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Development of Sustainable Chemo-enzymatic Processes for the Epoxidation of Terpenes

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Maximum effort

- Deadpool

Contents

Summary

Terpenes are by-products of the paper and pulp industry that are useful as fine chemicals, pharmaceuticals, flavours, fragrances, or monomers for polymers. Often, the terpenes are modified chemically to form terpenoids (oxidized terpenes) before being used industrially. Epoxidation, the process of adding molecular oxygen to an alkene, is usually the preferred type of modification. For terpenes, this is done using the Prilezahev method, which utilizes a peroxycarboxylic acid as the oxidant. This method has several risks such as detonation at high temperatures, use of harmful organic solvents, and generation of equal amounts of waste with respect to the final product. To avoid these issues, a green and sustainable alternative needs to be designed using the 12 principles of green chemistry, which governs a chemical process to produce minimal waste, make use of renewable substrates, and avoid harmful reaction conditions.

The present work focussed on the development of such a process and already incorporated the principles of green chemistry into the process design phase. To reach this goal, a chemo-enzymatic epoxidation process was envisioned, where a peroxycarboxylic acid is produced *in situ* using catalytic amounts of carboxylic acid in the presence of hydrogen peroxide and an enzyme (lipase). The resulting peroxycarboxylic acid, in turn, should spontaneously produce the epoxide with a regeneration of the carboxylic acid. The first step was to optimize this process for terpenes using the Taguchi method of robust design. Totally, eight process parameters were identified and optimized using this approach. The results indicated that the hydrogen peroxide concentration affected the process the most, while the type of terpene had minimal effect within a reaction time of 6 h to 8 h to attain maximum conversion. The optimized process was robust enough to epoxidize 20 substrates (terpenes and alkenes). After the process optimization, a validation run was performed in addition to a scale-up (1 cm^3 to 100 cm^3 and later to 3000 cm^3) and complemented by the design of a purification system to obtain pure terpene epoxide in yields greater than 72 %.

Following the optimization procedure for the chemo-enzymatic epoxidation of terpenes using lipases, the next step was to integrate hydrogen peroxide production within the chemo-enzymatic process to ensure high efficiency in production. The industrial method of anthraquinone (AQ) autoxidation for hydrogen peroxide production was chosen and scaled down to the laboratory level in order to achieve this goal. The first attempt was done in a one-pot stopped batch process. Such a combination was innovative and the first of its kind, but the process was incapable of producing epoxides at maximum conversion. Hence, a second prototype was designed to operate in a semi-continuous mode. In the improved version of the integrated process, the AQ and chemo-enzymatic epoxidation processes were performed separately and combined at the downstream end of the AQ process through a hydrogen peroxide reservoir. This not only en-

sured maximum hydrogen peroxide production, but also presented the possibility of integrating other processes that required hydrogen peroxide and a possibility of industrial scale operation. The final goal of this work was to replace the harmful and toxic organic solvent toluene with an alternative reaction medium, such as a deep eutectic solvent (DES). Several DES mixtures were tested and glycerol : choline chloride (GlCh) and sorbitol : choline chloride (SoCh) were successfully identified. The chemo-enzymatic epoxidation process was optimized again using the Taguchi method for these"traditional DES" based systems. 6 h to 8 h of reaction time was required for the complete conversion of substrates to products in this case. On further investigation of epoxidation reactions in traditional DES systems, there was the persistent issue of an unwanted by-product, glycerol and sorbitol esters for GlCh and SoCh, respectively. This problem was solved by changing the traditional DES to a "minimal" DES system comprising urea·hydrogen peroxide and choline chloride. On making this switch, the reaction was finished within 2 h to 3 h. Finally, the production process was again complemented with a clean and green purification procedure.

List of Publications

Sumanth Ranganathan and Volker Sieber (2017).
Development of Semi-Continuous Chemo-Enzymatic Terpene Epoxidation: Combination of Anthraquinone Autoxidation and the Lipase-Mediated Epoxidation Process.
In: Reaction Chemistry and Engineering, Vol.2.
DOI: 10.1039/c7re00112f.

Sumanth Ranganathan, Sandra Zeitlhofer, and Volker Sieber (2017).
Development of a Lipase-Mediated Epoxidation Process for Monoterpenes in Choline Chloride-Based Deep Eutectic Solvents.
In: Green Chemistry, Vol. 19.
DOI: 10.1039/c7gc01127j.

Sumanth Ranganathan, Johannes Tebbe, Lars O Wiemann, and Volker Sieber (2016).
Optimization of the lipase mediated epoxidation of monoterpenes using the design of experiments Taguchi method.
In Process Biochemistry, Vol. 51.
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Sumanth Ranganathan, Tobias Gärtner, Lars O Wiemann, and Volker Sieber (2015).
A one pot reaction cascade of *in situ* hydrogen peroxide production and lipase mediated *in situ* production of peracids for the epoxidation of monoterpenes.
In: Journal of Molecular Catalysis B: Enzymatic, Vol. 114.
DOI: 10.1016/j.molcatb.2014.12.008.

Chapter 1 Introduction

1.1 Products of the chemical industry

Since the beginning of time, human beings have been transforming objects of nature to forms that suited their needs better. Some examples include, baking bread, brewing beer, production of soap, *etc.* These transformation process involved following a sequence of steps to arrive at a desired product. Part of these transformations were performed in a large scale leading to the production of materials in useful amounts and the practice came to be known as industrial chemistry. The seeds of producing products at a large scale or industrial scale to meet the demands of the public was sown around the 1600s. The then Venetian Republic started their production and export of nitric acid, hydrochloric acid salts and dye stuff *etc.* Since then, there has been constant development in shaping the modern chemical industry, which until today, is responsible for producing more than 70,000 products [**?**, **?**]. This leads to the question of the types of products produced by the industry and how they can be differentiated. Generally, chemical products can also be classified into *differentiated* or *undifferentiated* (Table 1) [**?**].

Table 1: Classification of chemical products on the basis of the production volume [**?**, **?**]

Production volume	**Undifferentiated**	**Differentiated**
High	*Commodity* produced in large volumes from raw materials and sold universally with the same specifications. example (e.g.): ethylene, sulphuric acid, acetone *etc.*	*Pseudo commodity* similar to the commodities, but produced to fulfill a certain function rather than to a specific composition. e.g.: polyvinyl chloride, polyethylene, *etc.*
Low	*Fine chemical* produced to achieve a specific function in small volumes. e.g.: flavours, foods, fragrances, *etc.*	*Specialty chemical* are products of specific customer requirements that are produced in small volumes and purchased based on performance rather than composition. e.g.: catalysts, pharmaceuticals, pulp and paper chemicals, *etc.* [**?**, **?**]

The customer who purchases these products can also distinguish them as: *consumer products*

(need no further processing and can be used as purchased, like beer, toothpaste, *etc.*) and *producer products* (need to be processed by another industry prior to reaching the common public, such as ethylene is sold as a producer good, which can be used by the polymer industry to produce polyvinyl chloride.)
According to Cybulski *et al.*, there is no universal way to classify a chemical product [**?**]. However, both parties agree that the ideal way of classifying chemical products is based of the volume of production and its end usage.

1.2 Green chemistry

The foundations of the modern chemical industry were laid back in the 1800s, around the time of the industrial revolution. This meant that the processes and products were developed with the sole intention of maintaining the sophisticated lifestyle and well-being of human beings. No emphasis whatsoever was placed on the mode of production, the wastes that were generated and dumped into the environment, and non-polluting means of production [**?**]. The chemical industry functioned this way for a long time till the 1960s. In 1962, Ms. Rachel Carson published her book *Silent Spring*[1] detailing the fate of the environment due to polluting chemical processes and industries. 10 years later, Prof. Barry Commoner published his book[2] on the very same phenomenon discussing the technological, political, and social issues that contributed to the destruction of the environment. Both these books led to an increased awareness among the public regarding the chemicals in the living environment.
This in turn, led to the common conclusion that there should be proper and strict laws concerning the way products be manufactured and their effect on the environment be monitored closely [**?**, **?**, **?**].
This led to the concept of *"green chemistry"* around the 1990s. The concept was developed by the duo Paul Anastas and John C Warner in the year 1998 in their book *Green Chemistry: Theory and Practice*. In this book, they came up with twelve principles that governed the way chemistry was to be practiced in order to safeguard the environment [**?**, **?**, **?**]. This leads to the ultimate question: *What is Green Chemistry?* A plausible definition for the same can be given as follows:
"The efficient utilization of raw materials (preferably of renewable origin) that eliminates waste and avoids the usage of toxic or hazardous reagents and solvents during the production and application of chemical products" [**?**, **?**]. In simpler terms, this practice ensures the production of chemical products without harming the environment or the operator.

[1] Paperback book, Houghton Mifflin, Boston,1962
[2] *The Closing Circle: Nature, Man & Technology*, paperback, Random House Inc., 1971.

1.2.1 The 12 principles of green chemistry

The definition of green chemistry can be extended into 12 principles that ensure a clean synthesis of a chemical product [?, ?, ?]. An elaborate explanation of these principles are given below.

1. **Prevention**- In the literal sense, *Prevention is better than cure.* Preventing waste from being produced is better than dealing (treating/cleaning up) with it once it is formed [?, ?, ?].

2. **Atom Efficiency**- The synthetic methods should incorporate all reactants involved in the process into the final chemical product. One way of ensuring maximum atom utilization or atom efficiency in a synthetic reaction, is the term *"E-factor"*, which is defined as the total amount of wastes produced per kilogram of desired product. This E-factor depends on the specific industry segment from which the product is produced [?, ?, ?]. This is explained in table 2 [?, ?] below.

Table 2: E-factors in the chemical industry [?, ?]

Industry	**Product tons/year**	**Waste/Product (weight ratio)**
Oil refining	10^6 - 10^8	~ 0.1
Bulk refining	10^4 - 10^6	less than 1 - 5
Fine chemicals	10^2 - 10^4	$5 - 50$
Pharmaceuticals	10^0 - 10^3	$25 - 100$

3. **Less Hazardous Chemical Synthesis**- The methods of syntheses should produce or use substances that are less or not toxic at all to human health as well as the environment.

4. **Synthesis & Design of Safer Chemicals**- The chemical product produced should still maintain its potency, but should not be toxic.

5. **Usage of Safe Solvents and Accessories**- Using ancillary substances such as solvents, separating agents, *etc.* should be avoided or must be used only if absolutely necessary.

6. **Efficient Use of Energy**- Energy requirements for the designed/developed chemical processes should be minimal from the environmental and economic perspective, as in at ambient conditions of temperature and pressure.

7. **Utilizing Renewable Feedstock as Starting Material**- The feedstock should be of renewable origin rather than emptying, provided the process is technically and economically feasible. Presently, the major feedstock for synthesizing chemical products is obtained

from fossil fuels, which are non-renewable. Owing to the increasing chemical and energy demand, these resources are being depleted rapidly, dangerously nearing exhaustion [**?**].

8. **Reducing the Use of Derivatives**-Using protecting or de-protecting groups, blocking groups, *etc.* should be avoided or minimized, if possible. This is because of the additional steps required to produce the final product, which in turn, could produce waste.

9. **Catalysis**- Instead of using stoichiometric amounts of reactants, including a catalyst, preferably a selective one would be superior.

10. **Capable of Degradation**- The chemical products must be designed in such a way that when released into the environment post usage, it should be readily broken down to simpler products and not persist in the environment instead.

11. **Real Time Analysis to Monitor Synthesis Closely**- Methods to analytically determine the formation of hazardous substances should be developed for real time analysis with the additional monitoring and control steps.

12. **Practicing Safe Chemistry to Prevent Accidents**- The substances used in their actual or derivatized form in a chemical process must be chosen based on the basis of minimizing fire, explosion, or release into the environment.

