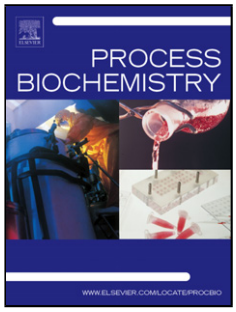

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Optimization of the lipase mediated epoxidation of monoterpenes using the design of experiments – Taguchi method

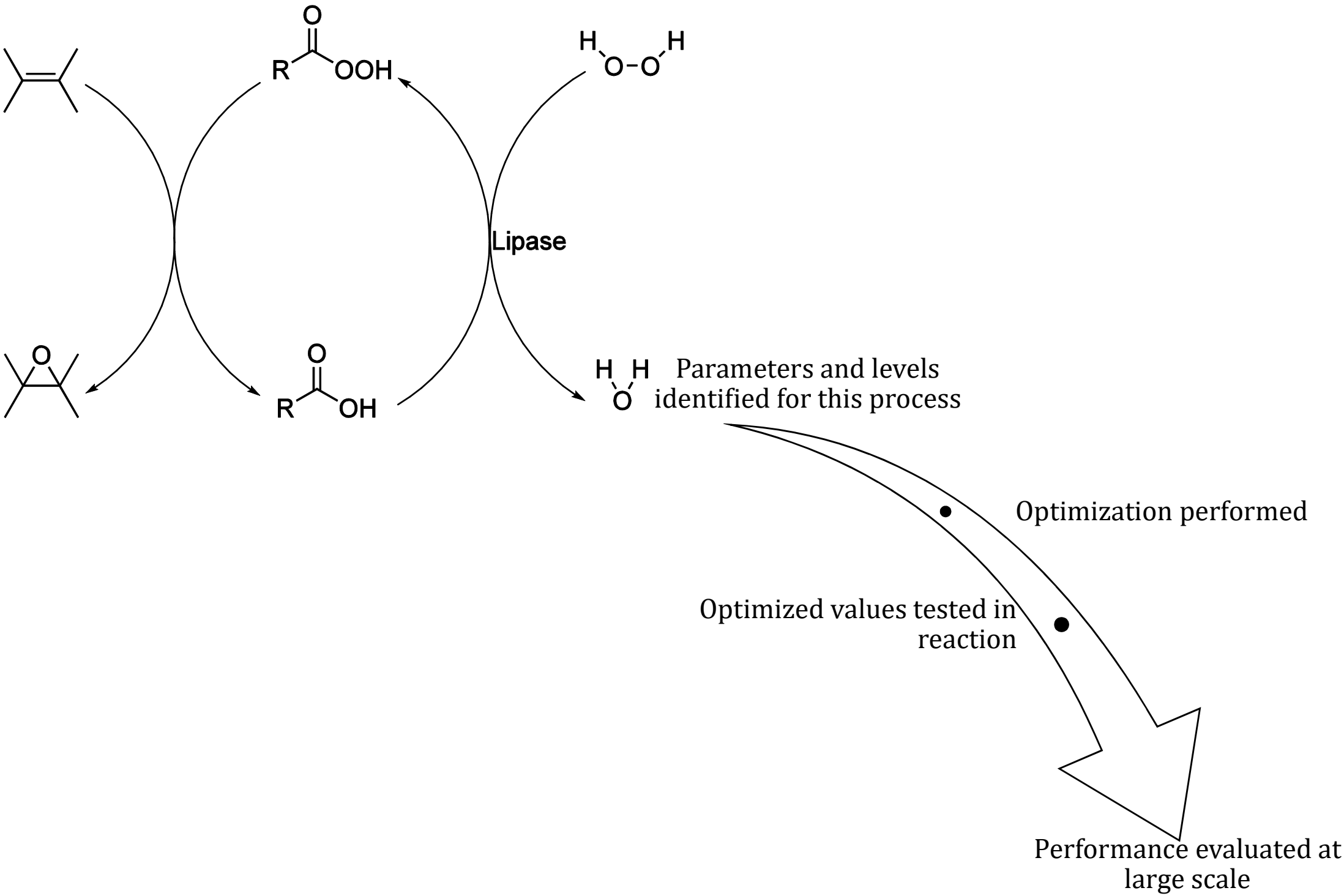
Sumanth Ranganathan[1], Johannes Tebbe[2], Lars O Wiemann[2,3], Volker Sieber[1,2]

1- *Chair of Chemistry of Biogenic Resources, Technical University of Munich, Schulgasse 16, Straubing 94315, Germany*
2- *Fraunhofer Institute of Interfacial Engineering and Biotechnology (IGB) Bio-, Electro and Chemo Catalysis BioCat Branch Straubing, Schulgasse 11a, Straubing 94315, Germany*
3- *Current address: evocatal GmbH, Alfred-Nobel-Str. 10, Monheim am Rhein 40789, Germany*

Email: sieber@tum.de

Phone: +499421187300

Fax: +499421187310


O
R
OOH
H
O-O
H
Lipase
O
O
R
OH
H H
O
Parameters and levels identified for this process
Optimization performed
Optimized values tested in reaction
Performance evaluated at large scale

Highlights

- Traditional lipase mediated epoxidation was extended for monoterpenes
- Design of experiments – Taguchi method used to optimize the process
- Hydrogen peroxide was determined to be the most important parameter
- Simple and easy product purification protocol developed

ABSTRACT

This work deals with the optimization of the *Candida antartica* lipase B (CALB) mediated epoxidation of monoterpenes by using the design of experiments (DoE) working with the Taguchi Method. Epoxides are essential organic intermediates that find various industrial applications making the epoxidation one of the most investigated processes in chemical industry. As many as 8 parameters such as the reaction medium, carboxylic acid type, carboxylic acid concentration, temperature, monoterpene type, monoterpene concentration, hydrogen peroxide concentration and amount of lipase were optimized using as less as 18 runs in triplicates (54 runs). As a result, the hydrogen peroxide concentration used was found to be the most influential parameter of this process while the type of monoterpene was least influential. Scaling up of the reaction conditions according to the findings of the optimization achieved full conversion in less than 6 hours. In addition, a purification process for the epoxides was developed leading to an isolated yield of ca. 72.3%, 88.8% and 62.5 % for α-pinene, 3-carene and limonene, respectively.

