Development of Lipase Mediated Epoxidation Process for Monoterpenes in Choline Chloride Based Deep Eutectic Solvents as well as solvent free conditions

SUPPLEMENTARY INFORMATION

OPTIMISATION USING THE TAGUCHI DESIGN

Choice of parameters and crossed array technique: The optimization of lipase mediated epoxidations was done for the GlCh and SoCh systems using the Taguchi crossed array method for optimization. When using the crossed array Taguchi method, the parameters must be categorized into two. They are explained below.

Inner array - represents the controllable parameters of the process. E.g. temperature, enzyme amount, type of reactant used and urea·H_2O_2.

Outer array - represents the uncontrollable parameters of the process. E.g. DES mixture

Once the parameters are categorized, the next step is to determine the array to be used. But it is vital that all experimental combinations of the inner array are tried out with every parameter(s) and level(s) of the outer array[59]. The inner and outer arrays and their levels used in this work are given in the table below.

Table 1: The different parameters and levels used during the optimization process are given. Since crossed array is used, their categorization is also given

Identifier	Parameter	Inner/outer	Level 1	Level 2	Level 3
A	Temperature	Inner	40 °C	50 °C	60 °C
B	Urea·H_2O_2	Inner	2 mmol	3 mmol	4 mmol
C	Enzyme	Inner	50 mg	75 mg	100 mg
D	Reactant	Inner	3-carene	Limonene	α-pinene
E	DES Mixture	Outer	GlCh	SoCh	Not applicable

From *Table **1***, it can be seen that there is one uncontrollable and four controllable parameters. So, theoretically, we could have combined the parameters and ran a normal L_{18} array, but instead, we used two L_9 arrays for the optimization of the two systems. The L_9 layout used is given below. The CALB enzyme used in this study was obtained from Chiral Vision (IMMCALB-T2-TXL, 15000 PLU/g). Therefore, 50 mg tests contained 750 PLU, 75 mg contained 1125 PLU and 100 mg tests contained 1500 PLU.

Table 2: L_9 orthogonal array showing the controllable parameters for the optimization of the epoxidation process in deep eutectic solvents (For detailed description of A-D, 1-3, please refer Table 1

Trial #	A	B	C	D
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2

7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

The justification for using two L_9 arrays is that for both cases a total number of 54 runs (2 L_9 arrays * 9 trials * 3 repetitions) was needed to arrive at a conclusion. The same number of runs would have been necessary if we chose an L_{18} array for process optimization (18 trials with 3 repetitions). Moreover, the method of analysis is still the same as well, i.e. larger is better[53]. Therefore, the crossed array technique using two L_9 (one for GlCh and the other for SoCh) arrays (Table 2) was used instead of the L_{18} array.

Optimization Results: All reactions were performed in the order described in Table 2 (once for GlCh and SoCh system) in triplicates. Minitab (version 17) software was used to analyze the results. The response variable used was: conversion of the monoterpenes (**1a-3a**) to their corresponding epoxides (**1b-3b**). The signal to noise ratio, a criterion used to evaluate the process, was set to "larger is better". This means that the largest response would yield the best outcome, which in this case, would be conversion of monoterpenes.

GlCh system: The result obtained from the software is given in the figure below Figure 1 and as a table (Table 3)