1.2.2 Why alternate reaction media?

Chemical reactions are heavily dependent on three main conditions: solvent, reaction temperature, and milieu of production. Of these three, the solvent to be used is to be optimized first [**?**]. The idea that solvents are necessary for chemical reactions to occur stemmed from Aristotle's proclamation *"corpora non agunt nisi fluida soluta"*[3] [**?**, **?**]. Any solvent can be used for this purpose; however, only a selected few such as volatile organic compounds (VOCs) are used for chemical synthesis in the industry. VOCs are compounds that are of anthropogenic or biogenic origin comprising of either alkanes, alkenes, aromatics, *etc.* On its release into the atmosphere, the VOCs undergo a number of physical and chemical transformations affecting the biosphere [**?**]. This situation needs to be changed as soon as possible because of the "greening" of chemical syntheses [**?**, **?**, **?**]. VOCs are toxic to the environment and the operator alike. Moreover, since VOCs are used in large amounts, this leads to the production of waste [**?**, **?**]. Solvents, if at all needed for the reaction to take place is to be selected based on *(i)* the likelihood for the actual synthesis to take place, *(ii)* the ease of product removal from the reaction mixture, *(iii)* cost, *(iv)* disposal and *(v)* hazardous nature (toxicity and flammability) of the solvent [**?**]. Presently the scientific community and the industry are researching green

[3] translation - "Compounds that are not fluid or not dissolved, do not react"

alternatives to replace the conventional and classical VOCs. Defining a solvent as "green" is one of the most difficult tasks as this involves a lot of constraints, which can be contradictory at times. Some of these constraints are: chemical efficiency (concerning reactions), safety at the operational environment (flash point, risk of peroxides, *etc.*), related health issues to the operator on exposure to these solvents, environmental effects on release into the environment, industrial constraints (recyclability, density, boiling point, freezing point, *etc.*) and cost. For example, water cannot be considered an ideal green solvent because of its high freezing point of 0 °C and high enthalpy of vapourization. Similarly, methanol is an inexpensive and readily available commodity chemical that is biodegradable and low resistivity, but is flammable, volatile, and harmful. If one were to choose either of these solvents, which one would it be? To help with the actual choice of solvents to be used in syntheses, a guide was published by Denis Prat and his co-workers, which split the solvents into the following categories:

- recommended
- needs to be substituted
- needs to be replaced
- banned [**?**, **?**, **?**].

1.2.3 Solvent-free conditions

According to the principles of green chemistry (section 1.2), it is essential that the generation of waste be avoided. More often than not, this is the reaction solvent. An estimate suggests that 56 % of the total mass used in pharmaceutical manufacturing is composed of solvents [**?**]. Designing and running reactions under solvent-free conditions would help avoid this situation. On performing the synthesis under these conditions, processes can be designed to be environmentally benign; handling costs for the reaction can be reduced; the work-up steps to obtain final product will be minimal, and the overall process would be cost effective [**?**, **?**]. However, on using solvent-free conditions, despite the components being highly concentrated, the reaction rate is still low due to the reduced availability of substrates. Such a system might need some activation to function efficiently. Microwaves, ultrasound, grinding, and heating are some of the activation energies that ensure the contact between reactants. Solvent-free reactions are classified into three categories based on the physical state of the reactants at the beginning of the reaction as: liquid-liquid, liquid-solid, and solid-solid. Although, solvent-free conditions provide several advantages it still has its limitations such as:

- heat and mass transfer issues associated with the reaction
- desired reaction capable of handling such high concentrations of reactants
- issues related to mixing for solid-solid reactions [**?**]

1.2.4 Water

Reactions needed to sustain life in the natural world are carried out exclusively in water, irrespective of whether the reactants are water-soluble or -insoluble. However, chemists tend to avoid this and perform their syntheses in organic solvents and even maintain it "dry". The reason for this is relatively simple: the chemical reagents used are not soluble in water and hence the reaction cannot take place [**?**]. Listed below are the advantages and disadvantages of using water as the reaction solvent [**?**].

Table 3: Advantages and disadvantages of using water as the reaction solvent in organic chemical synthesis [**?**]

Advantages	**Disadvantages**
•cheapest and most abundant resource	•solubility of non-polar reactants
•non-flammable and non-toxic	•issues concerning the purification of polar products post reaction
•can control exothermic reactions better than VOCs	•only selected reactions can be carried out e.g. Diel's Alder reactions, Michaels additions, and organometallic reacions, to name a few.

The work of Narayan *et al.* in 2005 explained that non-polar reactants, when stirred vigorously for short periods of time react *"on-water"* or on the surface of water rather than dissolving and then reacting *"in-water"* to yield products [**?**]. Although at first, both the terms seems to convey the same meaning, they are two different cases.

- in-water- Reactants and catalysts are dissolved in-water. For reactions in-water, the operations depend on: *(i)* hydrophobic effects, which determine the speed of the reaction, *(ii)* hydrogen bonding effects on reactants and intermediates that may add or oppose the hydrophobic effect, and *(iii)* polarity effects of water that might increase or decrease the rate of the reaction.

- on-water- Reactants and catalysts are present on the surface of water and sparsely soluble. These reactions are often carried out with insoluble reactants. The successful operation of such a process depends on: *(i)* trans-phase hydrogen bonding and *(ii)* insolubility and the nature of the reactant (solid or liquid).

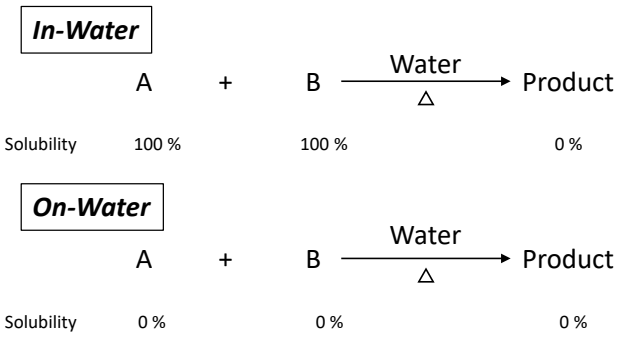

Figure 1: An ideal reaction showing the difference between *"in-water"* and *"on-water"* reactions [**?**]

Figure 1 shows an ideal reaction taking place *in-water* and *on-water* and in both cases, the product formed has no solubility in water. Assuming a 100 % yield, the reaction performed in-water should be having only the product, which, when purified would yield pure water afterwards. In the case of on-water reactions, the product will be left on the surface of water, the separation of which would yield pure water. However, this is the ideal case scenario. In real, trace amounts of all the reactants and products will be present in the water and this depends on the efficiency of the purification [**?**].

Sometimes, the reactions on water can be enhanced by using *micellar catalysis*. Surfactants, when used in solution such as water, tend to form aggregates called micelles, above a certain concentration called the critical micellar concentration. These micelles act as hydrophobic nanoreactors entrapping the non-polar reactants. They also facilitate a good mass transfer of catalysts, substrates, and products in and out of the micellar system. Surfactants such as Triton X-100, Brij-35, and TPGS-750-M *etc.* can be used to achieve this [**?**].

The major advantage of using water as the reaction solvent is that the negative environmental impact can be reduced. However, it must be noted that just by performing the chemical synthesis in water does not guarantee a green process. Issues such as atom efficiency, yield, and purification processes must be kept in mind when using water as the reaction solvent [**?**, **?**].

1.2.5 Supercritical Fluids

Super Critical Fluids (SCFs) are a new class of green solvents that are eyed as a potential replacement for the existing VOCs. A supercritical fluid is a substance that exists in an intermediate state resembling both a gas and a liquid at near or above critical temperature[4] and critical pressure[5], but below the pressure required to condense this substance into a liquid or deposit as a solid.

[4]Critical Temperature (T_c)- The particular temperature, characteristic to each gas, above which it is not possible to liquefy any gas.

[5]Critical Pressure (P_c)- The minimum pressure needed to liquefy a substance at its critical temperature (IUPAC goldbook)

Carbon dioxide (CO_2) under supercritical conditions is the most commonly used substance to produce a solvent. Besides CO_2, water, methanol, ethanol, acetone, or methane can be used to produce SCFs [**?**, **?**, **?**]. The table below gives an overview of the chemicals, both organic and inorganic that can be used to produce SCFs.

Table 4: List of inorganic and organic chemicals that can be used to produce SCFs [**?**]

SCF	**Name**	$\mathbf{T_c}$ (°C)	$\mathbf{P_c}$ (bar)	$\mathbf{d_c}$ ($g\,mL^{-1}$)	**MM** ($g\,mol^{-1}$)
NH_3	ammonia	132.4	113.2	0.235	17.03
Ar	argon	−122.5	48.6	0.531	39.95
CO_2	carbon dioxide	31.1	73.8	0.466	44.01
C_6H_6	benzene	289.5	49.2	0.300	78.11
C_2H_6O	dimethyl ether	126.9	54	0.242	46.07
CH_2F_2	difluoromethane	78.1	57.8	0.424	52.02
C_2H_6	ethane	32.2	48.7	0.207	30.07
C_2H_4	ethene	9.2	50.4	0.214	28.05
$C_2H_8N_2$	ethylenediamine	320	62.8	0.29	60.10
CHF_3	fluoroform	25.9	48.2	0.525	70.01
HBr	hydrogen bromide	90.0	85.5	n.a.	80.91
HCl	hydrogen chloride	51.5	82.6	0.42	36.46
HI	hydrogen iodide	150.7	83	n.a.	127.9
C_4H_{10}	isobutane	134.7	36.4	0.224	58.12
Kr	krypton	−63.76	54.9	0.912	83.8
CH_4	methane	−82.6	46.0	0.163	16.04
CH_3OH	methanol	239.5	80.8	0.273	32.04
C_4H_{10}	*n*-butane	152.0	38.0	0.228	58.12
C_6H_{14}	*n*-hexane	234.5	30.3	0.234	86.18
C_5H_{12}	*n*-pentane	196.6	33.7	0.232	72.15
N_2O	nitrous oxide	36.4	72.5	0.453	44.01
C_3H_8	propane	96.7	42.5	0.220	44.10
C_3H_6	propene	91.8	46.0	0.228	58.12
SF_6	sulphur hexafluoride	45.5	37.6	0.737	146.1
H_2O	water	374.0	220.6	0.322	18.02
Xe	xenon	16.6	58.3	1.099	131.3

d_c- density of the compound at T_c and P_c MM- Molar mass of the compound

Using SCFs listed in table 4 for chemical synthesis is comparatively advantageous to other

liquids frequently used as the reaction media. The advantages can be split into three categories as explained below:

- environmental

    1. does not produce smog on release into the environment
    2. most of the SCFs do not damage ozone layer
    3. CO_2 and water are not ecologically toxic
    4. there are no liquid wastes when using CO_2 and other volatile SCFs

- safety

    1. non-carcinogenic
    2. non-toxic; but not HCl, HI, HBr, ammonia
    3. nonflammable; CO_2, xenon, nitrous oxide, water, krypton, and fluoroform.

- process

    1. does not produce solvent residues (only gases and other volatile compounds)
    2. easy separation of products (only CO_2 and other volatile compounds)
    3. high rates of diffusion can be obtained when using SCFs in general
    4. are not viscous
    5. density as well as solvent power can be adjusted
    6. relatively cheap to produce (only CO_2, water, ammonia, argon, and hydrocarbons)

Although using SCFs provide a lot of advantages as mentioned above, there are also equal number of disadvantages that are associated with using SCFs. First and foremost, depending on the component used for producing SCFs, the process can get expensive, because of the extensive use of energy in terms of temperature and pressure. Secondly, using SCFs requires special equipment, which means the existing processes must be amended to accommodate the new design. Thirdly, some of the physical and chemical properties of SCFs can be hazardous to the operator/experimenter. Fourthly, it is evident that all SCFs are compressed gases, which implies that the potential energy in the vessel is large. Upon failure of the equipment, this can be released into the working environment leading to catastrophic events [**?**].

1.2.6 Ionic liquids

Ionic Liquids (ILs) are generally composed of ions (anions and cations) that are liquids at low temperatures ($< 100\,^{\circ}C$). E.g. molten sodium chloride is an ionic liquid when compared to sodium chloride dissolved in water, which is an ionic solution [?, ?, ?, ?, ?]. Typical cations for ionic liquids are the ones that contain nitrogen such as alkylammonium, N,N′-dialkylimidazolium, N-alkylpyridinium, and pyrrolidinium or phosphorous like alkylphosphonium. The most common choice of counterions to be used for preparing ILs include halides, BF_4^-, PF_6^-, CH_3COO^-, CF_3COO^-, NO_3^-, Tf_2N^- like $(CF_3SO_2)_2N^-$, $[RSO_4]^-$, and $[R_2PO_4]^-$. Some of the most commonly used ions to prepare ILs are represented in figure 2 [?]

