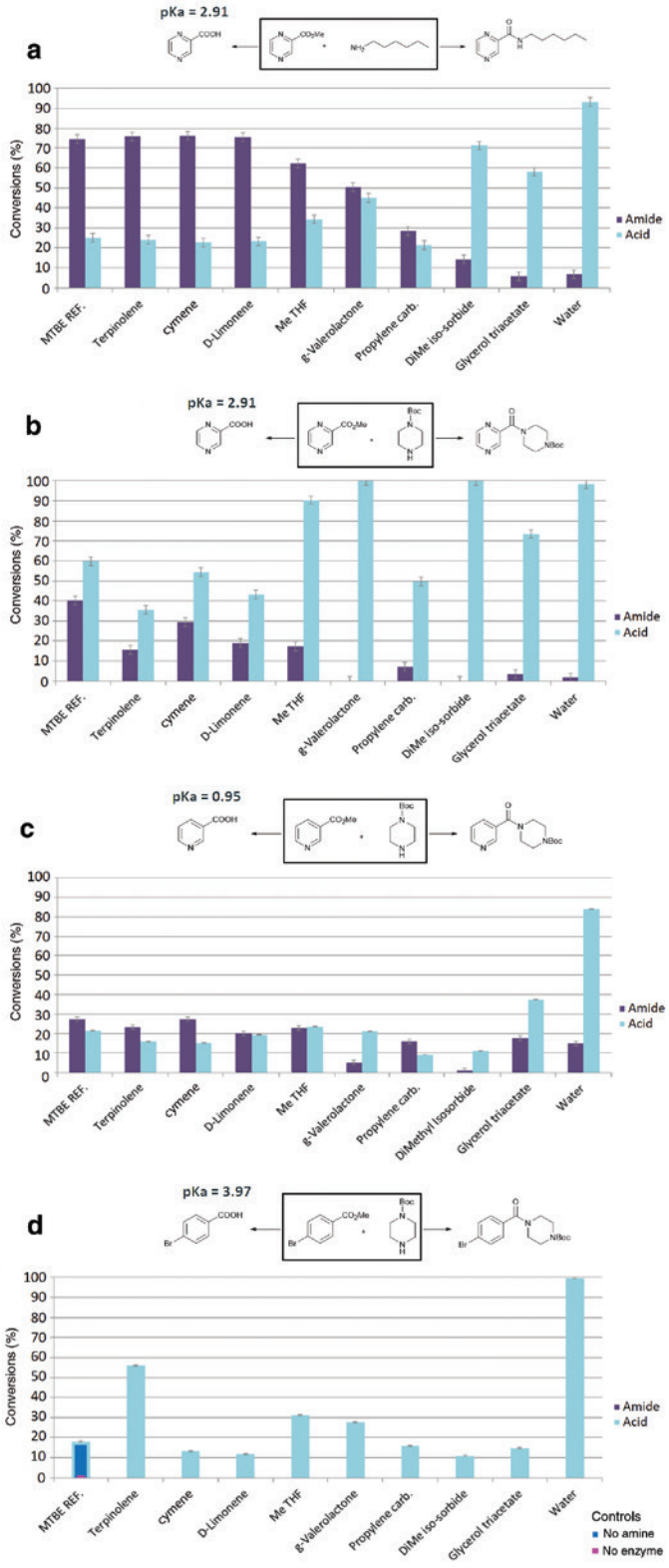

Fig. 4: Comparison of aminolysis (purple) versus hydrolysis (blue) after 6 h in the reaction of (a) methyl 2-pyrazinecarboxylate with n-hexylamine, (b) methyl 2-pyrazinecarboxylate with 1-Boc-piperazine, (c) methyl nicotinate with 1-Boc-piperazine and (d) methyl 4-bromobenzoate with 1-Boc-piperazine. In each case error bars were obtained by repetition of the reaction in MTBE (reference reaction) and extended across the dataset. In (d) no aminolysis was observed only hydrolysis of the ester, removal of the amine (dark blue) did not result in an increase in hydrolysis, while in the absence of enzyme (pink) no reaction took place.