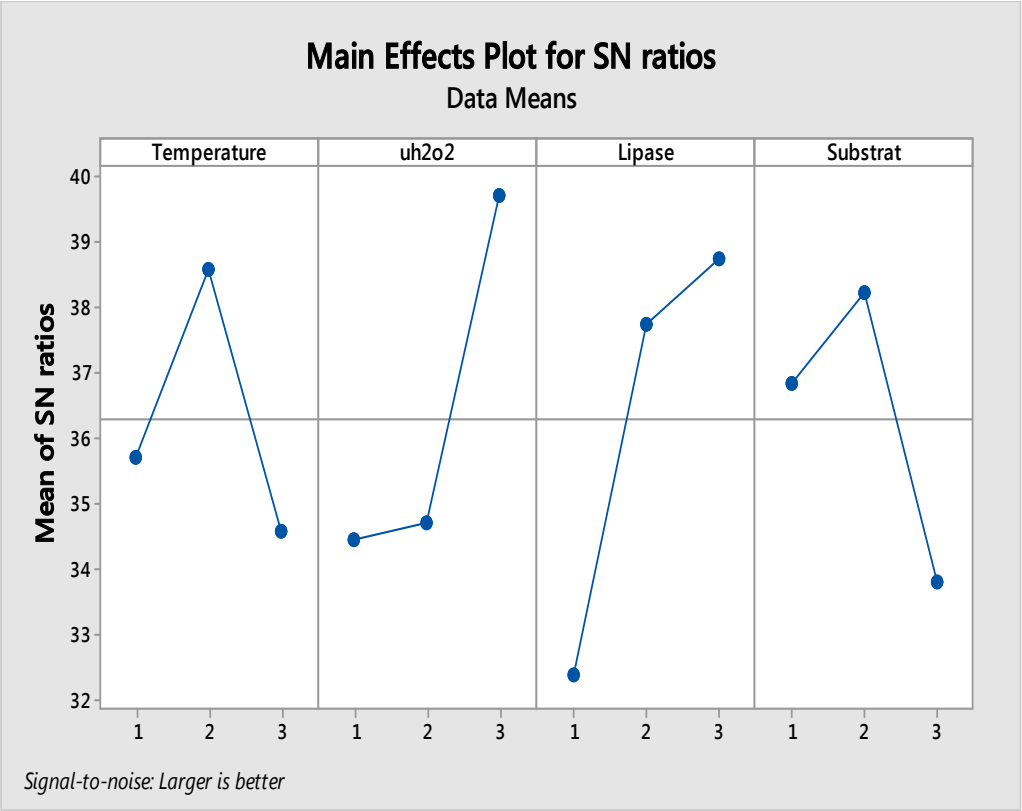

Figure 1: Main effects plot for the signal to noise (s/n) ratios of the GlCh system tested.

Table 3: The various s/n ratios obtained when using the different parameters and levels for GlCh as the reaction solvent. (Δ represents the numerical difference between the signal to noise ratios of the

various parameters and levels. Rank denotes the importance of the parameters in chronological order).

Level	Temperature (°C)	U·H_2O_2 (mmol)	Enzyme amount (mg)	Substrate
1	35.71	34.43	32.37	36.83
2	38.56	34.71	37.73	38.21
3	34.56	39.69	38.73	33.78
Δ	4.00	5.26	6.36	4.43
Rank	4	2	1	3

Our previous work in 2016[53] and the work of Björkling *et al.* in 1992[52] both show that H_2O_2 concentration is the pivotal parameter of the lipase mediated epoxidation process. Additionally, we also reported that lipase amount was ranked fifth in the list of the most important parameters. Surprisingly, this work does not comply with the aforementioned results, as the lipase amount had the maximum influence on the process followed by peroxide concentration. On using toluene, two phases (upper organic phase and lower aqueous phase) are obtained as in the case of DESs as well. But, the maximum content of water in the toluene system is 65% (35% aqueous H_2O_2), whereas, here it is definitely less than 65%. This is a known fact because any water in the system has to be generated *in situ* after the consumption of a hydrogen peroxide molecule for peroxy acid formation.

Soch system: The results obtained are given in Figure 2 as well as in Table 4.