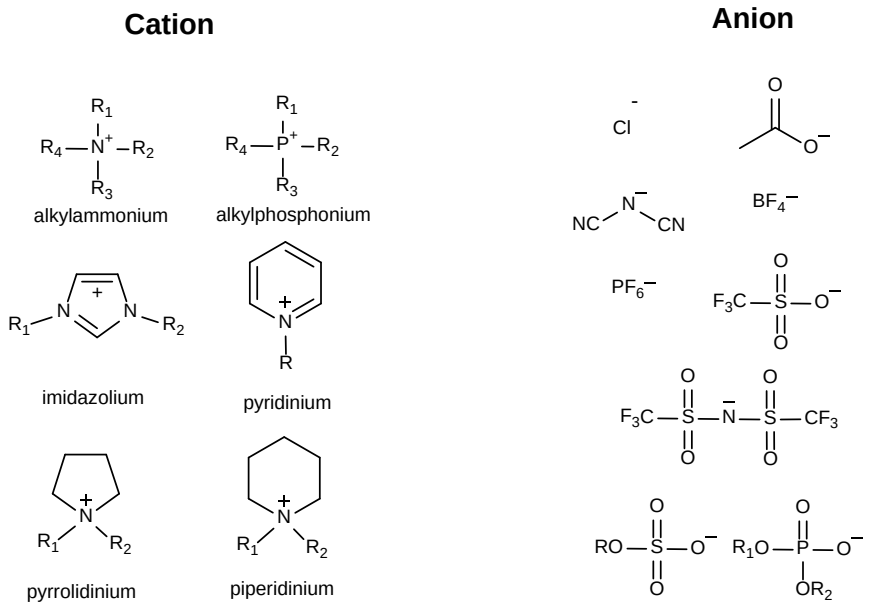

Figure 2: Chemical structures of commonly used anions and cations in synthesizing ILs [?]

The typical properties that ILs possess are listed below:

1. The liquids are non-volatile and non-flammable.
2. They have extremely wide ranges of temperature as a liquid and are mostly thermally stable.
3. Both organic and inorganic materials can be dissolved in ILs with relative ease.
4. They can be used as a highly polar and aprotic solvent (a solvent that is incapable of acting as a hydrogen-bond donor).
5. ILs are non-toxic [?, ?]

ILs are useful in synthesizing chemical products, acting as catalysts, medicine, CO_2 capture agents, and also as mineral extractors, plastics, batteries, super capacitors, lubricants and fuels [?]. One major factor to be considered when designing green chemical processes in ILs or any other alternate reaction media, is the ease of commercialization [?].

1.2.7 Deep eutectic solvents

The last type of alternate reaction media that has been described in literature are Deep Eutectic Solvents (DES) [?, ?, ?]. ILs, despite being promising alternatives to VOCs, are still not completely considered very environmentally friendly, which is the only drawback. Their "greenness" is challenged due to their poor biodegradability, biocompatibility, and sustainability. DES, considered as a class of ILs, have similar characteristics, but are cheaper (raw materials cost less), less toxic, and are often biodegradable [?]. DES are the association of two compounds: a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) through hydrogen bond interactions. The HBA is normally a quaternary ammonium salt, such as choline chloride (ChCl) and the HBD can be a compound with an alcohol, acid, or aldehyde functional group [?]. When the two are mixed and energy is supplied to the system, the combination of hydrogen bonding, van-der-Waals forces, and electrostatic forces between the halide anion and the hydrogen donor moiety decreases the freezing point of the mixture (compared to individual components) resulting in a liquid [?, ?, ?, ?, ?, ?, ?]. The first account of DES was reported by Abbott *et al.* in 2003. They reported the behaviour of 1 mol ChCl with 2 mol urea to have a melting point of approximately 12 °C; ChCl has a melting point of 302 °C and urea has a melting point of 133 °C [?]. Till date the DES have been used as a combined catalyst and solvent [?, ?], in biocatalysis [?], analytics [?], electrochemistry [?, ?] *etc.*

1.3 Renewables and their role in the modern chemical industry

Following the choice of a green solvent, the next step in the "greening" of a process is the selection of the starting material to be converted to product. The production of synthetic organic chemicals has always been performed by transforming carbonaceous feedstocks, of fossil fuel origin, which is an exhaustible or non- renewable resource [?]. This was not the case in the past, as human beings were dependent on renewable resources for their livelihood, especially energy [?]. At this point, it is necessary to differentiate a renewable resource from its non-renewable counterpart.

- Renewable resources- are quickly replaced or recycled by natural processes within the time-frame of human consumption or use. e.g. biomass.
- Non-renewable resources - are those that can be replaced by earth's processes that are

usually slow such that on consumption, the replacement would not be available within a useful time frame. e.g. fossil fuels [**?**].

Table 5 explains the transition between the forms of resources for energy in the United States.

Table 5: Time line of the transition between the sources of energy in the United States of America [**?**]

USA Energy Transitions		**Energy**	**Source**	**Type**
1st transition	1650 - 1900	wood to coal	biomass to fossil fuel	renewable to non-renewable
2nd transition	1900-1940	coal to oil	fossil fuel	non-renewable
3rd transition	1940 till date	oil to gas	fossil fuel	non-renewable
		oil & gas to renewable	non-fossil fuel	non-renewable to renewable
4th transition	not transitioned yet	non-fossil fuel	renewable	

Two things are clear from the table presented above. *(a)* the future generations will have to use non-fossil fuel sources of energy to maintain life on earth and *(b)* at an initial point of time, human beings did manage to survive by using renewable resources. Perhaps, with the population of today's world, making this switch might be difficult, but it is highly necessary [**?**].

Using resources that are petroleum-based has the following disadvantages: *(i)* global warming and its related issues, *(ii)* the soon-to-be exhaustion of the resource, *(iii)* price fluctuations due to geo-political tensions. Owing to the aforementioned issues related to using petroleum-based feedstock, it is the utmost need of the hour that the chemical industries shift their focus to the other abundant resource available—biomass [**?**]. This raises the question as to what can be defined as "biomass?" The book by Sillanpää in 2017, entitled *"A sustainable bioeconomy"*, provides a great insight into the use of biomass to build up a steady bioeconomy. There are several definitions for biomass given in the book and a generalized working definition can be given as:

"All organic and biodegradable matter of products, waste, and residues of biological origin from agricultural, forestry and related industries that is available on a recyclable and recurring basis."

Biomass comprises of cellulose, hemicellulose, and lignocellulosic materials, in addition to starch, lipids, and proteins.

Figure 3 shows the classification of biomass [**?**]. From figure 3, it is evident that biomass can be obtained from a variety of sources. It is estimated that 170 billion metric tons of biomass is

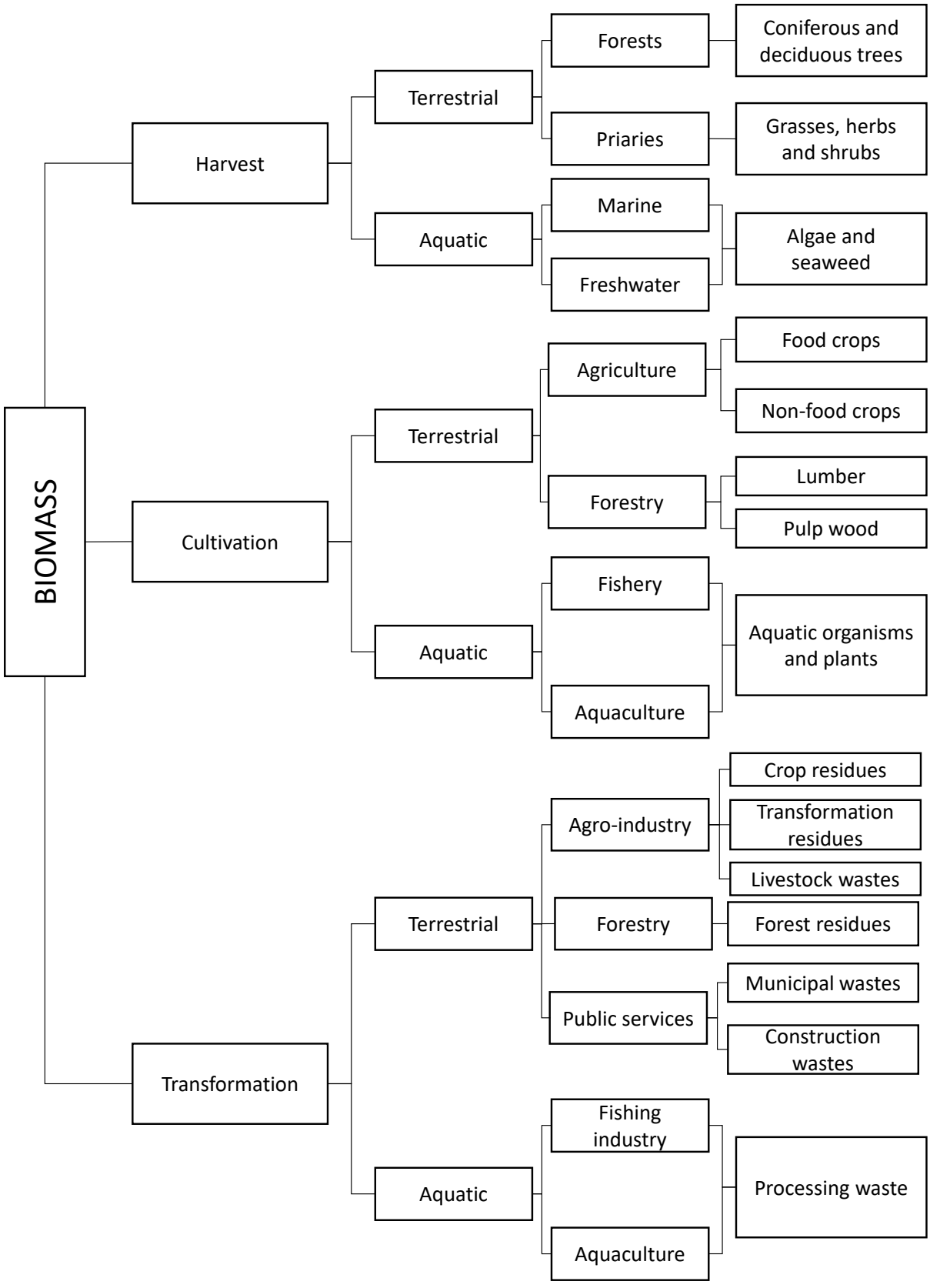

Figure 3: Classification of biomass to be used in the process industry [**?**]

produced every year, out of which 75 % are carbohydrates and the rest of the 25 % are lignins, lipids, other hydrocarbons, inorganic matter, *etc.* With a mere 5–7 billion metric tons being used for human consumption (food and non-food purposes), the rest of the biomass is available for transformation [**?**]. Wood, which is the primary form of biomass from the forest, can be divided into two types: *hardwood* and *softwood*. Depending on the type of wood, it has been used by humans for various purposes such as energy, ship building, flooring, furniture, *etc.* Irrespective of the type of wood, it consists of three polymers, *viz.* cellulose, hemicellulose and lignin. Additionally, wood also contains some extraneous components known as extractives (approximately 2 % to 5 %). The carbohydrates and lignin parts of the wood are converted to energy or platform chemicals using the concept of the biorefinery process. The biorefinery is a modified version of the traditional petroleum refinery and follows the sequence of steps shown in figure 4. The extractives on the other hand are chemically functionalized or modified to yield value added products [**?**].