Keywords: epoxidation, lipase, design of experiments, Taguchi method, monoterpenes, process optimization.

1. INTRODUCTION

Epoxides possess high polarities and ring strains making them a highly reactive species and very useful building blocks in organic synthesis. They are predominantly synthesized with the Prileschajew epoxidation method using peroxycarboxylic acids, that in turn attack the double bonds of alkenes [1, 2]. Peroxycarboxylic acids are extremely reactive, possess high oxidation potentials and are therefore recommended to be produced *in-situ* for safe operation of the epoxidation process [3]. The most commonly used substance for Prileschajew epoxidation is *meta*-chloroperbenzoic acid - a strong electrophile prone to detonation when exposed to shocks in the environment. In addition to the explosive nature, these reactions should be performed at a temperature range of 0-25°C [4].

Owing to the aforementioned operational hazards of using high amounts of this substance and the subsequent cleaning steps involved thereafter, chemo-enzymatic *in-situ* generation of peroxycarboxylic acids was developed by Fredrik Björkling and his co-workers in the early 1990s using lipases (glycerol ester hydrolases, E.C. 3.1.1.3). The process (Scheme 1[5]) was the first of its kind and subsequent works have been carried out using this protocol [6-10]; to name a few. Variations of this process have been reported by the works of Ankudey *et.al.* [11], when they used ethyl acetate as the solvent and acid donor for the epoxidation process. Another modification of the Björkling process was carried out by Klass & Warwel [12], where the researchers used dimethyl carbonate to epoxidize alkenes and carbon dioxide was obtained as the by-product. In addition to this, Baeyer Villiger Oxidation has also been done using the mechanism explained by Björkling and his co-workers [13-15].

Scheme 1 to be inserted here

Every process needs to be optimized for good yields and the process shown in Scheme 1 is no exception. On optimizing this process at a small scale (laboratory and pilot), the industrial production could be achieved with pure products being formed and less waste being generated. The outcome of an experiment highly depends on the careful design of the experimental process [16]. Generally, in the design of a statistically based experiment the first step is the choice of the performance characteristic or the response variable, which will be closely monitored. The second step is the identification of variables or factors that contribute to this response variable, which will be studied. The next step is the choice of different treatment stages or levels, at which these factors will be tested for individual experiments. The final step is the identification of uncontrollable factors or noise factors that may influence the process in any way [17]. The usage of statistical procedures follows the general principles of randomization, replication and duplication to predict the actual behavior of a process. Generally, Plackett-Burmann Design (PBD), Central Composite Design (CCD) and Box-Benkhen Design (BBD) have already been used to optimize several processes.

The optimization of the above mentioned lipase mediated epoxidation of alkenes has already been carried out with the traditional ‘alteration of one variable at a time’ [18, 19] and also using the response surface methodology approach [20, 21]. The disadvantage of the one variable at a time approach is that it generates large amounts of samples and waste, is extremely time consuming and also expensive. Although, the response surface methodology system is advantageous in minimizing the number of trials and predicting interactions of the variables used, the Taguchi method with orthogonal array design predicts a mean performance characteristic value close to the target value, instead of just adhering to traditional limits, which in turn improves the quality of the process/product [22] The present work deals exclusively with the optimization of this lipase mediated epoxidation process for such monoterpene substrates, esp. α-pinene, 3-carene and limonene using the Taguchi approach.

Once the process has been tested for these three substrates, the procedure will be expanded to other terpenes and alkenes as well. Monoterpenes are simple plant products that are found predominantly in essential oils, but also in waste streams of pulp and paper industries and are widely used in the food, paint and pharmaceutical industries. Their oxygenated versions, *viz.* monoterpene epoxides and the corresponding diols are building blocks and synthetic intermediates [10]. Another important aspect to consider is that in classical chemical epoxidation approach, various unwanted side products are generated [23]. Seven parameters at three different settings and one parameter at two different settings were tested for obtaining the maximum conversion. Scale-up of the optimized runs obtained from Taguchi method was investigated and found out to comply with the results.

2. MATERIALS & METHODS

2.1 Introduction – Taguchi method of experimental design

Many of the industrial processes of today use the technique that was developed by Dr. Genichi Taguchi [24]. The Taguchi method was developed on the foundations of robust design introduced in the 1950s and 1960s. Robust design can be defined as *"an engineering methodology for improving productivity during research and development so that high-quality products can be produced quickly and at low cost"*. This method can be applied to a range of problems and has already been used in the field of electronics, automotives, photography and many others [25].

On designing a process based on robustness strategy, the following approach is to be followed:

- Drafting of the P-diagram and classification of variables into noise (uncontrollable), signal (input) and response (output) factors (Figure 1)
- Use of orthogonal arrays for gathering usable information about the control factors by carrying out a minimal amount of experiments
- Determination of signal to noise ratio for determining the field quality through laboratory experiments. Because, with a decreasing mean, the standard deviation also decreases. The standard deviation cannot be reduced first and mean brought to the target value [26]. Hence, the signal to noise ratio is used.
- Use of this ratio in the specified way (larger the better, minimal the better and nominal the best) to determine the outcome of the process.

2.2 *Determining the signal to noise ratio*

There are many ways to define the signal to noise ratio. The three most important ones are described below.

Nominal is best $$\frac{Signal}{Noise} = 10log\frac{\dot{y}}{s_y^2}$$

Smaller the better $$\frac{Signal}{Noise} = -10log\frac{1}{n}(\sum y^2)$$

Larger the better $$\frac{Signal}{Noise} = -10log\frac{1}{n}\left(\frac{1}{y^2}\right)$$

Where, $\dot{y}$ is the mean of the data observed, s_y^2 is the variance calculated for y (observed data), n is the number of observations.