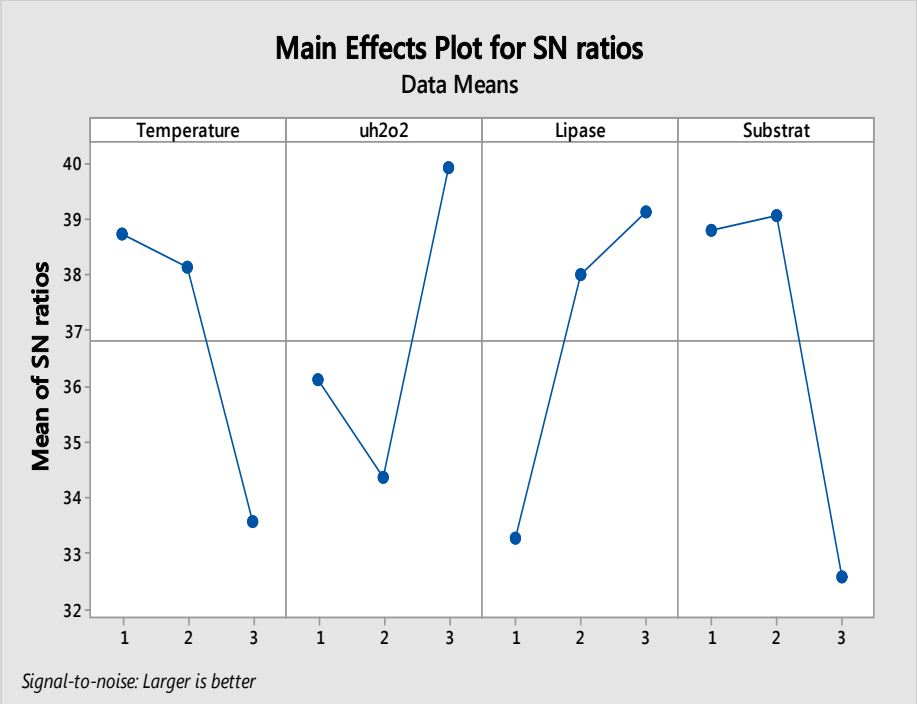

Figure 2: Main effects plot for the signal to noise (s/n) ratios of the SoCh system tested.

Table 4: The various s/n ratios obtained when using the different parameters and levels for SoCh as the reaction solvent

Level	Temperature (°C)	U·H_2O_2 (mmol)	Enzyme amount (mg)	Substrate
1	38.74	36.14	33.28	38.81
2	38.14	34.36	38.02	39.07
3	33.57	39.95	39.15	32.57
Δ	5.17	5.6	5.87	6.51
Rank	4	3	2	1

It can be seen that the temperature is of least importance for the SoCh system as well. We hypothesize the same reason as the GlCh system to be the major cause of this phenomenon, i.e. use of DES instead of organic solvent and use of hydrogen peroxide as a complex.

PURIFICATION PROCESS

The synthetic procedure used for producing 3-carene epoxide (**1b**) was: 15 mmol ChCl, 30 mmol U·H_2O_2, 10 mmol 3-carene, 2.5 mmol octanoic acid, 100 mg lipase and a reaction temperature of 60 °C for 3 h. The purification procedure was developed after an initial screening phase, which included the following steps:

- Decanting - The DES mixture and the epoxide produced were cooled down to -20 °C so that the decanting step could be made easy. After an overnight incubation at the aforementioned temperature, the un-polar phase was decanted into a fresh beaker. To this beaker, saturated (5 ml) sodium bicarbonate ($NaHCO_3$) solution was added and the phases separated. The aqueous phase was discarded and fresh sat. ($NaHCO_3$) solution was added and the process repeated for 5 times. An isolated yield of approximately 46 % was obtained.
- Addition of water (3 mass equivalents) - 3 mass equivalents (with respect to the DES individual components, i.e. CHCl and U·H_2O_2) of water was added to the DES and organic phase combination and vortexed vigorously for 30 seconds to 1 minute. The resultant mixture was filtered under vacuum and the lipase was recovered. The DES + water + organic phases were separated and the neutralization procedure was performed as in the previous case. An isolated yield of 82 % was obtained.
- Addition of water (10 mass equivalents) - 10 mass equivalents was added to the DES + organic phase and the lipase was removed using vacuum filtration. Neutralization procedure was followed as in the decanting step and an isolated yield of 87 % was obtained.
- Addition of $NaHCO_3$ to DES mixture - As $NaHCO_3$ is to be added for the neutralization procedure after phase separation, this test was performed using sat. $NaHCO_3$ (5 ml) directly instead of water. The neutralization procedure was followed after removing the lipase for two repetitions this time and the isolated yield was 60 %.

- Addition of ethyl acetate as extraction solvent - This test was carried out by adding 10 ml of ethyl acetate to the DES and vortexing the mixture for 30 seconds to 1 minute. The lipase was removed using vacuum filtration and the organic phase was subjected to the neutralization procedure. The isolated yield obtained when using this procedure was 82 %.
- Addition of n-heptane as extraction solvent - This test was carried out using n-heptane instead of ethyl acetate and the rest of the procedure was identical to the previous test. An isolated yield of 79 % was obtained.