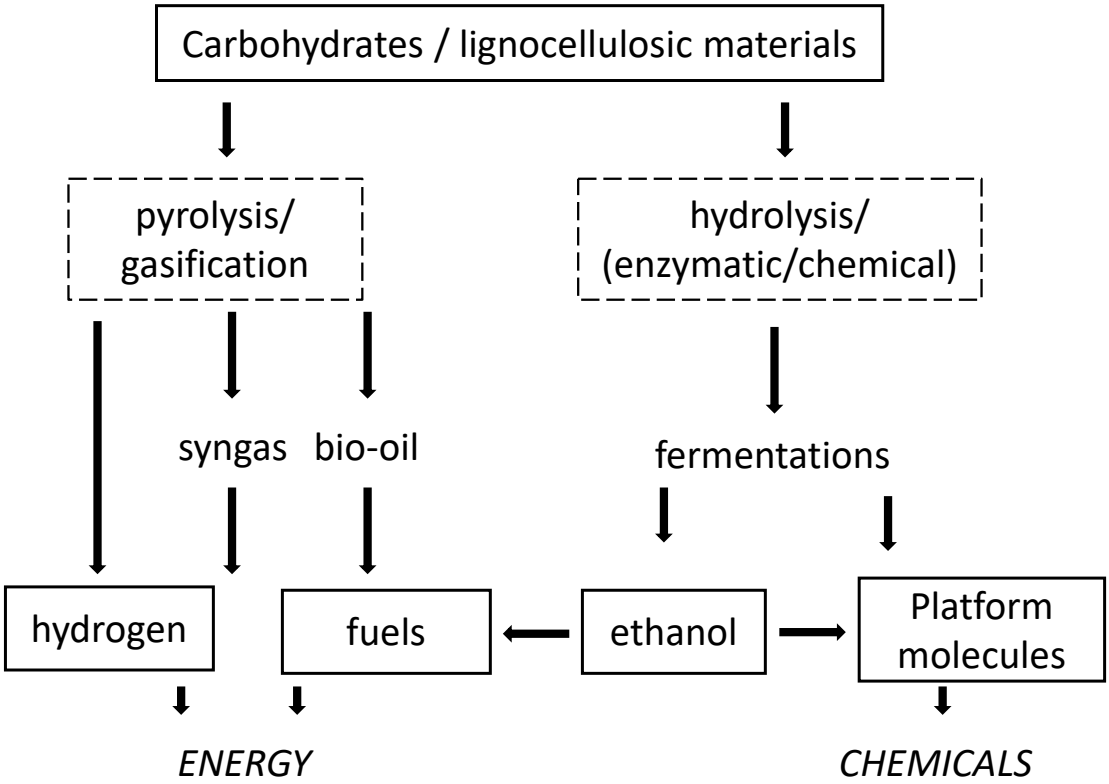

Figure 4: The biorefinery concept of producing energy or chemicals using biomass [**?**]

1.3.1 Terpenes

A critical component of this extractive fraction is the compound class called terpenes [**?**]. These are a class of hydrocarbons that are generally secondary metabolites of the plant kingdom made

of regularly ordered units of isoprene (a 5 carbon compound) arranged in a head to tail fashion, consisting of one or more double bonds. Of all the components of biomass, terpenes have the maximum energy output, however, they are produced in small amounts by the plants [**?**]. They are generally used as a flavouring, fragrance, cosmetic, or pharmaceutical material [**?**]. Some of the most commonly found terpenes and terpenoids are shown in figure 5 below.

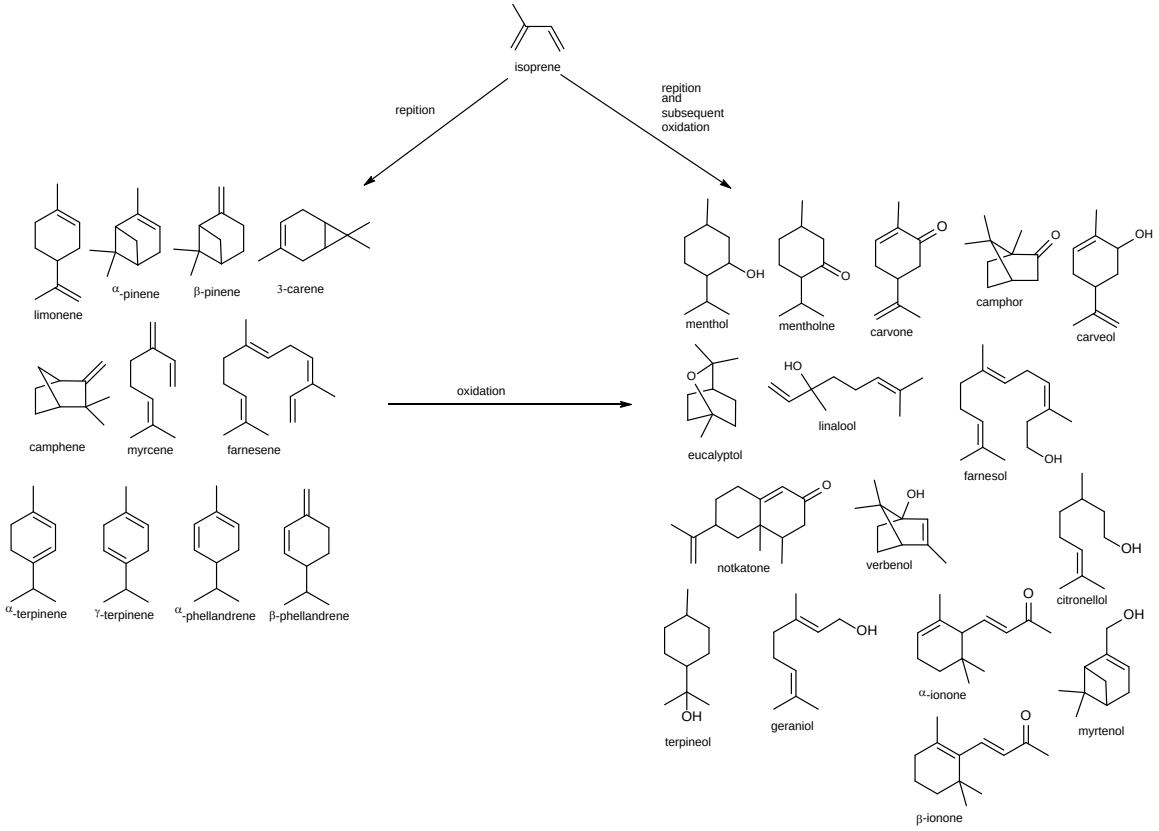

Figure 5: Chemical structures of some of the most commonly used terpenes and terpenoids in the industry. Figure modified from the version of Caputi & Aprea [**?**].

Depending on the number of isoprene units in the molecule, the compound can be classified as: *(i)* monoterpene (two isoprene units) e.g. limonene, *(ii)* sesquiterpene (three isoprene units) e.g. carophyllene, *(iii)* diterpene (4 isoprene units) e.g. class of taxane compounds, *(iv)* sesterterpene (5 isoprene units) e.g. geranylfarnesol, *(v)* triterpene (6 isoprene units) e.g. squalene, *(vi)* tetraterpene (8 isoprene units) e.g. lycopene and *(vii)* polyterpene (multiple isoprene units) e.g. rubber [**?**]. Terpenes can also be classified based on the number of rings a compound has as *(i)* acyclic (without any ring structure)e.g. myrcene, *(ii)* cyclic (with a single ring) e.g. terpinene, and *(iii)* bicyclic (with two rings) e.g. 3-carene [**?**].
Terpenes readily undergo oxidation naturally, or oxidized biosynthetically, or hydrated to yield a class of compounds called terpenoids, which are considered as important intermediates in the industry [**?**]. The most common mode to oxidize a terpene *in vitro* is to carry out an epoxida-

tion step followed by opening of the epoxide to an alcohol or a diol, or rearrange to a ketone or aldehyde [?, ?]. Section 1.4 would provide an overview of epoxides in general and the available epoxidation methods.

1.4 Epoxides and the epoxidation processes

Epoxides are an important class of chemicals that are one of the most commonly synthesized products in the chemical industry. They are predominant precursors that serve as a raw material for the surfactant, textile, cosmetic industries, to name a few. Epoxides are cyclic ethers that are produced by adding an oxygen atom to an alkene. The epoxidation process can either be direct as in the case of molecular oxygen, or by using bound oxygen from oxidants such as peroxycarboxylic acids, peroxides *etc.* The basic epoxide structure is depicted in figure 6 [?, ?].

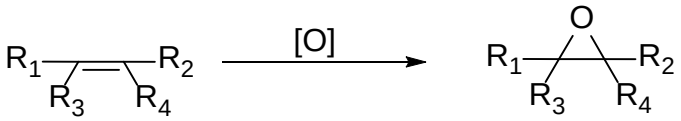

Figure 6: Basic structure of an epoxide, where, R_1, R_2, R_3, and R_4 are the different functional groups [?]

There are numerous ways of producing epoxides. The process of olefin epoxidation is extensively studied by the academic and industrial community [?]. Some of these methods are mentioned in the upcoming paragraphs.

1.4.1 Epoxidation using molecular oxygen

When considering an epoxidation process, the molecular oxygen mediated one is the most used method. This is because oxygen is a low cost and an environment friendly chemical. However, some organic solvent and oxygen mixtures pose the threat of spontaneous ignition at certain conditions of temperature and pressure [?, ?, ?]. The process is normally carried out in the presence of a metallic catalyst.
The metals for carrying out epoxidation reactions belong to two groups in the periodic table.

- Metals of the 4B-6B group of the periodic table - molybdenum (Mo) [?] , vanadium (V) [?], tungsten (W) [?], and titanium (Ti) [?]. These catalysts possess high selectivity[6], but, low activity[7].

- Metals of the 1B, 7B, and 8B group of the periodic table - cobalt (Co) [?], nickel (Ni) [?], manganese (Mn) [?], copper (Cu) [?], platinum (Pt) [?], and ruthenium (Ru) [?]. Unlike the previous category, these catalysts possess high activity, but are less selective [?].

[6] *Refers to the ratio of products obtained from given reactants*(IUPAC goldbook)
[7] also referred to as catalytic activity. *The rate of increase of a specific chemical reaction catalysed by an enzyme or a catalyst in general. It is measured in "katal"*(IUPAC goldbook)

The simplest method of producing an epoxide using oxygen as the oxidant is the Mukaiyama epoxidation[?]. In this process, an aldehyde is oxidized first to an activated oxygen species, of which one oxygen atom is then catalytically transferred to a metal catalyst to produce a carboxylic acid and an epoxide. The scheme for this reaction is given below (figure 7) [?, ?].

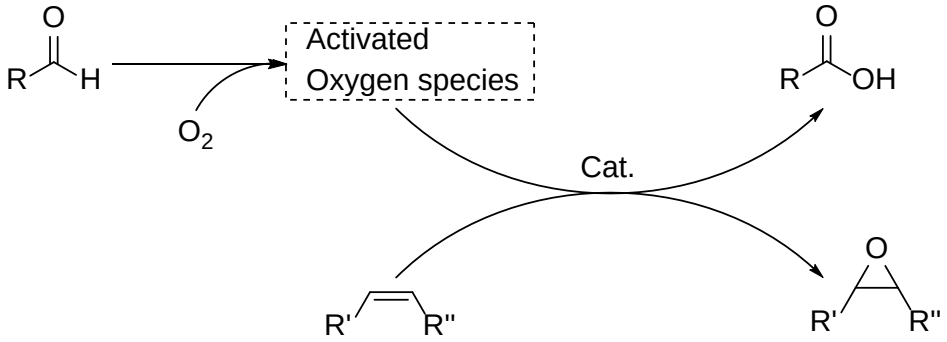

Figure 7: Mechanism of the Mukaiyama epoxidation using oxygen as the oxidant and aldehyde as the reducant for metal catalysed epoxidation of alkenes.(R, R′, and R″ are functional groups) [?, ?, ?]

1.4.2 Epoxidation using H_2O_2

The mechanism of an epoxidation reaction using H_2O_2 as the oxidant and titanium based catalyst is shown in figure 8 below. Epoxidation processes that use H_2O_2 as the oxidant are preferred to those that use oxygen or ozone. This is because of the fact that oxygen based processes (section 1.4.1) are prone to spontaneous ignition [?].

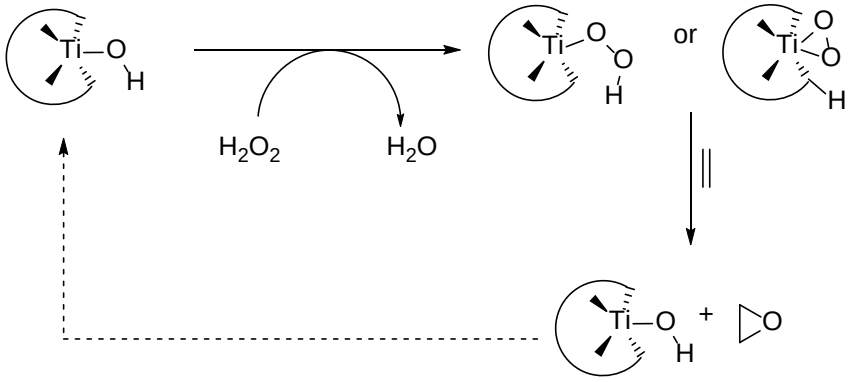

Figure 8: Epoxidation of ethylene using H_2O_2 catalysed by Ti(IV) containing polyoxometalates [?]