Nominal is most suitable when the output value needs to be around a certain value, e.g. ratio of nitric acid and hydrochloric acid in aqua regia mixture. Smaller the better is to be used

when an output characteristic needs to be minimized, e.g. electromagnetic radiations from telecommunication equipment. Larger the better is to be used when a response needs to be maximized without compromising the process reliability, e.g. yield of a certain chemical process [25].

The approach of this method is primarily focused on determination of the optimal variable settings of process, thus achieving improved performance, in addition to reducing variability in the process with the help of orthogonal arrays [27]. The Taguchi method considers three stages in the development of a process- system design, parameter design and tolerance design. During the system design stage, the experimenter determines the basic configuration of the process. In the parameter design stage, values specific to the system are assigned in a nominal manner, so that the variability from uncontrollable variables (noise variables) is minimized. Tolerance design is used to indicate the best tolerances for the selected parameters [28, 29]

Figure 1 to be inserted here

2.3 Chemicals

3-Carene, α-pinene was obtained from Sigma Aldrich Co. LLC. Toluene was purchased from Chem Solute, Germany. Ethyl acetate and acetonitrile were purchased from Carl Roth, Germany. Hydrogen peroxide (35%) was bought from Avantomaterials, Netherlands. Lipase enzyme (CALB, 7500 TBU/g) for the reaction was purchased from Chiral Vision, Netherlands (Order No: CALB-T2-150XL).

2.4 Reaction Conditions

The reaction was carried out in 1 mL gas chromatography vials. There were 8 parameters that were to be optimized in this work and are described in (Table 1). The parameters and the

various levels listed below in Table 1 were later used in an orthogonal array for process optimization using the Taguchi Method. Final run of the process using optimized parameters was carried out in 100 mL round bottomed flasks. Temperature was controlled in an oil bath using Heidolph Magnetic Stirrers (MR series) fitted with a Pt 1000 temperature sensor purchased from Heidolph industries, Germany. The reaction contents were stirred using a magnetic stirrer at 500 rpm.

Table 1 to be inserted here

2.5 Analytics

The analytics of the epoxidation process was monitored using gas chromatography coupled with mass spectrometry. The gas chromatograph (GC-QP 2010, Shimadzu) was coupled with an autoinjector (AOC-5000, Jain Compipal) and was fitted to a mass spectrometer (GC-MS-QP2010 Plus, Shimadzu). The column that was used for measurements was a BPX5 column (SGE Analytical Science, Australia) of 0.25 mm diameter with a thickness of 0.25 µm and a total length of 30 m. Helium was used as the carrier gas and the temperature profile used for the analysis is given below:

- *Gas chromatography:* Start at 60°C with a holding time of 1 min, increase to 170°C at a rate of 10°C/min and finally increasing the temperature to a maximum of 270°C with a holding time of 3 min at a rate of 70°C/min
- Mass spectrometer: Ion source was maintained at a temperature of 200°C and the interface temperature was 250°C

The analysis was done using the software provided by Shimadzu, *GC-MS Postrun analysis* and the mass to charge ratio (m/Q) of all compounds used in this work were compared to the

database of *National Institute of Standard and technology (NIST) library-version 08*. The samples for analysis were prepared in ethyl acetate of LC-MS grade and to avoid the saturation of the MS detector, a solvent cut at 3.9 min was implemented with the help of the software. The different retention times for the monoterpenes and their corresponding epoxides on using the above mentioned procedure is given below in Table 2.

Table 2 to be inserted here

The epoxides of alpha-pinene, 3-carene and limonene after purification were also analyzed using proton (^{1}H) Nuclear Magnetic Resonance (NMR). It was performed on a Bruker Avance 400 (1H: 400.13 MHz, 13C: 101 MHz, T=300 K). The residual peak of the solvent ($\delta CDCl_3$: H7.26; C77.0) was used as the internal reference and chemical shifts have been reported in δ [ppm]. All resonance multiplicities are designated as s (singlet), d (doublet), t (triplet), m (multiplet) and coupling constants J in Hertz [Hz].

3. RESULTS & DISCUSSION

3.1 Choice of reaction medium for optimization

To test the best reaction medium for the chemo-enzymatic epoxidation process, all other parameters except the reaction medium were kept constant and the best functioning system, i.e. the reaction medium, was fed into the design. The following reaction conditions were used for the initial screening of various solvents: Substrate– limonene, substrate amount– 100 mM, hydrogen peroxide (35%) amount- 150 mM, acid type- octanoic acid at 50 mM, lipase– 30 mg, temperature- 60°C. The different solvents tested for this purpose and their logP values

[30] are given in Table 3. A reaction time of 24 h was used with sampling done at 1, 3 and 6 h and the reaction was followed for the production of limonene epoxide.

Table 3 to be inserted here

Although all solvents yielded a conversion of more than 90%; acetonitrile, methylcyclohexane, n-heptane, xylene and toluene had the maximum (i.e. full) conversion after 24 h (Figure 2).

Figure 2 to be inserted here

In the work of *Björkling et al*. [5] in 1992, it was reported that toluene, xylene and nitromethane were ideal for high yields. However, in order to choose the two best solvents, the conversion of those that generated 100% conversion was analyzed in more detail in this work (Figure 3). Acetonitrile and toluene were shown to have the best conversion after 3 h, complying only partially with the findings of Björkling *et al.* Hence, these two solvents were chosen to be fed into the Taguchi design. Björkling *et al.* had also shown high concentrations of hydrogen peroxide being important for the formation of the peroxycarboxylic acid. This information was helpful in choosing the hydrogen peroxide concentration (levels) for the Taguchi design that was appropriate for the process described in this work.

Figure 3 to be inserted here

3.2 Results of the Taguchi Method

The first step in the Taguchi method as mentioned in Section 2 was the identification of controllable and uncontrollable factors. The controllable factors are given in the materials and methods section (Table 2), while the uncontrollable factors could be attributed to water content in the immobilized lipase, type of immobilization of the lipase etc. The reactions were

carried out according to the Taguchi method's orthogonal array design and were selected using the software *Minitab v 17.0*.

