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Biocatalysis in bio-derived solvents: an improved approach for medium optimisation

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An improved multi-parameter approach to the correlation of enzymatic activity and solvent properties is proposed. The development of the correlation facilitated the introduction of renewable solvents in a CALB catalysed synthesis of fatty esters.

The development of effective enzymatic catalysis has great potential for the improvement of industrial chemical processes.[1] Both process efficiency and environmental footprint could benefit from the use of biocatalysts as well as product claims notably 'natural'.[2] One of the obstacles that has often prevented industry from using this methodology is the difficulty in matching the enzyme's preferred reaction conditions to those of a typical organic chemical process.[3] Traditionally, water is the solvent employed in bio-transformations. Despite its strong environmentally favourable features with respect to safety, cost and sustainability, its use in many of industrial applications is problematic due to: a) solubility problems that may require the addition of an organic water-soluble cosolvent; b) difficulties in isolation and purification steps; c) difficult enzyme recovery; d) water affecting the reaction chemistry; e) cost and environmental impact of treating contaminated aqueous waste. These issues may be addressed by pursuing non-aqueous biocatalysis, a well established technology in the academic literature, yet not common in industrial application.[1,4] An analysis of the literature shows that there has been a somewhat random approach to the choice of organic solvent in enzymatic screening, reflecting the difficulty in predicting which type of solvent could be the most suitable for a specific bio-process.[5,6]

The little work that has been done on using so-called 'green' solvents has primarily focused on ionic liquids and supercritical CO$_2$.[7] Very recently some attention has been given to the use of bio-derived solvents.[8] In particular the use of bio-derived solvents could provide an effective alternative media without compromising on the safety and the environmental acceptability of the process.[9]

Laane *et al.* (1987) first suggested that log P (namely the solvent's ability to partition between an octanol/water mixture) was a suitable solvent parameter for organic medium optimisation in biocatalysis.[10] Several studies have followed, looking at different parameters for defining a robust correlation between solvent properties and biocatalytic performance.[11 12]

Herein, an improved multi-parameter approach to the analysis of the behaviour of *Candida Antarctica* Lipase type B (Novozyme 435) in 24 different organic solvents is reported. A kinetic screening was carried out on an esterification reaction to form a commercially valuable fatty ester, hexyl laurate, widely used in personal care and cosmetics' formulations.[13]
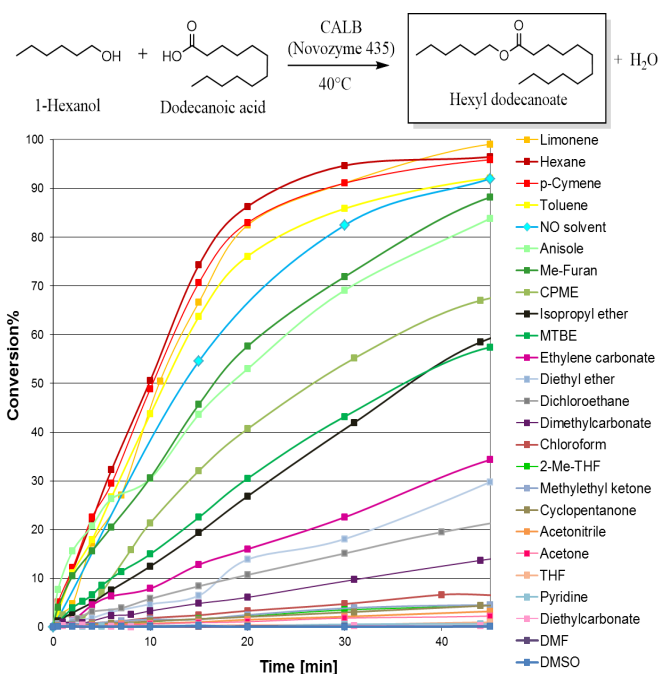

Fig. 1 Kinetic screening of CALB in organic solvents

As a control, catalyst-free and solvent-free reactions have been undertaken. In absence of the biocatalyst no conversion

was observed. Whilst, experiments performed in a catalysed solvent-free system demonstrated reaction rates between those conducted in toluene and Me-furan. The catalysed solvent free system set a benchmark between solvents that enhance or inhibit the rate of reaction. At first glance and from a green chemistry stand point a reaction that requires no solvent is preferable. However, significant quantities of auxiliaries are required for the extraction, purification and analysis of the products, thus reducing the perceived advantages of a solvent-free system.[9]

The solvents of study were chosen to provide a range of log P values from -2 to +5, encompassing conventional solvents and including a few bio-based - or potentially bio-based - ones. Unsurprisingly, hydrophobic solvents, with higher log P values, appeared to have the best asset for this reaction.