However, the reactions with H_2O_2 are catalysed by any one of the metals mentioned previously (section 1.4.1). As seen from the figure, the reaction proceeds in two steps.
Step 1: Activation of the titanium hydroxo moeity by H_2O_2 to form titanium peroxo and hydroperoxo intermediates.

Step 2: Transfer of an oxygen atom from the catalyst to ethylene to form ethylene oxide. On doing so, the initial state of the catalyst is achieved once again, thus completing the catalytic cycle. A modified scheme from Jimenez-Lozano *et al.* for such a reaction is given in figure 8 [**?**].

Another example of an epoxidation process using H_2O_2 was reported by Cokoja *et al.* in a biphasic system in 2015. The researchers used immobilized perrhenate ($Re_2O_7(OH_2)_2$) in an aqueous phase as the catalyst. This catalyst was capable of forming hydrogen bonds with H_2O_2 to form supramolecular ion pairs in a hydrophobic ionic liquid that favored epoxide formation. The mechanism of the reaction is given in figure 9 [**?**].

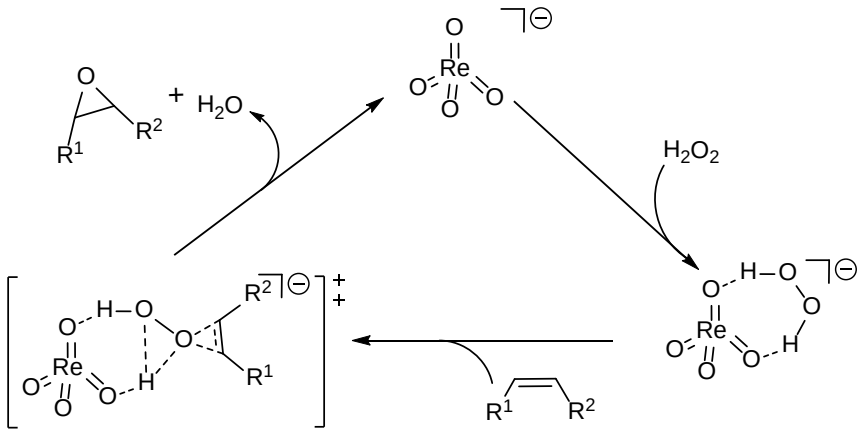

Figure 9: Epoxidation of alkenes in ILs using H_2O_2 and perrhenate ions [**?**]

The advantages of using H_2O_2 as the oxidant for epoxidation are as follows:

- water is the sole by-product
- there is a high content of oxygen active species (for carrying out the epoxidation reaction)
- is inexpensive compared to other oxidants such as organic peroxides (section 1.4.4) or peroxycarboxylic acids (section 1.4.6) [**?**, **?**]

1.4.3 Epoxidation using halohydrin

Hypohalous acids and their salts can also be used to epoxidize olefins. The mechanism for the epoxidation of butene is given in figure 10). The first step in the epoxidation by halohydrin is the addition of hypochloric acid to butene. This forms a halohydrin and by an elimination step the halogen atom is released yielding an epoxide.

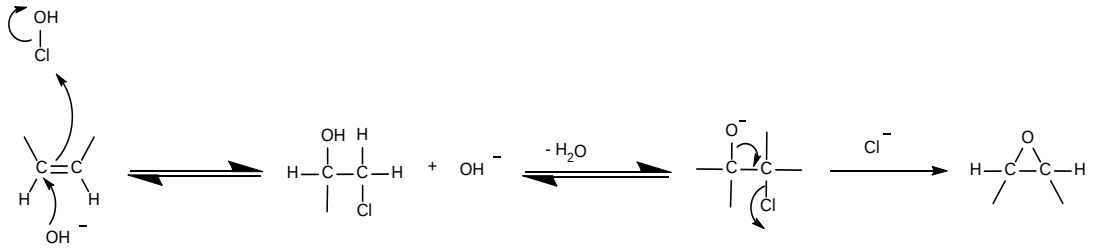

Figure 10: Mechanism of the halohydrin mediated epoxidation of butene [**?**]

It must be noted that this process can only be carried out for electron deficient alkenes and is highly stereospecific[8] (*i.e.* a *cis*-olefin leads to a *cis*-oxirane) [**?**, **?**].

1.4.4 Epoxidation using peroxides

A peroxide compound is named so because it contains a –O-O– group known as the peroxo group. For organic peroxides, this group is attached to at least one carbon atom. The simplest member of this group is hydrogen peroxide. Organic peroxides are industrially important compounds because of their use in epoxidation processes. Most of these peroxides are generated *in situ* because of detonation issues [**?**]. It is worth mentioning here that epoxidation processes with hydroperoxides are restricted to allylic groups[9]. This method is used exclusively when stereospecificity is expected and required in the final product. *tert*- butyl hydroperoxide (TBHP) is one of the most commonly used organic peroxides for epoxidation reactions. It is used at the industrial level to produce propylene oxide from propylene [**?**, **?**].

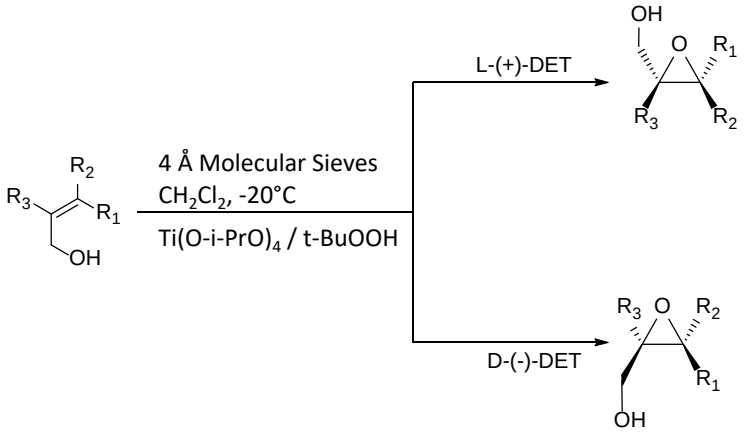

Figure 11: Sharpless epoxidation of an alkene using TBHP as the oxidant in the presence of *meso*-diethyl tartarate and titanium isopropoxide catalyst. Scheme taken from [**?**]

The Sharpless epoxidation uses TBHP in the presence of enantiopure diethyl tartrate and

[8] *The preferential formation of one isomer over the other in a chemical reaction.*(IUPAC goldbook)

[9] *Refers to the group CH_2=$CHCH_2$ and derivatives formed by substitution. A functional group such as -OH attached to the allylic group is referred to as an allyl alcohol*(IUPAC goldbook)

titanium isopropoxide based catalyst to produce epoxides with predictable stereochemistry. The general reaction scheme of this reaction is shown above (figure 11). A detailed description of the mechanism can be obtained from [**?**, **?**]

1.4.5 Epoxidation using ozone

In addition to using molecular oxygen to epoxidize alkenes, it is also possible to use ozone (O_3) in the presence of a catalyst for the same purpose [**?**, **?**, **?**, **?**]. In 1994, Atkinson *et al.* used ozone to epoxidize a variety of alkenes at room temperature in a pressurized vessel. The reaction produced hydroxyl (OH) free radicals, hence, cyclohexane was used as a scavenger to prevent unselective reactions [**?**]. A plausible mechanism for the reaction was given by Waller *et al.* for the epoxidation process using ozone and a metal porphyrin complex (Fe(TMP)Cl) as the catalyst (figure 12). The authors reported that the ozone was used more as a co-oxidant rather than the actual oxidant itself.

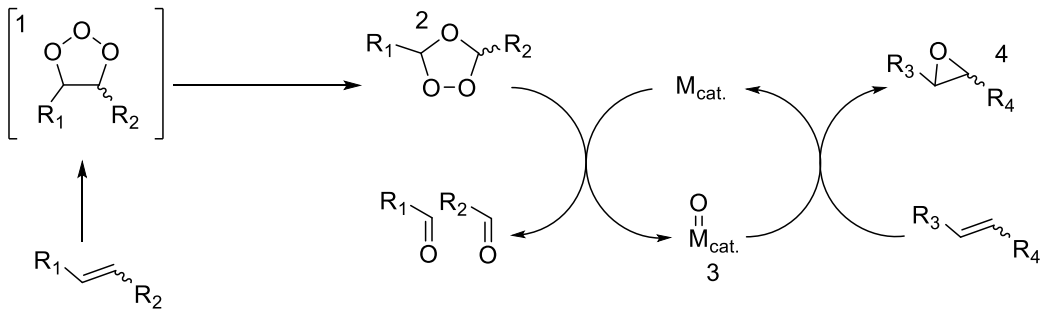

Figure 12: Mechanism of the ozone mediated epoxidation of alkenes using a prophyrin catalyst (Fe(TMP)Cl). [**?**]

From figure 12, it can be seen that the ozone first reacts with an alkene (containing functional groups R_1 and R_2) to form the primary ozonide (**1**), which then rearranges to form a secondary ozonide (**2**) resembling an organic peroxide. This reacts with the Fe(TMP)Cl to from the active catalyst (**3**) that transfers an oxygen atom to the alkene (containing functional groups R_3 and R_4) to form an epoxide (**4**) [**?**].

1.4.6 Epoxidation using peroxycarboxylic acids

Of all the epoxidation methods discussed so far, the Prilezhaev epoxidation process is the simplest method to epoxidize alkenes [**?**]. This reaction was reported for the first time by Prilezhaev in 1909. The reaction is carried out in a neutral organic solvent in the presence of a peroxycarboxylic acid and an alkene at a reaction temperature range of $-20\,°C$ to $25\,°C$. The most commonly used peroxycarboxylic acid in the industry for this purpose is *meta*-choro perbenzoic acid (*m*-CPBA). Additionally, performic acid and peracetic acid can also be used for such

reactions. Owing to their unstable nature and affinity for spontaneous detonation, the use of large amounts of peroxycarboxylic acid is generally not allowed. Instead, the peroxycarboxylic acid is added in aliquots to the reaction or is generated *in situ* [?, ?, ?, ?]. The mechanism of the *m*-CPBA mediated epoxidation of an alkene with functional groups R_1, R_2, R_3, and R_4 is shown in figure 13 below.

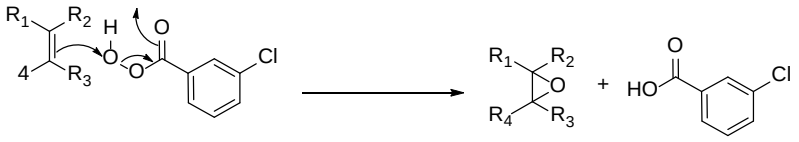

Figure 13: Epoxidation of an alkene using the Prilezhaev method [?].

1.4.7 Shi Epoxidation

The Shi method of epoxidation is a special means of obtaining epoxides using chiral dioxirane species generated *in situ* from oxone ($KHSO_5$) and a ketone derivative of fructose acetal in acetonitrile. This method is also considered to be a complimentary reaction of the Sharpless epoxidation explained in figure 11. The reaction was first reported by Shi *et al.* in 1996, who reported an enantiomeric excess[10] of 89 % to 95 % for a variety of alkenes tested [?, ?]. The mechanism of this epoxidation method is given in figure 14 below.