For 1 parameter at 2 levels and 7 parameters at 3 levels, the array selector suggested a L_{18} array, which means 18 experiments were to be carried out in triplicates to obtain the optimum settings for the process (Table 4).

Table 4 to be inserted here

The experiments were done in the same sequence as described in the table and were repeated three times; at the end of which conversion was monitored for each. Conversion values for the different set of experiments obtained is shown in Figure 4.

Figure 4 to be inserted here

The main effects for the various parameters and levels are given below. The conversion values were used to calculate the signal to noise ratios. The characteristic used here was the "larger the best", and the signal to noise ratios obtained is given in Table 5. The signal to noise ratio and the various ranks determined for the process were done using the software *"Minitab® 17.1.0"* and are shown in Figure 5.

Table 5 to be inserted here

Rank 1 implies maximum impact on the process and rank 8 implies minimum impact of the process. So, from the above table, it can be concluded that the maximum impact on the process in decreasing order is: hydrogen peroxide concentration > substrate concentration > type of carboxylic acid used > temperature > enzyme amount > carboxylic acid amount > reaction medium used > substrate type used.

Figure 5 to be inserted here

When choosing the right level for each of the parameter, the one with the highest value needs to be chosen; as it affects the process and maintain it at the maximum production efficiency. On using this concept, the final conditions for testing at optimum conditions would be: Hydrogen peroxide concentration-500 mM, substrate concentration-100 mM, substrate type-limonene, temperature- 40°C, carboxylic acid type- octanoic acid (C8), enzyme amount-60 mg, solvent type-toluene and carboxylic acid concentration- 70 mM. The test run with the optimized conditions was done using these parameters and levels but in a scaled up fashion.

If a full factorial experiment were to be carried out with the same amount of parameters, then more than 4000 experiments were to be done, and on making it three times to minimize the error the numbers keep increasing and so does the costs and the amount of waste. Hence, the design of experiments using the Taguchi method could be considered as a great way to extract information on process optimization by reducing costs and by making the process more robust even when considering variations in the operating conditions with regard to uncontrollable factors.

3.3 Scale-up based on the results of the Taguchi method

The scale up was done for the optimized reaction conditions in a volume of 100 mL and based on the results from the Taguchi method explained in the previous section. In order to test the variability of the process, all three monoterpenes were used in the epoxidation process. The concentration of limonene, 3-carene and α-pinene was 200 mM. The conversions obtained with the optimized parameters along with changed monoterpene concentrations are shown in Figure 6.

Figure 6 to be inserted here

It can be inferred that the process can be carried out with monoterpenes at 200 mM concentration as well. At the end of the reaction, hydrogen peroxide concentration was checked for all three processes and residual amounts could be discovered.

In order to test for the adaptability of the process further, 3-carene (300 mM) was tested using the same set of optimized values. The results showed incomplete conversion (up to 70%) to its corresponding epoxide and the reaction was left to run for another 16 h. In the end, there was no characteristic improvement in the conversion; even though hydrogen peroxide was detectable in the medium. A possible explanation could be the inactivation of the enzyme, however, a reusability test with the same enzyme showed a complete conversion of starting material (Results not shown). The exact reason for this phenomenon is still not known and needs to be investigated further. Furthermore, control reactions with no enzyme, acid or hydrogen peroxide showed no conversion after 16 h reaction time.

3.4 Purification of Monoterpene epoxide

The scaled up process of monoterpene epoxidation was then subjected to a purification step. According to Arata & Tanabe [31], the epoxides of terpenes are highly sensitive compounds in a basic medium. On using a strong base such as 10 M sodium hydroxide solution, they immediately undergo ring opening to form diols. Hence a weaker base such as sodium bicarbonate (saturated amounts), was used for more than 5 to 7 times to completely neutralize the residual acid concentration that was used in this process, *viz.* the octanoic acid (C8) as a sodium salt. On developing this process to industrial efficiency, this salt could be used as a valuable by-product of the process. The exact procedure of carrying out this purification step is shown in Figure 7.

Figure 7 to be inserted here

The isolated yields of the whole process of epoxidation are 72.3% for alpha pinene, 88.8% for 3-carene and a combined isolated yield (mono and di-epoxides) of 62.5% for limonene. In the case of limonene, the ratio of mono to di-epoxide was 80: 20 (%). The ratio of the mono-epoxide isomers was 55 % *cis* and 45% *trans*. For limonene di-epoxide, 4 different diastereomers could be obtained theoretically. The ratio of these four predicted from GC-MS software Postrun analysis, is: 4, 40, 19 and 37%. Although all three reactions yielded a 100% conversion (GC-MS and NMR spectra attached as supplementary information), the subsequent steps involving neutralization of the octanoic acid with saturated sodium bicarbonate as well as non-optimized manual handling led to the loss of some product as well.

4. CONCLUSION

The process conditions of the lipase mediated epoxidation of monoterpenes were optimized successfully in the tested range using Taguchi method of robust design. A total of 8 parameters (1 parameter at 2 levels and 7 parameters at 3 levels) were successfully optimized in this research. However, a point of concern is that interactions were not accounted for when using the Taguchi method. Nevertheless, the efficiency of the system was tested for volumes of up to 100 mL and was found to comply with the findings of optimization. With our optimization we were able to reach full conversion of substrates after 4 – 6 h, compared to the 6 - 24 h required in the process by Björkling *et al.*, which even showed incomplete conversion in certain cases. A simple and efficient purification method for the epoxides was developed and carried out using the two phase extraction setup using a weak base such as saturated amounts of sodium bicarbonate. Though it can be argued that a stronger base such as sodium or potassium hydroxide needs to be used, the consequences of the epoxide ring being opened to a diol cannot be overlooked, even if it had to be at the expense of product(s) loss. The purification system is to be tested for other alkene epoxides and the monoterpene epoxides as well. The idea of this optimization procedure was to make the product robust in such a way

that by changing the substrate alone, the process could be used to produce its corresponding epoxide.

CONFLICT OF INTEREST

The authors wish to declare no financial or commercial conflict of interest.

