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Synthesis of cholesterol-reducing sterol esters by enzymatic catalysis in bio-based solvents or solvent-free

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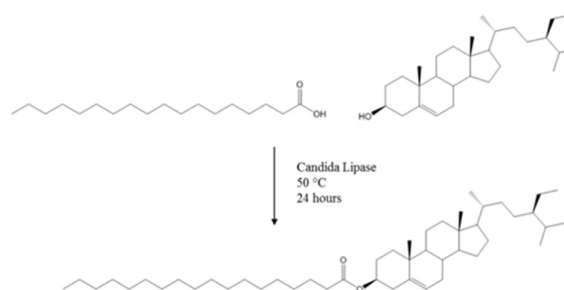
Enzymatic synthesis of a β -sitosterol ester in bio-based solvents was compared with conventional solvents. Limonene and *p*-cymene gave higher initial reaction rates than *n*-hexane, and comparable conversions after 24 hours (~75%). Importantly, a solvent-free system yielded the highest conversion (88%).

Due to their structural similarities, β -sitosterol has been proven to reduce absorption of cholesterol in the gut.¹⁻⁴ However, in order to use phytosterols as food additives, their properties need to be improved. β -Sitosterol has poor solubility in both water and fat.⁵ A high melting point also makes β -sitosterol unsuitable for formulation into foods that require refrigeration.⁵ Esterification of β -sitosterol with a food grade fatty acid is a common method used to improve these characteristics. β -Sitosterol esters are now frequently used as natural hypo-cholesterolemic food additives.^{6,7}

Classically, phytosterols would be reacted with food grade fatty acids or fatty esters *via* acid catalysed esterification or base catalysed transesterification.^{8,9} These reactions are performed at high temperatures which is energy intensive and can cause degradation of the product or lead to by-product formation. In most cases, a post-reaction workup dilutes the catalyst into a low concentration acidic or basic aqueous phase. Catalyst reuse is not possible without extensive drying, and as such neutralisation and disposal is often carried out instead.^{8,9} As the β -sitosterol esters need to be a food-grade product, sufficient purification must be carried out to meet regulatory barriers.¹⁰

Alternatively, the lipase-catalysed esterification of β -sitosterol with fatty acids has been reported for industrial use (Scheme 1).¹¹ Many lipases have been studied in this reaction giving varying yields based on the nature of the enzyme and of the substrates employed.¹²⁻²³ In this work we consider the use of bio-based solvents to complement bio-catalysis. Bio-based

solvents are wholly or partially produced from biomass feedstocks.²⁴ They can be equivalent to conventional solvents (e.g. bio-ethanol) or neoteric molecules.²⁵



Scheme 1. Lipase catalysed esterification of β -sitosterol with steric acid.

To the best of the authors' knowledge, the lipase-catalysed sterol esterification has not been reported in a range of bio-based solvents.²⁶ Herein, the lipase catalysed esterification of β -sitosterol was investigated in green and sustainable bio-based solvents to give a nutraceutical product. Three readily available lipases were selected for initial studies and the most active enzyme was subsequently used for kinetic studies in a range of bio-based solvents. The objective was to test a range of solvents with dissimilar properties in order to examine and understand the relationship between solvent properties and reaction efficiency.

Lipase from *C. rugosa* and lipase from *C. antarctica*, CAL-A and CAL-B were investigated. *C. rugosa* and CAL-A were selected due to their high performance for this type of reaction in the literature, while CAL-B was selected due to its broad applicability.^{17,19,20} An enzyme-free control experiment was also performed to confirm the necessity of a catalyst. The experiment was carried out in *n*-hexane as the reaction was previously reported to perform well in this non-polar solvent.^{17,20} The results of this study showed that CAL-A had the best performance reaching a conversion of 74% after 24 hours and was therefore selected for subsequent reactions (see the ESI).

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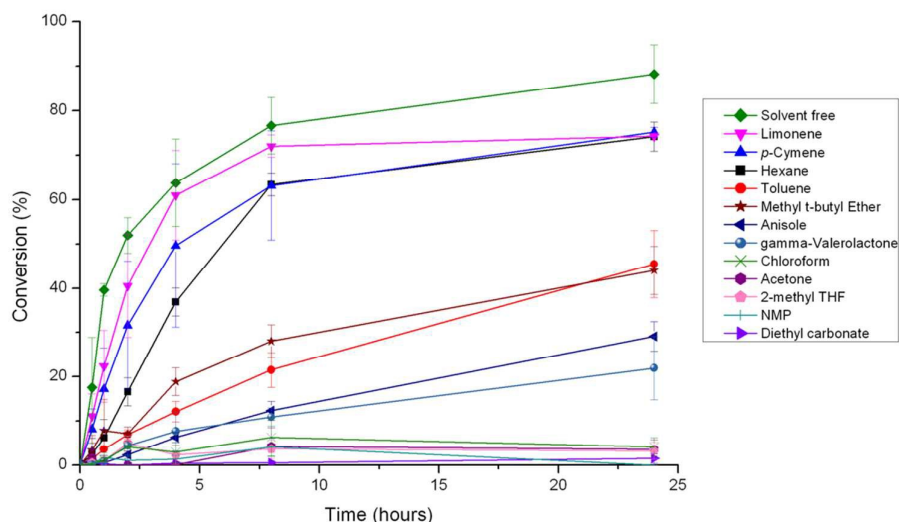


Fig. 1. Kinetic screening of CAL-A catalysed esterifications of β -sitosterol with stearic acid in different solvents.