This first observation was assessed through a correlational analysis, in which the initial reaction rate was compared against log P in order to verify the relevance of the latter. In addition, a regression analysis using the 'least squares' method was employed to investigate the statistical significance of six solvent parameters and the combination thereof: α (hydrogen bond donating ability), β (hydrogen bond accepting ability), π^* (polarisability), δ_H^2 (square of Hildebrand's solubility parameter), Vm (molar volume), ln[w] (measured water content).[14] By a reiterative process of excluding the variables carrying the highest risk of misdirecting the correlation, a statistically relevant correlation was found when combining hydrogen basicity and molar volume.

Figure 2 and Table 1 present a comparison of the outcomes from the two approaches: a) the traditional initial rate vs log P correlation, b) the newly proposed correlation between initial rate vs β and Vm.
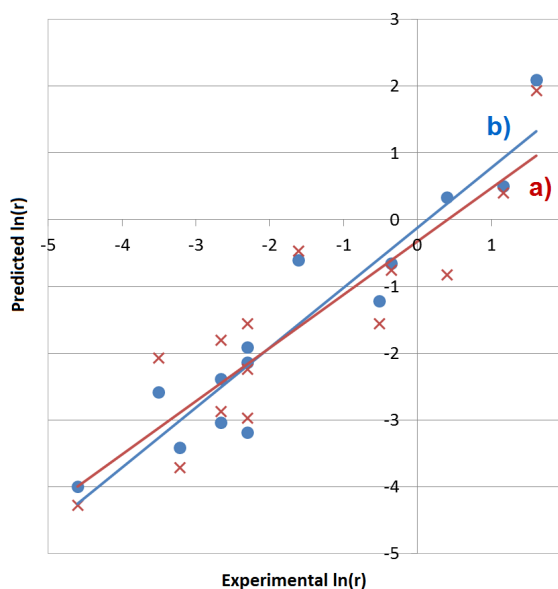

Fig. 2 Comparison of predicted rate values (y) against experimental ones (x) through the a) traditional and b) new model. Only the solvents with known and verified solvent parameters have been used in the correlation analysis. The initial rate in the ln(r) values expressed in mM/min.

Table 1 Statistical parameters of the two correlations

Partition coefficient ($LogP$)	Combined β and Vm
$ln(r) = -2.59 + 1.16logP$	$ln(r) = -3.70 - 4.53\beta + 0.044Vm$
$y/x = 0.80$	$y/x = 0.90$
$R^2 = 0.79$	$R^2 = 0.90$
$intercept = 0.33$	$intercept = 0.12$

The devised two-parameter-based correlation shows a marked improvement of the statistical parameters over the previous one. The ratio between the predicted and the experimental rate values is closer to 1, as well as the trend-line's R^2 value, corresponding to about 10% improvement. Moreover, the natural intercept of the trend-line is much closer to zero is desirable.

It is important to note that log P was defined by Kamlet *et al.* (1984) as:[15]

$$logP = 0.24 - 3.38\beta + 0.0266Vm - 0.96\pi^*$$

The fact that the $\dfrac{\beta}{Vm}$ ratio from the correlation equation is comparable to the one from the log P definition confirms that log P is indeed a statistically significant parameter for studying the medium effects on biocatalysis. The new approach suggests that π^* has a negligible contribution and that a more reliable correlation can be obtained by excluding this parameter.

High hydrogen basicity and small molar volume mean strong solvent-solvent interactions. These may create an organised cybotactic region and impose an entropic penalty on the kinetics of the reaction. In fact, high solvent structuredness can hinder the solutes from easily diffusing in and out the active site's environment, thereby impacting on the formation of the Michaelis complex (acyl-enzyme), which is commonly considered to be the rate determining step.[5][16]

The prevailing effect of the molar volume over the polarisability coefficient may be explained through its influence on how the solvent interacts and diffuses into the enzyme's active site. Bulky and poorly interactive solvents are less likely to diffuse into the active site and thus disrupt its polarity and configuration. This interpretation finds support in concepts presented by Ottolina *et al.* (1994) and Serdakowski & Dordick (2007) on how the solvent's properties and geometry can interact with the active site.[17,18] As a result, solvents possessing a low β and high Vm values are expected to be ideal for this type of reaction.