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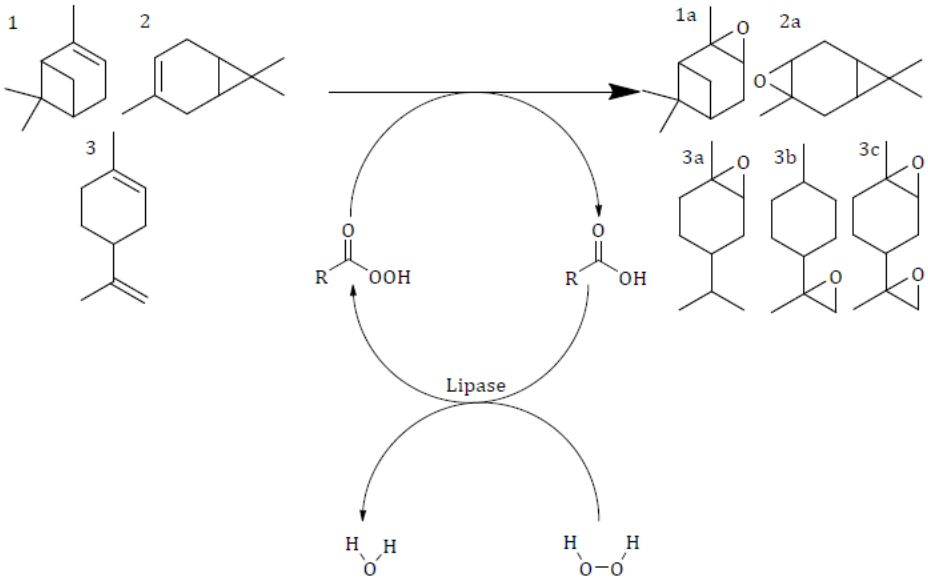

Scheme 1: Lipase mediated epoxidation of monoterpenes using peroxycarboxylic acids according to the method of Björkling et.al, 1992. (1 – α-pinene, 2- 3-carene and 3-limonene; 1a- α-pinene epoxide, 2a- 3-carene epoxide, 3a and b- limonene monoepxoide and 3c- limonene diepoxide)

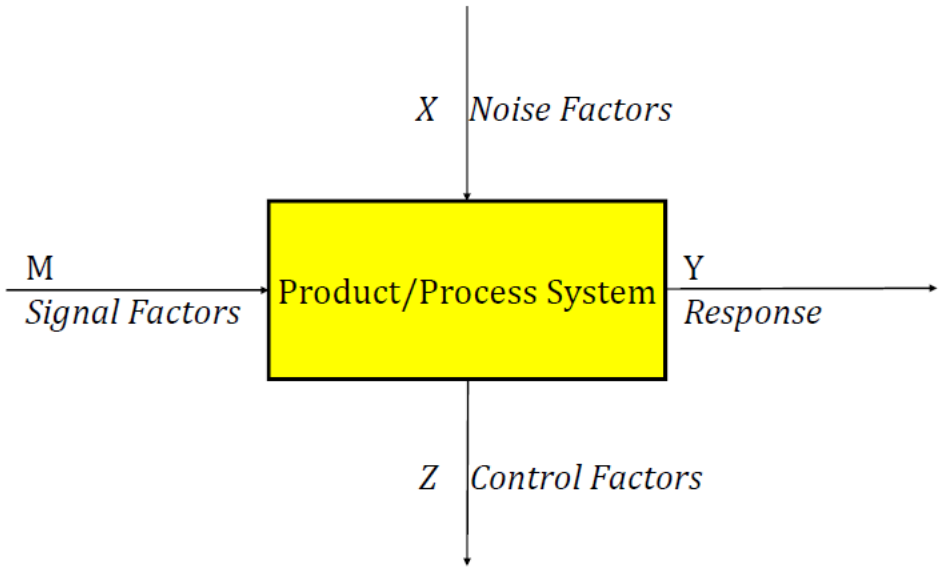

Figure 1: Parameter diagram for product/process system

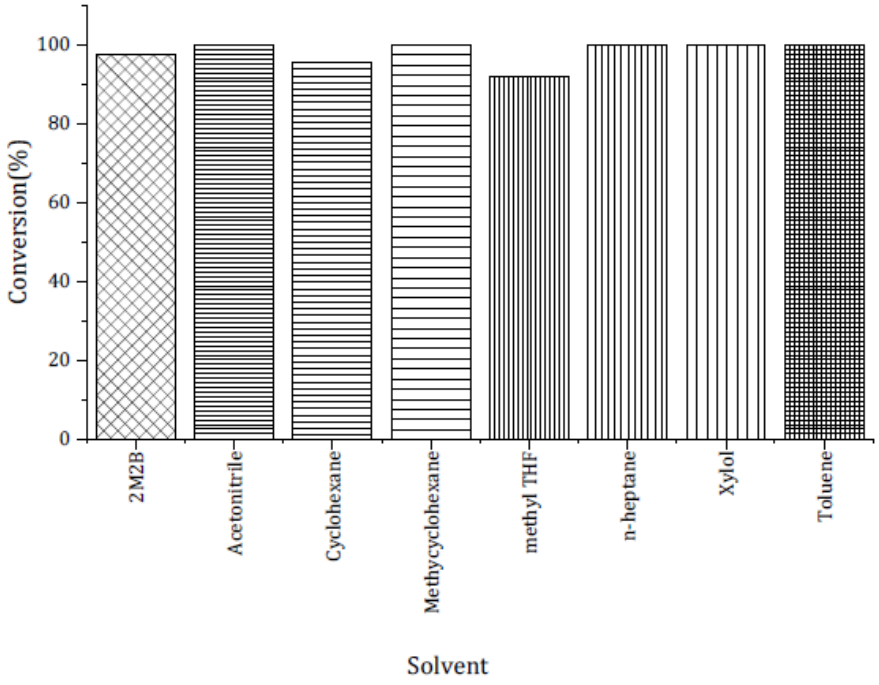

Figure 2: Results of solvent screening for the conversion of monoterpenes to monoterpene epoxide over a period of 24 h.