To investigate the possible use of bio-based solvents for this reaction, 8 solvents were chosen and compared with a number of conventional solvents. Solely aprotic solvents were selected because protic solvents such as alcohols could act as competitive inhibitors. The majority of the bio-based solvents selected have had their green credentials discussed and ranked in a recent solvent selection guide.²⁷ All are classed as recommended or problematic, with the latter classification predominantly as a result of their high boiling point impairing recovery. None of the bio-based solvents used in this work are considered hazardous or highly hazardous. The progression of the β -sitosterol esterification with stearic acid was monitored by GC-FID. The conversions over time are shown in Fig. 1. The reaction can be seen to progress rapidly in limonene and *p*-cymene, both derived from the essential oil of citrus waste.²⁸ These solvents work as well as, if not better than, the conventional solvent *n*-hexane. In *n*-hexane, good conversion is obtained but the reaction progresses more slowly than with the citrus-derived solvents.

Recent work focussed on enzymatic esterification reactions to form aliphatic esters has found that a hydrophobic environment (*i.e.* a hydrocarbon solvent) outperformed solvent-free systems.²⁹ However, no solvent is the preferred option since the effective concentration of reactants are much higher. This current study demonstrated that the reaction of β -sitosterol with fatty acids in solvent-free conditions provided the fastest rate and highest yield of β -sitosterol esters. This is likely due to the large hydrocarbon regions of the reactants. Furthermore, the high enzyme loading used throughout this study could have lessened the mass transfer difficulties often

associated with solvent-free reactions.³⁰ The amount of enzyme use is expected to permit adequate contact between the substrates and catalyst. At larger scales, mass transfer issues are likely to be more significant. Another potential issue with the solvent-free system at larger scales is that higher chain length fatty acids will remain solid at low temperature, suggesting the need for a solvent may become more important. Future work shall include an optimisation of the catalyst loading in the best performing solvents and for the solvent-free reaction, ensuring a less wasteful and more cost-effective procedure. Nonetheless, if the solvent-free reaction performs well this would circumvent the primary limitation of the high boiling citrus based solvents, where solvent removal is energy intensive unless the products can be precipitated from solution.

Table 1 gives the experimentally determined initial reaction rates in each solvent. The relationship between reaction rate and different solvent properties was investigated. There were no statistically significant correlations with either dipolarity (π^*), hydrogen bonding (donating ability, α ; accepting ability, β), or molar volume individually (see the ESI). However, the results did show a correlation with $\log P_{ow}$, which is in turn dependent primarily on hydrogen bond accepting ability (β), but also dipolarity and molar volume all in combination.²⁹ This is consistent with research on the CAL-B catalysed production of hexyl laurate which found that solvents with high $\log P_{ow}$ values provided the greatest reaction rates.²⁹ Some outliers were present for solvents with a low $\log P_{ow}$. The main outlier was methyl *t*-butyl ether, which displayed higher conversions than expected based on its low lipophilicity.

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Table 1. Solubility parameters and initial rates of reactions for different solvents.

Reaction solvent [†]	α	β	π^*	Log P_{ow}	Initial rate of reaction (mmol/hour)
Solvent free					
None	n/a	n/a	n/a	n/a	0.1057
Bio-based solvents					
Limonene	0.00 ³¹	0.00 ³¹	0.16 ³¹	4.57 ³²	0.0599
<i>p</i> -Cymene	0.00 ³¹	0.13 ³¹	0.39 ³¹	4.10 ³³	0.0464
Diethyl carbonate	0.00 ^{††34}	0.40 ^{††34}	0.45 ^{††34}	1.21 ³²	0.0004
Propylene carbonate	0.00 ³⁵	0.38 ³⁵	0.90 ³⁵	-0.41 ³²	0.0025
Acetone	0.08 ^{††34}	0.48 ^{††34}	0.71 ^{††34}	-0.24 ³³	Negligible
2-MeTHF (2-methyltetrahydrofuran)	0.00 ³⁶	0.58 ³⁶	0.53 ³⁶	1.26 ³⁷	Negligible
γ -Valerolactone	0.00 ³⁶	0.60 ³⁶	0.83 ³⁶	-0.13 ³⁷	0.0048
Cyrene (dihydrolevoglucosenone)	0.00 ³⁸	0.61 ³⁸	0.93 ³⁸	-1.52 ³⁹	Negligible
Conventional solvents					
<i>n</i> -Hexane	0.00 ^{††34}	0.00 ^{††34}	-0.08 ^{††34}	4.00 ³³	0.0161
Toluene	0.00 ³¹	0.12 ³¹	0.50 ³¹	2.73 ³³	0.0092
Methyl <i>t</i> -butyl ether	0.00 ⁴⁰	0.45 ⁴⁰	0.25 ⁴⁰	0.94 ³³	0.0099
Anisole	0.00 ^{††41}	0.32 ^{††41}	0.73 ^{††41}	2.11 ³³	0.0061
NMP (<i>N</i> -methyl-2-pyrrolidinone)	0.00 ³⁸	0.75 ³⁸	0.90 ³⁸	-0.46 ³⁷	0.0013
Chloroform	0.20 ^{††41}	0.10 ^{††41}	0.58 ^{††41}	1.97 ³³	0.0020

[†]The greenness of solvent entries are colour-coded according to the assessment developed in ref. ²⁷. ^{††}Polarity values are an average of different dye sets.