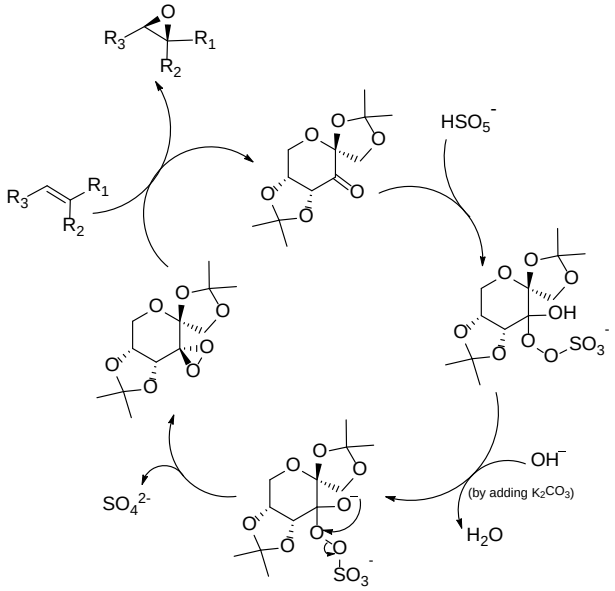

Figure 14: Shi epoxidation mechanism using fructose acetal and oxone [?, ?]

[10]For a mixture of (+) and (−) enantiomers, with composition given as the mole or weight fractions $F_{(+)}$ and $F_{(-)}$ (where $F_{(+)} + F_{(-)} = 1$), the enantiomer excess is defined as — $F_{(+)}$ $F_{(-)}$ — (and the percent enantiomer excess by 100.

1.4.8 Jacobsen-Katsuki epoxidation

This type of epoxidation is carried out using an oxomanganese salen N,N′-ethylene-bis (salicylideneaminato) complex. An example of this type of epoxidation is shown in the figure 15 below.

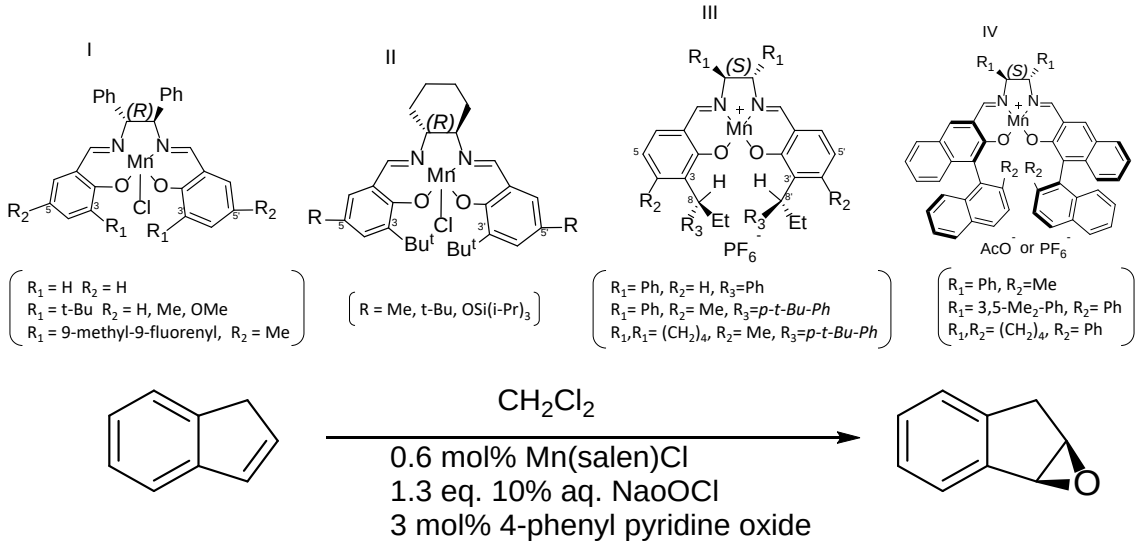

Figure 15: Jacobsen catalyst (I and II) and Katsuki catalyst (III and IV) that are used for the Jacobsen-Katsuki epoxidation process with an example of the epoxidation reaction [**?**]

The Jacobsen-Katsuki catalysts are of two types:

1. Jacobsen's catalysts with two stereogenic centers (I and II, figure 15)

2. Katsuki's catalysts with four stereogenic centers (III and IV, figure 15)

It is to be noted that this type of epoxidation is performed only when a specific isomer of the epoxide is required [**?**, **?**, **?**].

1.4.9 Epoxidation using enzymes

A variety of enzymes[11] can be used either directly, or indirectly to epoxidize fatty acids in nature. Some of these enzymes are cytochrome P450 monooxygenase, diiron center oxygenase, lipoxygenase, lipases *etc.* The cytochrome P450 monooxygenase (EC: 1.14.x.y) is one of the oldest and largest enzyme class distributed throughout all living forms. It carries out the catalysis by incorporating a single oxygen atom into the reactant and produces water as the by-product. In nature, this enzyme can either hydroxylate or epoxidize a fatty acid. It also

[11] mostly protein macromolecules that function as (bio)catalysts by increasing the rate of a specific reaction. (IUPAC goldbook)

needs a cofactor[12] to oxidise its reactant, which is expensive, when required for *in vitro* synthesis. Moreover, P450s cannot function in organic solvents.

Peroxygenases (EC 1.14.x.y) belong to the P450 family of enzymes and are haem containing membrane bound enzymes. Unlike the P450 monooxygenases, these enzymes do not need cofactors to initiate oxygen transfer. These enzymes can also catalyse reactions *in vitro* in pure organic solvents.

In addition to the peroxygenase and monooxygenase, hydrolases such as lipase (EC 3.1.1.3) and carboxyl esterases also produce epoxides from unsaturated fatty acids and H_2O_2 using the Prilezhaev mechanism (section 1.4.6) [**?**]. A detailed account of such a process is given in section 1.5.1.

1.5 Issues associated with the existing modes of epoxidation

Each of the epoxidation processes explained in section 1.4 are efficient and established methods to epoxidize alkenes, each with their share of pros and cons that are to be considered when epoxidizing alkenes at an industrial level.

Table 6: Pros and cons of the existing epoxidation processes

Epoxidation Method	**Pros**	**Cons**
Oxygen (section 1.4.1)	•cheap and abundant resource •minimal wastes produced •industrially accomplished (gaseous alkenes)	•flammable gas •performed in pressurized vessels •capable of detonation
H_2O_2 (section 1.4.2)	•inexpensive resource •produces water as by-product •industrially accomplished (for liquid alkenes)	•can explode at high temperatures •requires complex catalysts •uses harmful organic solvents
Halohydrin (section 1.4.3)	•simple two step reaction •industrially accomplished (propene)	•produces equimolar waste •halogenated acids, organic solvents, and alkaline conditions
Peroxide (section 1.4.4)	•enantiopure synthesis •industrially accomplished (gaseous and liquid alkenes)	•drastic reaction conditions •requires complex catalysts and suitable for lab-scale applications

[12]Organic molecules or ionic substances required by an enzyme for its activity. Normally, the cofactor binds to an inactive enzyme and produces the fully functional and active enzyme.

Ozone (section 1.4.5)	•synthesis takes place at room temperature 25 °C •stereoselective synthesis	•ozone is a toxic, flammable, and corrosive gas[13] •requires pressurized reactors for reaction to occur
Peroxycarboxylic acids (section 1.4.6)	•industrially accomplished for liquid alkenes •stereoselective synthesis	•produces equimolar waste •uses organic solvents in excess
Shi epoxidation (section 1.4.7)	•enantioselective synthesis •simple reaction conditions	•uses harmful organic solvents •lab-scale synthesis and specific for certain alkenes
Jacobsen-Katsuki epoxidation (section 1.4.8)	•enantioselective synthesis •works with a wide range of alkenes	•developed only for lab-scale applications •uses harmful organic solvents
Enzymatic epoxidation (section 1.4.9)	•mild reaction conditions	•more expensive than chemical catalysts
cytochrome P450	•stereospecific •can be obtained from microbes, plants, and animals	•requires cofactor •cannot catalyse a reaction *in vitro* in organic solvents
peroxygenase	•requires no cofactor and can work in pure organic solvents	•is of eukaryotic origin; recombinant expression is a bottleneck.

1.5.1 Chemo-Enzymatic Epoxidation Process

Of all the epoxidation methods described in this work, the enzymatic means of epoxidation is considerably more green than the chemical processes. This is because enzymes are environmentally benign catalysts that need mild reaction conditions which are in accordance with the principles of green chemistry (section 1.2). The chemo-enzymatic epoxidation process was first described by Fredrik Björkling *et al.* in the year 1992 [**?**]. In their work, they reported the epoxidation of alkenes using a lipase from *Candida antartica*.

Lipases are enzymes that belong to the hydrolase family (triacylglycerol acylhydrolase, EC 3.1.1.3) that act on carboxylic ester bonds. They are of industrial significance because of their hydrolysis, esterification, and transesterification reactions in non-aqueos media. Lipases are spread throughout the animal and plant kingdoms and are important for hydrolyzing glycerides to fatty acids and glycerol [**?**, **?**, **?**, **?**]. This enzyme is capable of producing peroxycarboxylic acid in organic solvents when catalytic amounts of carboxylic acids are reacted with H_2O_2. The

[13]Materials and Safety Data Sheet (MSDS)

scheme of this reaction is given in figure 16 [?].

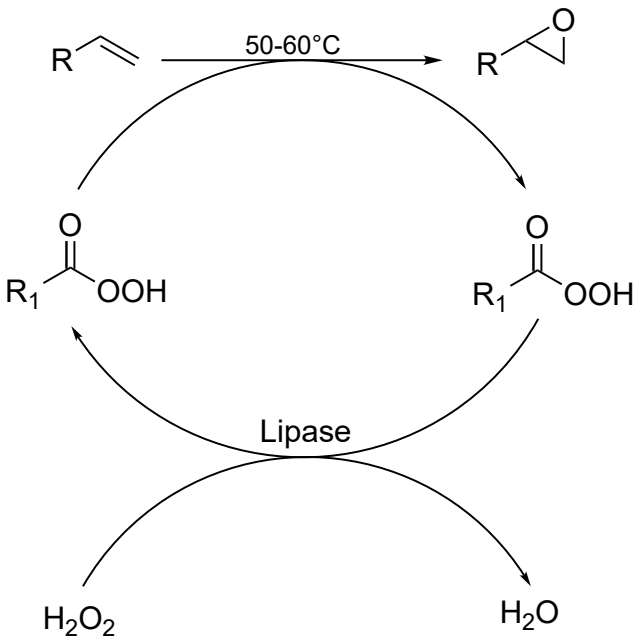

Figure 16: Lipase mediated epoxidation of alkenes in organic solvents according to the method of Björkling *et al.* R and R_1 are functional groups [?]

It is to be noted that the lipase, *viz. Candida antartica* lipase B (CALB), is used to prepare the percarboxylic acid. The epoxidation step is spontaneous and follows the Prilezhaev mechanism explained in section 1.4.6. The advantages of carrying out the synthesis in this manner is that only catalytic amounts of carboxylic acid are needed to produce the epoxide. Hence, the chances of detonation and build up of equimolar amounts of waste is avoided. Warwel and Klaas reported the use dimethyl carbonate as a solvent to produce epoxidized fatty acid methyl esters [?]. Ankudey *et al.* reported the use of ethyl acetate, an ester, as a solvent and carboxylic acid donor in the presence of Urea · hydrogen peroxide ($U{\cdot}H_2O_2$) to produce epoxides [?]. Since the report of Björkling *et al.* in 1992, the process has been used to produce epoxides by a vareity of researchers [?, ?, ?, ?, ?, ?].

1.6 Process Optimization

The lipase mediated epoxidation of alkenes shown in figure 16 was an innovative and simple way to produce epoxides in a non-polluting way. If this process needs to be industrialized, there are several stages in between that require a certain set of skills, techniques, and multidisciplinary teams, till the first plant be operational. Optimization techniques will be needed during three stages of process development:

1. During the early part of process development, *i.e.* when the reaction is investigated in detail.

2. To test for robustness in order to adhere to a specific quality constraint.

3. When optimizing conditions for established procedures.

The optimization strategy needs to be used in every stage of process development and it is almost exclusively done using the statistical design of experiments (DoE) [**?**].
DoE is a technique used to obtain as much data about a reaction system with as few experiments as possible [**?**]. DoE is also used to determine the effect of a certain variable (parameter that is controlled by the experimenter; also known as *signal*) has on the outcome of the process. A biological, clinical, or a chemical trial, when performed a number of times, the expectation is that the response would be the same throughout. However, this is not the case, as there will always be a variation in the outcome because of the variations. These variations in the outcome of an experiment are called errors and they are of two types: *(a) experimental error* is the variation in a response of a trial under the exact experimental conditions and *(b) Measurement error*- is the variability of a response when measurements are taken repeatedly. It is to be mentioned that in reality, the experimental error is the primary causative agent of fluctuation rather than the measurement error. Therefore, the aim of DoE is to approach the ideal case scenario by minimizing noise ($\approx$ experimental error) and separate it from the signal [**?**].
The traditional method of process optimization uses the one variable at a time (OVAT) approach. As the name suggests, OVAT is carried out by varying one parameter constant and keeping the other parameters and measuring the response. This is disadvantageous as a pseudo-optimum state would be reached and there will be huge amount of wastes produced if every combination were to be tried out. DoE overcomes these drawbacks and helps the experimenter to arrive at the actual optimum reaction conditions [**?**, **?**, **?**]. There are several ways to perform a DoE, but this work focuses on the Taguchi method of experimental design for process optimization because of its industrial implementation [**?**].