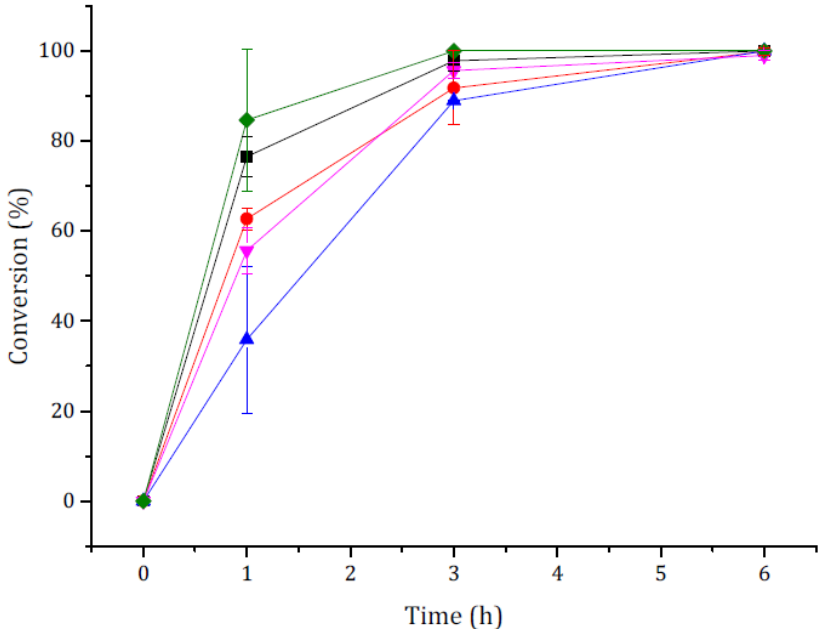

Figure 3: Kinetics of limonene conversion over a time period of 6 h using acetonitrile (square), methylcyclohexane (circle), n-heptane (triangle), xylene (inverted triangle) and toluene (diamond)

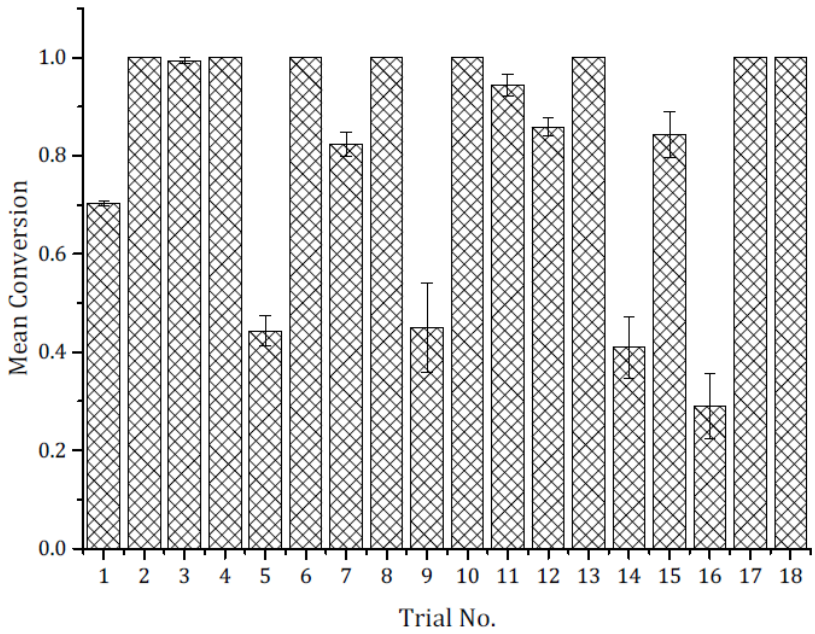

Figure 4: Conversion obtained by trying out different formulations of parameters and levels as suggested by the orthogonal array design (L_{18}).

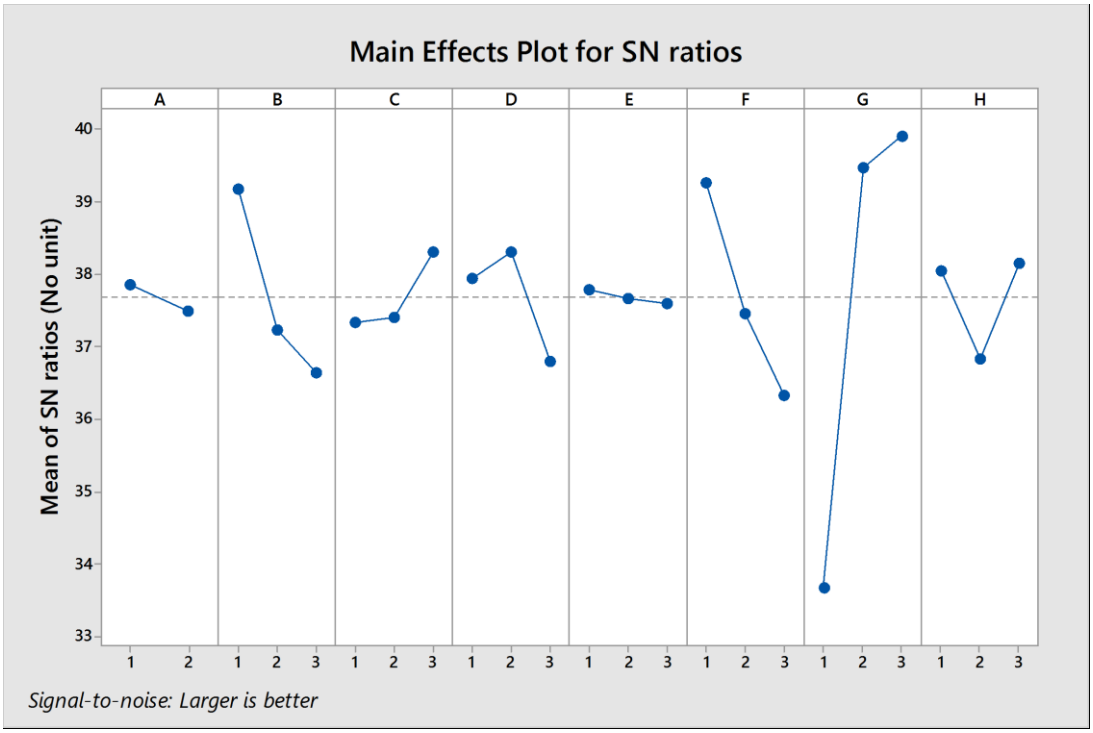

Figure 5: Signal to noise ratios for various parameters and levels tested using the L18 orthogonal array and "larger the better" characteristic. Dashed line implies the mean signal to noise ratio for all 18 trials. (A-H parameters tested at different levels 1, 2 and 3 (Table 1))