PHASE BUILDUP AFTER THE ADDITION OF n-HEXANE

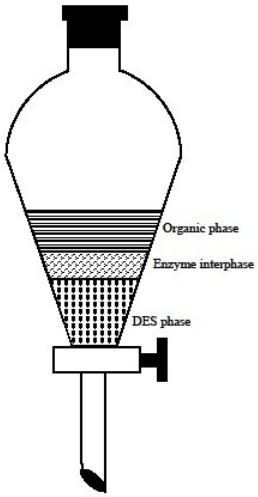

Figure 3: Separating funnel with DES (bottom most layer, diamonds), lipase enzyme (interphase, slanted bricks) and the ethyl acetate phase (top-most, horizontal lines) consisting of terpene epoxide and octanoic acid.

DETECTION OF SORBITOL AND GLYCEROL ESTER IMPURITIES

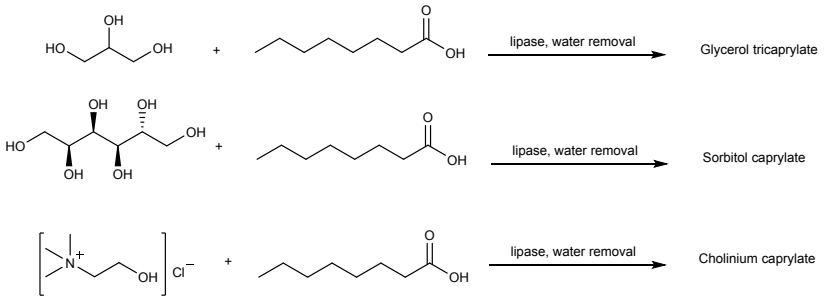

Figure 4: Formation of caprylate esters using lipases and DES mixes as the reaction medium for the epoxidation of terpenes

The total amount of this impurity, identified as glycerol tricaprylate corresponds to a maximum of 1 - 2.5 %, relative to the final product. The un-polar nature of this compound could be the reason for the

compound being extracted along with the desired products in n-hexane as the organic solvent. A strange phenomenon was also observed when using the SorCho system. There were monoesters of sorbitol and octanoic acid which were also visible on the chromatogram (not shown). Although the amount of impurity is minimal (1-2.5 % of the final product), it cannot be overlooked as it would imply purification effort for its removal. Accordingly, DES mixtures incapable of any ester formation would be preferable in comparison to the GlCh and SoCh systems.

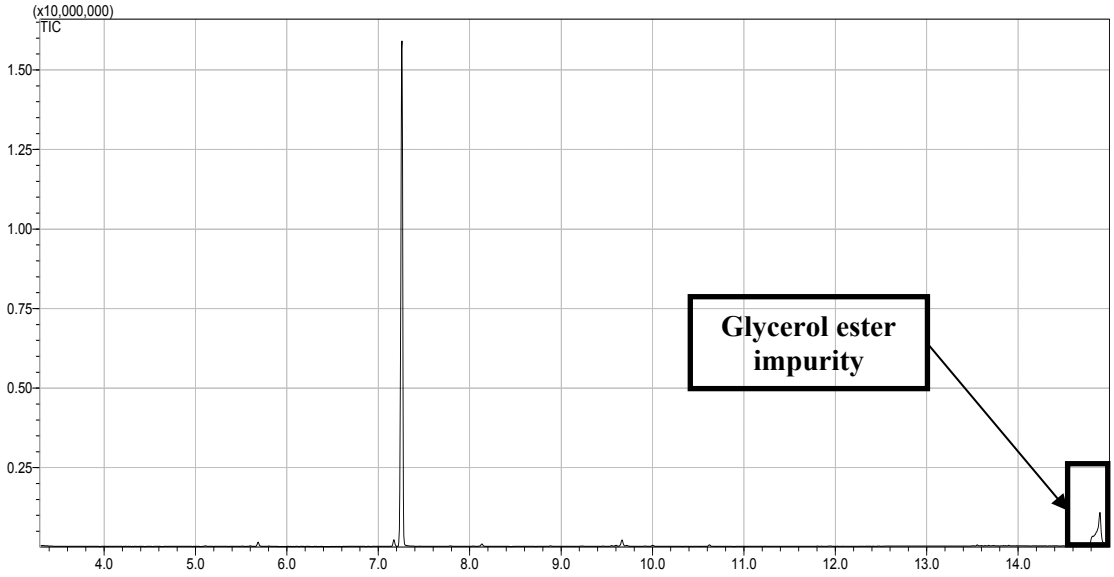

Figure 5: GC-MS chromatogram of the purified 3-carene oxide with the impurity towards the far end of the chromatogram