1.6.1 Taguchi method of robust design

The Taguchi method of robust design was developed by Dr. Genichi Taguchi of Japan to improve the quality of products. It is a method that is based on the statistical DoE to establish the optimum process settings. The objective of using this design is to make a product or process resistant to variations or noise and always maintain productivity standards [**?**]. Noise factors can be classified into the following categories:

- External variations- represents the environment in which the process is carried out and determines the final outcome of a process as well. e.g. temperature, humidity, dust, *etc.*

- Unit-to-unit variation- is the inevitable and unavoidable variation in any process and is because of variations in equipment, material, or processes.

- Variations due to deterioration- A product that is solid or a volatile liquid might deteriorate over time leading to diminished product performance.

The strategy of the Taguchi design is based on orthogonal arrays and fractional factorial, wherein not all combinations of the factors and levels are tested in order to obtain a robust process. A robust process maybe defined as *"an engineering strategy that can be used to improve productivity during the initial stages of research and development in order to produce high quality products within a short period of time and at low cost."* The following approach is to be followed when using the robustness approach to design a process.

- Drafting a P-diagram to classify variables into input (signal), output (response), and uncontrollable (noise) factors (figure 17).

- Using orthogonal arrays to gather information about control factors by performing a minimal number of experiments.

- Determine signal-to-noise ratio (S/N) after evaluating laboratory experiments.

- Using this S/N value to determine the outcome of the process.

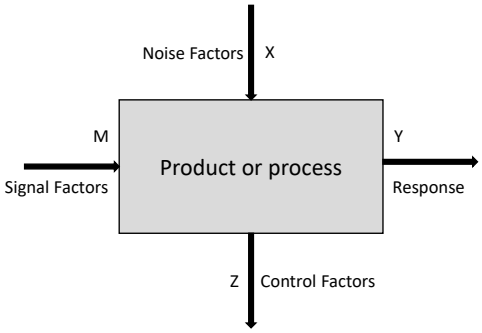

Figure 17: P-diagram or parameter diagram used to design a robust process[**?**]

From figure 17, it can be inferred that there are three types of parameters that control the outcome of the process.

1. *Signal*- designated by the letter M in the figure. These are parameters that are selected and set by the experimenter who is designing the product or the process in order to express the intended value of the process or the product. e.g. speed setting on a ceiling fan determines the amount of breeze.

2. *Noise*- designated by the letter X denote the factors that are not in control of the experimenter and cause the response (Y) to deviate from the expected outcome. e.g. power fluctuations in the supply.

3. *Control*- is denoted by the letter Z. This represents the set of parameters set by the designer, who will vary it at different settings (levels) to affect the outcome of the process.

As mentioned above, it is essential to determine the S/N to determine the outcome of the process. In the equations 1.1, 1.2, and 1.3, $\bar{y}$ refers to the mean of the data that were under observation, $s_y{}^2$ is the variance, and 'n' denotes the number of observations.
Equation 1.1 refers to the criteria *"nominal is best"*. In this setting, the experimenter is interested in achieving a particular target value. Equation1.2 refers to the *"the smaller the better"* criteria. In this setting, the experimenter wishes to reduce the response value. Equation 1.3 refers to the *"the larger the better"* characteristic. As the name suggests, this setting is used when the experimenter wishes to increase the response of a process [**?**, **?**, **?**, **?**]. Mostly for a chemical process, the *larger the better* criterion is used to maximize the yield of a process. The criteria to determine S/N is mentioned in the equations below.

$$\frac{Signal}{Noise} = 10 * log\left(\frac{\bar{y}}{s_y{}^2}\right) \tag{1.1}$$

$$\frac{Signal}{Noise} = -10 * log\left(\frac{1}{n}(\sum y^2)\right) \tag{1.2}$$

$$\frac{Signal}{Noise} = -10 * log\left(\frac{1}{n}\right)\left(\frac{1}{y^2}\right) \tag{1.3}$$

1.6.2 H_2O_2 production in general

Section 1.5.1 describes a lipase mediated epoxidation process that functions effectively where each component has its own role. In figure 16, alkene plays the role of a substrate, enzyme plays the role of the catalyst, carboxylic acid can be considered as the co-catalyst and H_2O_2 is used as the co-substrate. Of these compounds, only H_2O_2 has to be replenished regularly in order to maintain maximum productivity, failing which,the reaction will end due to the insufficiency of H_2O_2 in the system. The H_2O_2 needed for the process can be added at regular intervals, or generated *in situ*. Adding aliquots of H_2O_2 to the reaction mixture over specific periods would be the easiest solution to ensure continuous production of epoxide. However, this is a tedious task and there might be deterioration effects (as explained in section 1.6) due to long-term storage, leading to variation in epoxide production. To avoid this and achieve constant amounts of epoxide over time, a H_2O_2 generation system must be integrated to the process design. Of all the several ways used to produce hydrogen peroxide such as enzymatic,

electrochemical, chemical, and photocatalytic, only the anthraquinone (anthraquinone (AQ)) mediated autoxidation process is the one that is capable of industrial scale production [**?**, **?**, **?**]. Hence, only this part is covered in this thesis.

1.6.3 Anthraquinone autoxidation process for manufacturing H_2O_2

The AQ mediated autoxidation process was used to produce H_2O_2 for the first time by BASF company in Germany using the methodology of Riedl and Pfleiderer [**?**]. The production cycle consists of four stages:

- **Stage 1: Reduction**- alkyl AQ is dissolved in a mixture of hydrophobic and hydrophillic solvent. The dione (AQ) is hydrophobic and the hydrogenated version of the quinone is hydrophillic. In order to dissolve both compounds effectively, a mixture of solvents such as aromatic solvents and trialkyl phosphates needs to be used. The mixture of these two is called a working solution. The reactants are then exposed to a hydrogen environment in the presence of palladium (Pd), nickel (Ni), or ruthenium (Ru) for the reduction to take place.

- **Stage 2: Filtration of the catalyst particles**- In order to prevent degradation of the H_2O_2 produced in the next step, it is absolutely necessary to remove the catalyst before the oxidation step [**?**]. The scheme for producing H_2O_2 is given in figure 18.

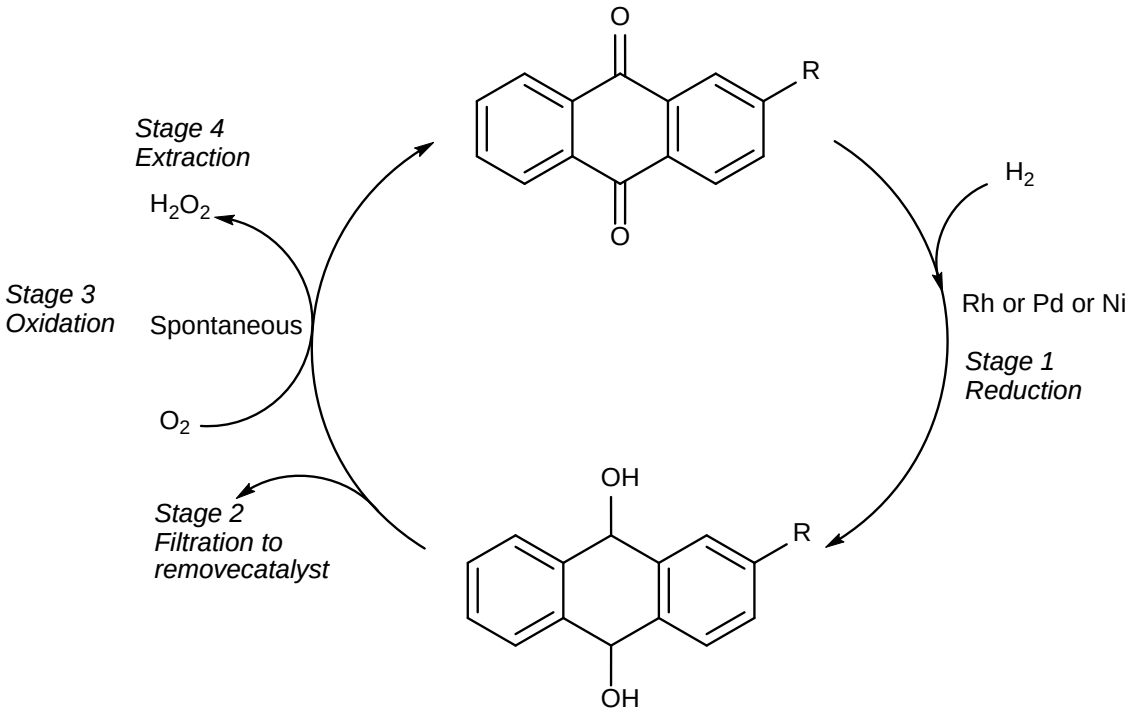

Figure 18: Scheme of the Riedl & Pfleiderer AQ autoxidation process to produce H_2O_2

- **Stage 3: Oxidation**- During the oxidation step, the hydrogenated quinone along with the working solution, now devoid of any catalyst, is oxidised using either air or pure oxygen. Owing to the safety issues associated with using a pure supply of oxygen gas, it is often avoided in large scale applications. The hydrogenated anthraquinone is oxidized to AQ yielding hydrogen peroxide.

- **Stage 4: Extraction of liquid H_2O_2**- is the last stage of the process. Here, the AQ, the H_2O_2 produced, and the working solution are treated with water to remove the H_2O_2. After this, the cycle continues with the reduction step all over again. The concentration of the peroxide can be adjusted to the users' needs. Typically, this is in the range of 15 % to 40 %. Owing to the explosive nature of the peroxides on exposure to heat, concentrating the peroxide beyond 50 % is not advised [**?**, **?**, **?**, **?**].

1.7 Objectives of this work

The objective of this work is to develop a chemo-enzymatic mediated epoxidation process using the enzyme catalyst CALB for a variety of terpenes. To achieve this goal, the first step is to optimize the process. Statistical DoE can help in designing a robust process, *i.e.*, a process that is resistant to variations and is capable of producing epoxides with the same efficiency. This is done using the Taguchi method of robust design. Following the optimization procedure, the process needs to be tested with the freshly optimized parameter set and scaled-up to test if the findings comply with the actual process. Additionally, to complete the process development step, a purification procedure needs to be designed as well to obtain epoxides of high purity.
Following the optimization, the next step is to integrate a H_2O_2 production process with the chemo-enzymatic one to ensure continuous production of epoxides. The AQ autoxidation process of H_2O_2 manufacture was chosen owing to its pre-existing industrial acclaim. First, the coupling is done in a one-pot stopped batch process, which would prove that a coupling of this sort is possible. Following this, the processes are coupled in a semi-continuous manner, where the AQ process and the CALB process function independently and are coupled through a H_2O_2 reservoir. The setup is designed such that it can be industrialized in the near future.
Since the chemo-enzymatic process uses organic solvents to produce epoxides, the process loses its green quotient. In an attempt to reduce the toxic impact of the process on the environment and the operator, a non-toxic alternate media needs to be used. First, the possibility of running the reaction in DES is to be investigated. Following which, several DES are to be screened and the best functioning systems are to be chosen. After which, the process is to be optimized for terpenes in DES, as this is a very new system. Finally, the purification process also needs to be developed for this system to complete a green process.