Case study: From solvent properties to green solvent-selection

The green credentials of solvents are gaining increasing weight in the solvent-selection process, in which a balance between reaction efficiency and eco-efficiency should always be sought.[9] The literature reports a number of studies in which hydrolases catalysed reactions were carried out in bio-based or potentially bio-based solvents. Among these, the use of 2-methyltetrahydrofuran is the most prominent.[19] However, the lipases' strongly hydrophobic medium requirements have not yet been met by a bio-solvent.

A recent research has found citrus-derived alkenes (limonene and *p*-cymene) as being suitable solvents for solid-acid catalysed acylation reactions.[16] The low basicity and low Hildebrand's parameter of these bio-solvents were ideal, favouring the reaction. Notably, the joint effect of β and δ_H^2 was related to the solvent's associating ability and in particular to its capacity to accommodate a bulky transition state. The considerations presented in that case resemble the ones deduced from the lipase screening in this study. Similarly the combination of β and Vm account for the solvent's structured-ness and its entropic and enthalpic contribution to the reaction's kinetics. This fact has inspired the first application of limonene and p-cymene, along with other bio-based solvents, in lipase catalysis within this study.

The biocatalysed synthesis of hexyl laurate was performed in nine organic solvents that currently have the potential to be 100% derived from bio-resources. Among these, limonene and p-cymene as natural extracts from food waste, 2-methylfuran and 2-methyltetrahydrofuran as sugar-derivatives, four carbonates that can be obtained from bio-alcohols and carbon dioxide, and acetone as a fermentation by-product.[20] Figure 3 highlights the performance of each of these solvents which is expressed as the logarithm of the initial rate of reaction.

Accordingly with the previous considerations, the solvents derived from citrus waste provided for the highest initial rate and almost 100% conversion after 40 minutes (as seen in Figure 1). The remarkable reaction performance obtained in these lowly interactive and bulky solvents has confirmed the considerations derived from the correlation study. Moreover, the effect of catalyst load and reusability in green solvents has been investigated and it is made available in the supplementary information.

Among the other solvents, 2-methylfuran gave the highest initial rate followed by the bulkier cyclic carbonates. Once more, decreasing rates of reaction have been observed for the increasing solvent's hydrophilicity. A parallel between hexane and p-cymene reveals a similar reaction efficiency, yet the latter is highly superior as for its sustainability and health & safety credentials.[16]
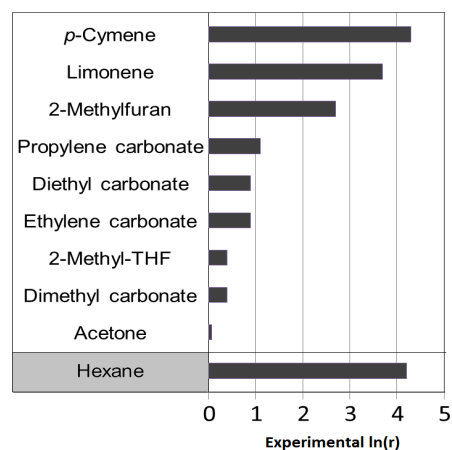

Fig. 3 Bio-based solvents' performance compared to a common oil-derived solvent. The initial rate in the ln(r) values expressed in mM/min.

In conclusion, an improved correlation was found relating the biocatalytic performance and the solvent properties based on hydrogen-bond accepting ability and molar volume. Compared to previous literature models the solvent's molar volume becomes a meaningful parameter. This fact could relate to its participation in log P and therefore in the ability of the solvent to extract structural water from the enzyme. Moreover, it is expected to play a role in penetrating the active site and thus influencing the solutes diffusivity. The high rate and yield of reaction in citrus-waste derived solvents and the almost complete activity retention after addition of fresh reagents provide the basis for a highly effective and sustainable process for industrial fatty ester synthesis.

Acknowledgements

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