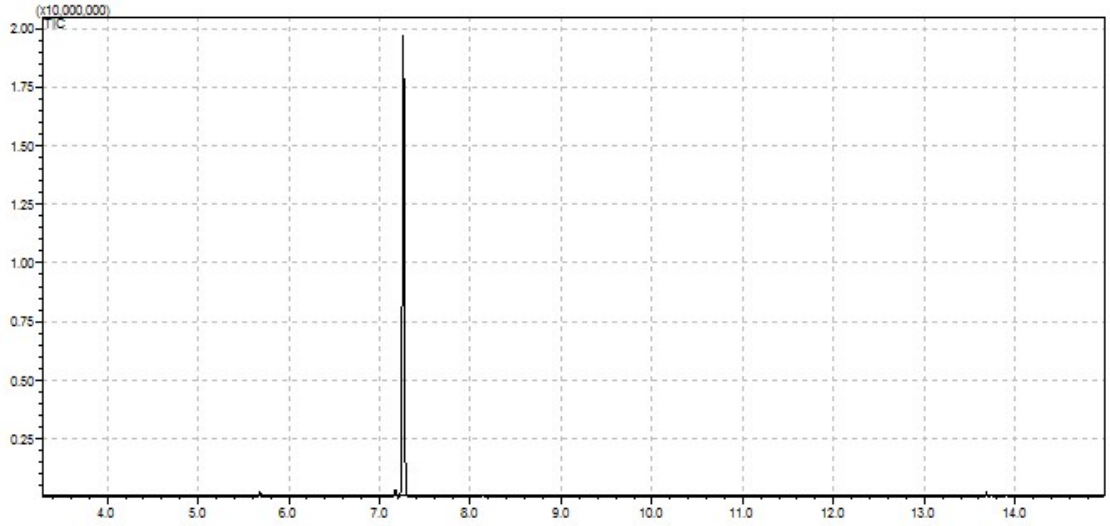

Figure 6: GC-MS chromatogram of 3-carene epoxide on using the ChCl:U·H_2O_2 DES mixture as the reaction solvent for epoxidation

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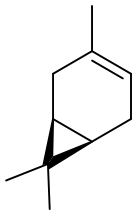
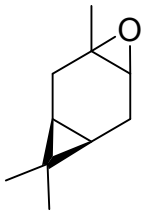
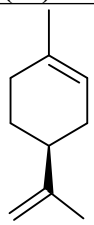
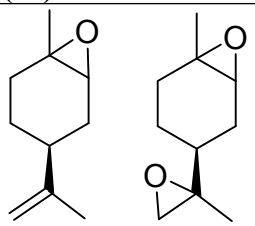
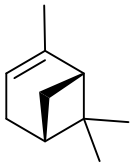
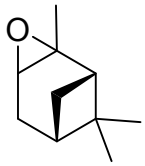
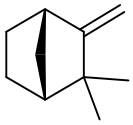
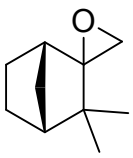
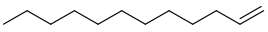
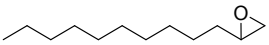
Summary of conversion on using different reaction media for various reactants

Table 5: Conversion obtained for various reactants 1a-5a on using different organic solvents (a-toluene[53], b – solvent free conditions, c – conventional- GlCh DES system, d – conventional- SoCh DES system and e – minimal DES system.

Entry	Substrate	Product	Time (h)	Conversion (%)
1	 (1a)	 (1b)	5[a]	100
			8[b]	
			8[c,d]	
			2[e]	
2	 (2a)	 2b) (2c)	4[a]	100
			8[b]	82.80
			8[c,d]	98.77, 99.27
			3[e]	100
3	 (3a)	 (3b)	4[a]	100
			8[b]	5.56
			8[c,d]	83.17, 66.67
			3[e]	100
4	 (4a)	 (4b)	24[c,d]	99.29, 94.42
5	 (5a)	 (5b)	24[c,d]	58.31,75.37