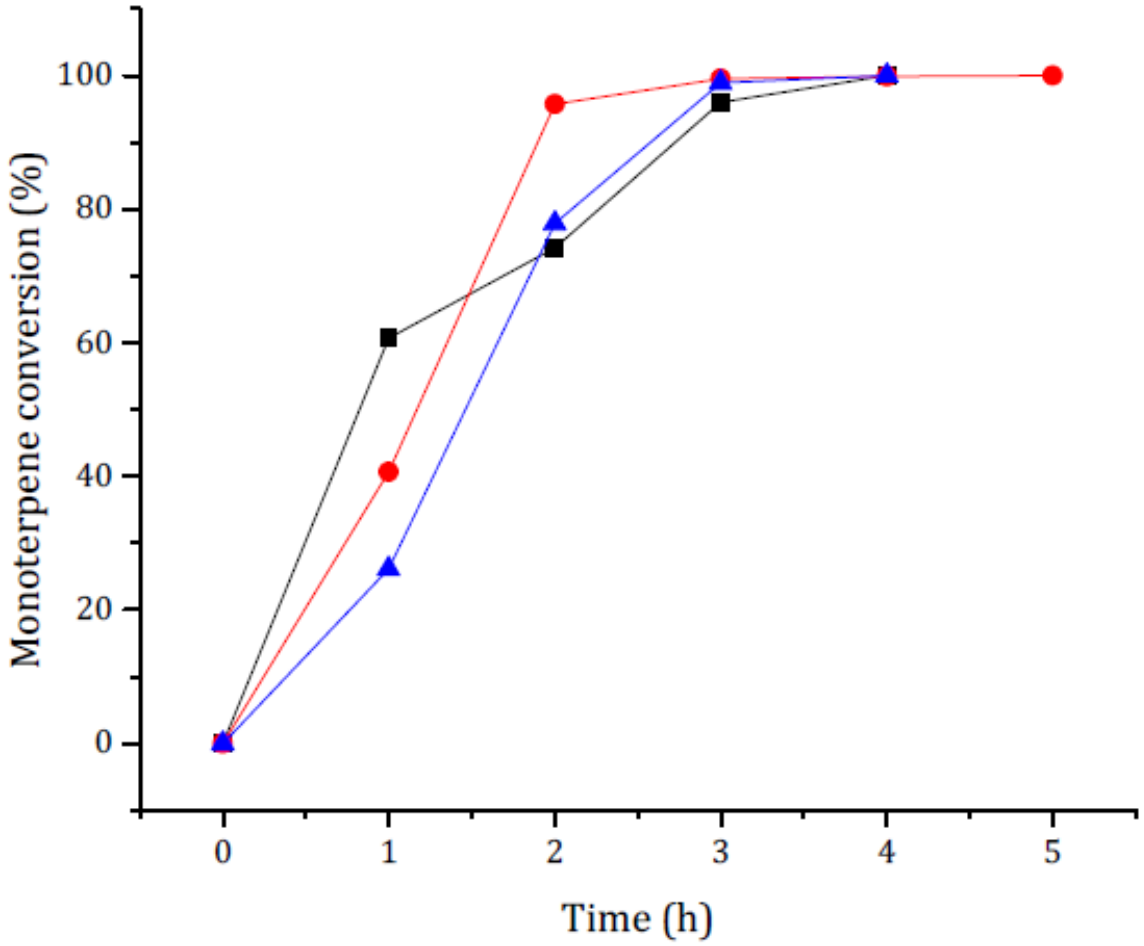

Figure 6: Conversion profile of limonene-200 mM (triangle), alpha-pinene-200 mM (square) and 3-carene-200 mM (sphere) using optimized parameters from Taguchi method. (Note: Conversion for the limonene reaction refers to the epoxidation of the molecule as such with two olefin bonds being epoxidized.)