In Fig. 3d, this observation was verified with a structurally similar but less reactive ester, pyridine carboxylate **12**. The reaction was monitored in the same set of solvents and compared between PSL and CALB to assess capability. While PSL showed activity against this substrate pair in all solvents, CALB was shown to be inactive. Across the plots of Fig. 3, it can be noticed that some reactions start with a rapid initial rate of reaction and after 3 h they either plateau or adopt a different gradient. This effect was also observed in other reactions, see Supporting Information. Current literature describes this phenomenon as caused by the competition from the hydrolytic cleavage of the ester [9]. The addition of molecular sieves to the reaction was previously proven to control moisture and avoid accumulation of methanol in the medium, and thus used to prevent this competition from taking place.

The competition between aminolysis (amide formation) and hydrolysis (carboxylic acid formation) was investigated and summarised in Fig. 4, for which the relative conversions to amide or acid are presented at a 6-h time-point against 10 solvents. In general, the results suggest that the intensity of this effect may be influenced by both the solvent and the substrate combination used. Solvents are presented along the bar chart in order of increased hydrophilicity, with MTBE and water as benchmarks for comparison at the two sides.

Across the four plots of Fig. 4 solvent influence varies significantly, yet some general consideration can be made: (1) the more non-polar solvents (MTBE, terpinolene, p-cymene, D-limonene) facilitate the aminolysis giving the highest amide to acid ratios – this trend is particularly evident in Fig. 4a; (2) methyl-THF and γ-valerolactone show the highest variability in behaviour, but seem to favour hydrolysis (3) comparisons between dried and wet solvents have been undertaken and no difference was observed, suggesting that the water partaking in hydrolysis is already present in the enzyme formulation (Figs. S3 and S4 Supporting Information).

Figure 4 can also be analysed in terms of substrate specificity. In Fig. 4a and b, the same ester was reacted with different amines showing that the least reactive one was more easily subjected to competition from hydrolysis. Different esters were also investigated in order to assess whether their stability was a factor in the trends observed. Acid dissociation constant pKa was used to account for the reactivity of the carbonyl group, where higher pKa meant more efficient delocalisation of the negative charge of the carboxylate ion by the aromatic ring. In Fig. 4b and c, a switch from pyrazine carboxylate **11** to pyridine carboxylate **12**, i.e. a less reactive carbonyl group (acid pKa 0.95), led to a decrease in the overall conversion of the ester across the solvent spectrum, but had little effect on the aminolysis to hydrolysis ratio. In contrast, the use of 4-bromobenzoic ester **14** was expected to give higher conversions due to its more electron-positive carbonyl (acid pKa 3.97), but instead gave no sign of aminolysis as shown in Fig. 4d. Partial hydrolysis was observed in this latter case, equivalent to the control tests where no amine was added to the MTBE medium (Fig. 4d) – for more control tests see Supporting Information.

Conclusions

In conclusion, this work provides additional guidelines for conscious selection of solvents and substrate combinations for PSL catalysed amide synthesis. Renewable terpene-based solvents (terpinolene, p-cymene, D-limonene) were proven to be reliable suitable media for PSL amidation. Such solvents could provide a safer and more sustainable alternative to traditional non-polar solvents including toluene. The main observation in terms of substrate scope, verified both with PSL and CALB, is that once the enzyme binds a bulky para-substituted substrate, it is subsequently unable to accommodate anything other than a small reagent in the active site, thus discriminating against the aminolysis and favouring the hydrolysis. This mechanism of binding supports the widely accepted 'Ping Pong – Bi Bi' mechanism used to describe enzyme kinetics. Typically, the enzyme would first bind the ester, release the alcohol, and only then bind the nucleophile for acylation – be it the amine, alcohol or water [15, 16]. Ester stability (pKa) does not appear to drive selectivity, but rather to influence overall conversion rate.