Greater than expected product formation was also observed in γ -valerolactone despite its negative log P_{ow} value (Fig. 1 and table 1). The performance of methyl *t*-butyl ether is unusual given its low log P_{ow} , but not unprecedented because considerable yields were also seen by Panpipat *et al.* using CAL-A to catalyse reactions in this solvent.²⁰

Solvents with a high log P_{ow} displace the layer of water loosely solvating the enzyme, but as they are hydrophobic they do not have the ability to strip the enzyme-bound water.³⁰ This is expected to shift the equilibrium of esterification forwards without losing enzyme activity. It is understood that hydrophobic organic solvents reduce the flexibility of the enzyme by locking it into its active form.³¹ This can explain the kinetic benefit of using lipophilic solvents. Although limonene, *p*-cymene and *n*-hexane have very similar log P_{ow} values, the initial reaction rate in *n*-hexane is notably lower than in the other two solvents. The poor solubility of the reactants in the less polar *n*-hexane is likely to exacerbate the effect of its lesser lipophilicity.^{32,33} The two unsaturated citrus-based solvents may interact to some extent with the polar regions of the sterol and fatty acid, facilitating their solvation enough not to hinder the reaction.

In *p*-cymene, the initial reaction rate increases with increasing temperature (see ESI). It has been shown that CAL-A

is very stable at high temperatures and suggested that its optimum temperature is above 90 °C.³⁴ Nonetheless, it also performs well in mild conditions and 50 °C was the maximum temperature selected in order to allow for a range of other enzymes to be tested, as well as a range of solvents (2-MeTHF, acetone, chloroform *etc.* all have relatively low boiling points). The effect of acyl chain length on the rate of esterification in *p*-cymene was also investigated (see ESI); and the reaction rate improves with acyl chain donors of longer length which is consistent with previous observations in the literature.²⁰

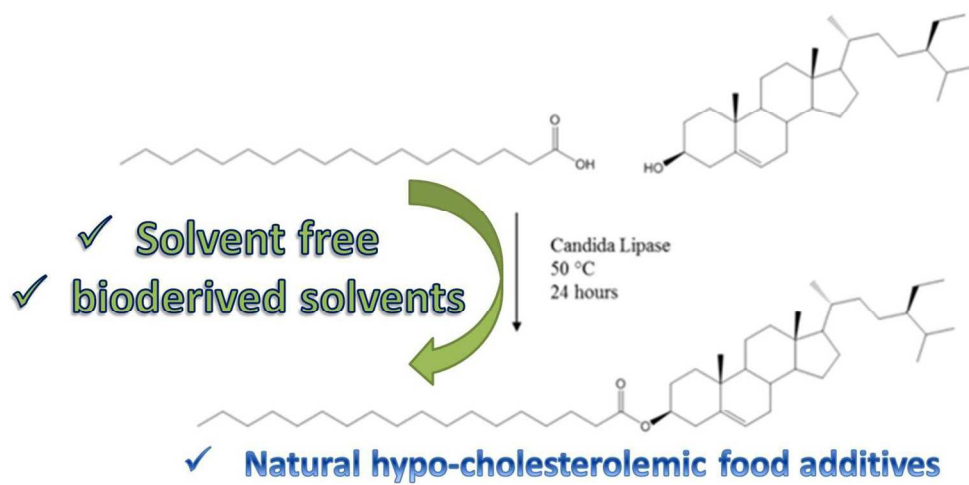
Conclusions

The present work has investigated the influence of bio-based solvents on the synthesis of sterol esters. Generally, the initial reaction rate is proportional to solvent log P_{ow} as is typical of enzymatic catalysis. Esterifications in general benefit from low polarity solvents (and specifically those with poor hydrogen bond accepting ability, β),^{35,36} and log P_{ow} is inversely proportional to hydrogen bond accepting ability (β).²⁸ It has been demonstrated that green bio-based solvents such as limonene and *p*-cymene are suitable alternatives to *n*-hexane and other conventional solvents for the biocatalysis of β -sitosterol with fatty acids to form a β -sitosterol ester. Since β -sitosterol esters are important nutraceutical products, the

solvents used in the esterification reactions have to be food-certified, which paves the way for the use of natural, bio-based solvents for this type of application. Importantly, a solvent-free system yielded the highest conversion (88%) to the sterol ester. The use of a solvent-free system not only reduces the energy associated with solvent removal but can lead to a green biocatalytic route to β -sitosterol esters which could act as effective natural hypo-cholesterolemic food additives.

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