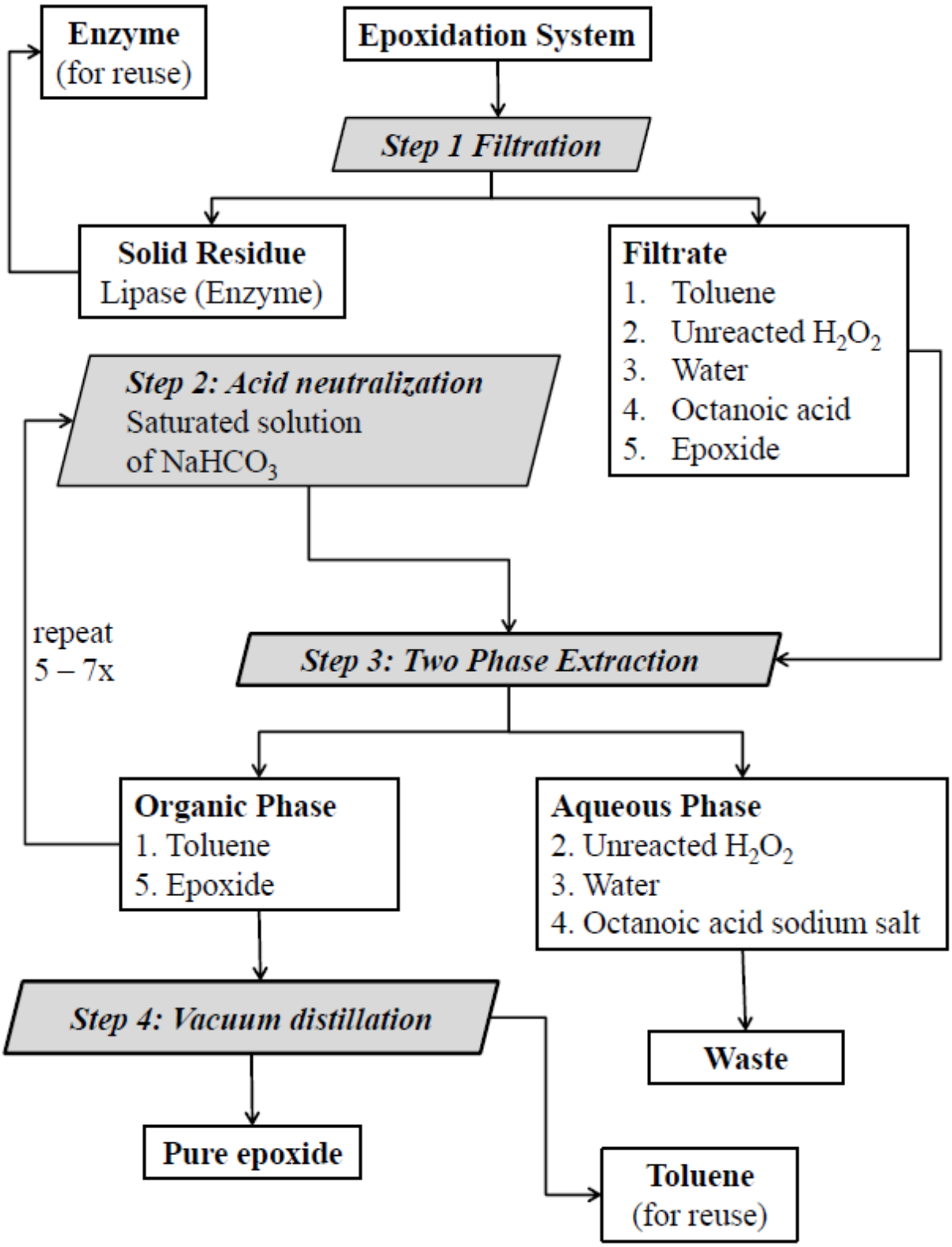

Figure 7: Epoxide purification process after the use of optimized lipase mediated epoxidation systems

List of Tables

Table 1: List of parameters and the levels for the optimization of lipase mediated epoxidation of monoterpenes

	Parameter	**Level 1**	**Level 2**	**Level 3**
A	*Reaction Medium*	toluene	Acetonitrile	-
B	*Carboxylic acid type*	octanoic acid (C8)	decanoic acid (C10)	lauric acid (C12)
C	*Carboxylic acid concentration*	30 mM	50 mM	70 mM
D	*Temperature*	20°C	40°C	60°C
E	*Monoterpene type*	limonene	3-carene	α-pinene
F	*Monoterpene concentration*	100 mM	200mM	300 mM
G	*Hydrogen peroxide concentration*	100 mM	300 mM	500 mM
H	*Lipase amount*	20 mg	40 mg	60 mg

Table 2: Retention times of the various monoterpenes and their subsequent epoxides obtained by GC-MS analysis

S.No	**Compound**	**Retention Time (min)**
1	Limonene	5.735
2	Limonene epoxide	7.272

3	Limonene-diepoxide	9.554
4	3-carene	5.809
5	3-carene epoxide	7.945
6	α-pinene	4.392
7	α-pinene epoxide	6.802

Table 3: Different solvents tested and their log P values according to [32].

S.No.	**Solvent**	**Log P**
1	2-methyl-2-butanol	0.89
2	Acetonitrile	-0.34
3	Cyclohexane	3.44
4	Methylcyclohexane	3.88
5	Methyl tetrahydrofuran	1,26
6	n-heptane	4.66
7	Xylene (isomeric mixture)	3.12 – 3.2
8	Toluene	2.73

Table 4: L18 orthogonal array for the optimization of 1 parameter at 2 levels and 7 parameters at 3 levels for the optimization of lipase mediated epoxidation of monoterpenes (For detailed account of the parameters and levels, please refer Table 1)

Trial. No.	**Solvent**	**Acid type**	**Acid concn.**	**Temperature**	**Substrate**	**Sub. Concn.**	**H_2O_2 concn.**	**Enzyme amount**
1	1	1	1	1	1	1	1	1

2	1	1	2	2	2	2	2	2
3	1	1	3	3	3	3	3	3
4	1	2	1	1	2	2	3	3
5	1	2	2	2	3	3	1	1
6	1	2	3	3	1	1	2	2
7	1	3	1	2	1	3	2	3
8	1	3	2	3	2	1	3	1
9	1	3	3	1	3	2	1	2
10	2	1	1	3	3	2	2	1
11	2	1	2	1	1	3	3	2
12	2	1	3	2	2	1	1	3
13	2	2	1	2	3	1	3	2
14	2	2	2	3	1	2	1	3
15	2	2	3	1	2	3	2	1
16	2	3	1	3	2	3	1	2
17	2	3	2	1	3	1	2	3
18	2	3	3	2	1	2	3	1

Table 5: S/N ratio for different trials calculated using the "larger the better" characteristic. A-H all parameters tested (Table 1) (Values obtained from *Minitab 17.0* software)

Level	A	B	C	D	E	F	G	H
1	-2.13*	-0.83*	-2.66	-2.06	-2.21*	-0.73*	-6.32	-1.95
2	-2.50	-2.76	-2.60	-1.69*	-2.33	-2.54	-0.53	-3.16

3	-	-3.36	-1.70*	-3.20	-2.41	-3.68	-0.09*	-1.84*
Delta	0.37	2.54	0.96	1.51	0.20	2.95	6.23	1.32
Rank	*7*	*3*	*6*	*4*	*8*	*2*	*1*	*5*
*	*Optimized set of values for the process*							

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