This study highlights the significant potential for bio-based solvents (included limonene which can be extracted from orange peel waste) to be utilised in combination with PSL enzymatic catalysts to promote amidation reactions for the synthesis of pharmaceutically relevant fine chemicals. This therefore enhances

the green credentials of such processes and opens new doors for the application of bio-based solvents in pharmaceutical synthesis. This work also demonstrates a need to further investigate enzyme activity in relation to para- or 4-substituted substrates. A priority in PSL chemistry remains a methodology to tackle the competing hydrolysis reaction. Further protein immobilisation purification and optimisation developments [17], coupled with a clearer understanding of chemical potential will aid in furthering the uptake of PSL in fine chemical manufacturing.

Acknowledgements: Andrew Hunt would like to acknowledge the financial support of the Thailand Research Fund (Funder Id: http://dx.doi.org/10.13039/501100004396, RSA6280031) and Khon Kaen University. Financial support from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation is gratefully acknowledged. The research leading to these results has received funding from the Innovative Medicines Initiative (http://www.imi.europa.eu) Joint Undertaking under Funder Id: http://dx.doi.org/10.13039/501100010767, grant agreement no. 115360, resources of which are composed of the financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies in kind contribution.

References

[1] A. K. Ghose, V. N. Viswanadhan, J. J. Wendoloski. *J. Comb. Chem.* **1**, 55 (1999).
[2] J. S. Carey, D. Laffan, C. Thomson, M. T. Williams. *Org. Biomol. Chem.* **4**, 2337 (2006).
[3] C. A. Montalbetti, V. Falque. *Tetrahedron* **61**, 10827 (2005).
[4] J. H. Clark, D. J. Macquarrie, J. Sherwood. *Green Chem.* **14**, 90 (2012).
[5] A. Iemhoff, J. Sherwood, C. R. McElroy, A. J. Hunt. *Green Chem.* **20**, 136 (2018).
[6] A. Gallant Lanctôt, T. M. Attard, J. Sherwood, C. R. McElroy, A. J. Hunt. *RSC Adv.* **6**, 48753 (2016).
[7] G. Paggiola, A. J. Hunt, C. R. McElroy, J. Sherwood, J. H. Clark. *Green Chem.* **16**, 2107 (2014).
[8] A. Pellis, F. P. Byrne, J. Sherwood, M. Vastano, J. W. Comerford, T. J. Farmer. *Green Chem.* **21**, 1686 (2019).
[9] B. M. Dorr, D. E. Fuerst. *Curr. Opin. Chem. Biol.* **43**, 127 (2018).
[10] N. J. Turner, E. O'Reilly. *Nat. Chem. Biol.* **9**, 285 (2013).
[11] A. Aires-Trapote, P. Hoyos, A. R. Alcántara, A. Tamayo, J. Rubio, A. Rumbero, M. J. Hernáiz. *Org. Process Res. Dev.* **19**, 687 (2015).
[12] S. Van Pelt, R. Teeuwen, M. Janssen, R. Sheldon, P. Dunn, R. Howard, R. Kumar, I. Martínez, J. Wong. *Green Chem.* **13**, 1791 (2011).
[13] A. Maraite, P. Hoyos, J. D. Carballeira, Á. C. Cabrera, M. B. Ansorge-Schumacher, A. R. Alcántara. *J. Mol. Catal. B: Enzym.* **87**, 88 (2013).
[14] S. Kim, Y. K. Choi, J. Hong, J. Park, M. J. Kim. *Tetrahedron Lett.* **54**, 1185 (2013).
[15] As identified by industrial partners within European consortium CHEM21 – www.chem21.eu (accessed Aug 2019).
[16] S. K. Karmee, R. van Oosten, U. Hanefeld. *Tetrahedron: Asymmetry.* **22**, 1736 (2011).
[17] https://echa.europa.eu/substance-information/-/substanceinfo/100.015.140 (accessed May 2019).
[18] M. P. Bousquet-Dubouch, M. Graber, N. Sousa, S. Lamare, M. D. Legoy. *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1550**, 90 (2001).
[19] A. Cornish-Bowden. *Fundamentals of Enzyme Kinetics*, 4th ed., Wiley-Blackwell, London (2012).
[20] Y. Cao, Y. Zhuang, C. Yao, B. Wu, B. He. *Biochem. Eng. J.* **64**, 55 (2012).

Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/pac-2019-0808).