Chapter 2 Materials and Methods

2.1 Materials

The following materials were used throughout this thesis for conducting experiments. All materials were used as such, unless stated otherwise. 3-carene oxide was produced in house as mentioned in section 2.2.1.3 and purified according to the protocol developed in this work (see section 2.2.1.12).

2.1.1 Chemicals

Table 7: List of chemicals

Chemical	**Manufacturer**	**Purity**
(+)limonene	Acros Organics, Germany	96 %
(+)limonene oxide (isomeric mix)	Sigma Aldrich, Germany	97 %
(+)3-carene	TCI Chemicals, Germany	>90 %
(+)α-pinene	Sigma Aldrich, Germany	>97 %
(+)α-pinene	TCI Chemicals, Germany	98 %
(*1R*)-(-)nopol	Sigma Aldrich, Germany	96 %
(*1S*)-(-)verbenone	Sigma Aldrich, Germany	95 %
(*R*)-(-)carvone	Sigma Aldrich, Germany	98 %
(*S*)-(-)perillic acid	Sigma Aldrich, Germany	95 %
(*S*)-*cis*-verbenol	Sigma Aldrich, Germany	95 %
1-dodecene	Sigma Aldrich, Germany	≥99 %
1-octene	Sigma Aldrich, Germany	98 %
2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma Aldrich, Germany	≥98 %
2-ethyl anthraquinone (EAQ)	Sigma Aldrich, Germany	≥97 %
2-methyl-2-butanol	Alfa Aesar, Germany	≥ 98 %
2-propanol (technical grade)	Th.Geyer &CoKG, Germany	min. 99 %
3-carene	Sigma Aldrich, Germany	≥ 90 %
acetonitrile	Th.Geyer &CoKG, Germany	Technical grade
aqueous H_2O_2 (aq.H_2O_2)	Fischer Scientific, Germany	30 %
aq.H_2O_2	Fischer Scientific, Germany	35 %
aq.H_2O_2	Carl Roth, Germany	50 %

camphene	Sigma Aldrich, Germany	95 %
cardanol	Industrial sample from New Zealand	>90 %
chloroform-d	Sigma Aldrich, Germany	99.8 atom %D
cyclohexane	Carl Roth, Germany	≥99.5 %
cyclohexane	VWR Chemicals, Germany	min. 99 %
cyclohexene	Sigma Aldrich, Germany	99 %
decanoic acid	Merck KGaA, Germany	≥98 %
D(-)fructose	Sigma Aldrich, Germany	≥99 %
D-sorbitol	Applichem GmbH, Germany	min. 99 %
ethyl acetate (chromatography grade)	Th.Geyer &CoKG, Germany	min. 99.95 %
ethylene glycol	VWR Chemicals, Germany	≥99.5 %
farnesene, mixture of isomers	Sigma Aldrich, Germany	No information
farnesol (mixture of isomers)	Sigma Aldrich, Germany	≥95 %
geranic acid, technical grade	Sigma Aldrich, Germany	85 %
geraniol	Sigma Aldrich, Germany	98 %
glycerol	Carl Roth, Germany	≥99 %
isoprene	TCI Chemical, Germany	>99 %
L-(+)-tartaric acid	Sigma Aldrich, Germany	≥ 99.5 %
laevulinic acid	Sigma Aldrich, Germany	99 % (food grade)
L-alanine	Sigma Aldrich, Germany	≥98 %
L-arginine mono hydrochloride	Sigma Aldrich, Germany	≥98 %
L-aspartic acid	Sigma Aldrich, Germany	98 %
lauric acid	Merck KGaA, Germany	≥99 %
L-carveol, mixture of *cis* & *trans*	Sigma Aldrich, Germany	≥95 %
L-cysteine	Sigma Aldrich, Germany	≥98 %
L-glutamic acid	Sigma Aldrich, Germany	≥99 %
L-glycine	Sigma Aldrich, Germany	≥99 %
L-histidine	Sigma Aldrich, Germany	≥99 %
linalool (mixture of isomers)	Sigma Aldrich, Germany	95 %
L-methionine	Sigma Aldrich, Germany	≥98 %
L-tyrosine	Sigma Aldrich, Germany	≥99 %
L-valine	Sigma Aldrich, Germany	≥98 %
malonic acid	Sigma Aldrich, Germany	99 %
mesitylene	Sigma Aldrich, Germany	98 %

methyl cyclohexane	Sigma Aldrich, Germany	≥99 %
methyl tetrahydrofuran	Sigma Aldrich, Germany	≥99.5 %
myrcene (Stabilized)	Sigma Aldrich, Germany	≥95 %
myrtenol	Sigma Aldrich, Germany	≥95 %
n-heptane	Carl Roth, Germany	≥99 %
n-hexane	Th. Geyer GmbH & CoKG, Germany	>95 %
octanoic acid	Carl Roth, Germany	≥99.5 %
octanoic acid	Sigma Aldrich, Germany	98 %
oleic acid	Carl Roth, Germany	≥99 %
potassium carbonate, anhydrous	Sigma Aldrich, Germany	99.9 %
sodium bicarbonate	Sigma Aldrich, Germany	≥99.5 %
sodium hydroxide (pellets)	Applichem GmbH, Germany	min.99 %
sodium sulphite, anhydrous	Carl Roth, Germany	≥98 %
styrene	Sigma Aldrich, Germany	≥99 %
tert.-butyl anthraquinone (tBAQ)	TCI Chemical, Germany	98 %
toluene	Merck KGaA, Germany	≥99.9 %
toluene (GC Ultra Grade)	Carl Roth, Germany	≥99.8 %
tributyl phosphate	Sigma Aldrich, Germany	97 %
urea	Sigma Aldrich, Germany	molecular biology grade
U·H_2O_2	Sigma Aldrich, Germany	97 %
U·H_2O_2	Alfa Aesar, Germany	97 %
valeric acid	Merck KGaA, Germany	≥98 %
xylene	Carl Roth, Germany	≥98.5 %
xylitol	Sigma Aldrich, Germany	≥99 %
zinc bromide	Sigma Aldrich, Germany	≥98 %
zinc chloride	Strem Chemicals, Germany	min.97 %(ALS)
α-ionone	Sigma Aldrich, Germany	≥90 %
α-methylstyrene	Sigma Aldrich, Germany	99 %
α-phellandrene	Sigma Aldrich, Germany	≥85 %
α-pinene oxide	Sigma Aldrich, Germany	97 %
α-terpinene	Sigma Aldrich, Germany	≥95 %
α-terpineol	Sigma Aldrich, Germany	90 %
β-citronellol	Sigma Aldrich, Germany	96 %
β-ionone	Sigma Aldrich, Germany	≥96 %

2.1.2 Catalysts

In this work, several enzymes, and palladium in different formulations were used that are mentioned in the table below. The catalysts were used as such without any modification or pre-treatment, unless stated otherwise.

Table 8: List of catalysts

Enzyme/Catalyst	**Manufacturer**	**Units/loading**
amano lipase PS, from *Burkholderia cepacia*	Sigma Aldrich, Germany	$\geq 30\,000\,\mathrm{U\,g^{-1}}$
alcohol oxidase	Sekisui Diagnostics, UK	$7\,\mathrm{U\,mg^{-1}}$
CALB Immo plus	c-LEcta GmbH, Germany	$11\,600\,\mathrm{PLU\,g^{-1}}$
CALB Immo plus	c-LEcta GmbH, Germany	$16\,700\,\mathrm{PLU\,g^{-1}}$
CALB 10L (liquid formulation)	Fermenta Biotech, India	$10\,000\,\mathrm{TBU\,cm^{-3}}$
CALB-IMMCALB-T2-150XL	Chiral Vision, the Netherlands	$15\,000\,\mathrm{PLU\,g^{-1}}$
$\mathrm{CALB_{TA}}$10000	Fermenta Biotech, India	$10\,000\,\mathrm{PLU\,g^{-1}}$
CALB (Novozyme 435)	Sigma Aldrich, Germany	$\geq$5000 $\mathrm{TBU\,g^{-1}}$
esterase from pig liver (lyophilized powder)	Sigma Aldrich, Germany	$\geq 50\,\mathrm{U\,mg^{-1}}$
glucose oxidase	Sigma Aldrich, Germany	250 kU
lipase from *Candida rugosa* (lyophillized powder)	Sigma Aldrich, Germany	$15 - 25\,\mathrm{U\,mg^{-1}}$
lipase from porcine pancreas Type II	Sigma Aldrich, Germany	$100 - 500\,\mathrm{U\,mg^{-1}}$
palladium on activated carbon (Pd/C)	Sigma Aldrich, Germany	10 % Pd basis
Palladium on alumina (Pd/Al_2O_3)	Strem Chemicals, Germany	0.5 % Pd basis
Pd/Al_2O_3	VWR chemicals, Germany	5 % Pd basis
peroxidase from horseradish (HRP)(Type VI)	Sigma Aldrich, Germany	$\geq 500\,\mathrm{U\,mg^{-1}}$

2.1.3 Miscellaneous materials

- micropipettes of various sizes (10 µL, 100 µL, and 1000 µL)
- micropipette tips of various volumes (0.5 µL to 10 µL, 10 µL to 100 µL, 100 µL to 1000 µL)
- beakers of different volumes (5 $\mathrm{cm^3}$ to 800 $\mathrm{cm^3}$)
- round bottomed flasks of different volumes (5 $\mathrm{cm^3}$ to 1000 $\mathrm{cm^3}$)

- reflux coolers
- Tygon F-4040-A (inner diameter 3.2×10^{-3}m, outer diameter 6.4×10^{-3}m, thickness 1.6×10^{-3} m) for organic solvent resistance
- GC vials (1.5 cm^3 total volume)
- glass vials (5 cm^3 to 20 cm^3 total volume)
- disposable polystyrene cuvettes, $10 \times 4 \times 45\,mm^3$
- separating funnels of various sizes
- Buchner funnel and flask
- filter papers (size: 150 mm, medium filtration rate, particle retention: 5 µm to 8 µm)
- Whatmann filters of 0.21 µm
- silicone oil and sand for heating applications
- double deionized distilled water (dd.H_2O) for diluting H_2O_2 solutions and preparing buffers

2.1.4 Instruments

The following instruments were used throughout the duration of this work.

Table 9: List of instruments

Equipment	**Model**	**Manufacturer**
autosampler	AOC-5000	Jain Compipal, Germany
gas chromatograph	GC-QP-2010	Shimadzu, Germany
gas chromatograph column	BPX5 (0.25 mm diameter, 30 m length, 0.25 µm)	SGE Analytical Science, Austria
heating block	MR Hei-Connect	Heidolph, Germany
mass spectrometer	GC-MS-QP2010 Plus	Simadzu, Germany
membrane pump	SIMDOS 10	KNF Neuberger Inc., United States of America
metal block thermostat	custom made for Schott bottles	VLM, Germany
nuclear magnetic resonance (NMR) apparatus	Avance 400	Bruker, Germany

rotary evaporator	Hei-Vap Advantage	Heidolph. Germany
stainless-steel wire mesh	wire diameter 0.12 mm, mesh size 0.20 mm	Metallwaren-Riffert, Austria
Ultra-Violet (UV) *vis* spectrophotometer	UV-1800	Shimadzu, Germany
vacuum pump	whisper-quiet	Vacuubrand, Germany
analytical balance	Quintix	Sartorius Stedin, Germany

2.1.5 Softwares

The following software were used to complete this work.

Table 10: List of softwares

Software	**Purpose**
ChemDraw	drawing chemical structures and schemes
Mestrenova	for evaluating NMR spectra
Microsoft Excel (2017)	for calculations
Microsoft Powerpoint (2017)	for preparing process schemes
Microsoft Word (2017)	preparing manuscripts for publication
Minitab (version 17.0)	Generation of orthogonal arrays Taguchi method evaluation
Postrun analysis (Shimadzu)	Gas chromatography-Mass spectrometry (GC-MS) analysis of compounds

2.2 Methods

2.2.1 Synthetic methods

2.2.1.1 Solvent free synthesis of terpene epoxides

In order to check if a solvent is necessary for epoxidizing terpenes, the first set of tests were carried out in the absence of any solvent. The first set of tests were carried out using aq.H_2O_2 and U·H_2O_2 as the peroxide source. The reaction conditions are given below:

1. **aq.H_2O_2 as peroxide source:** The initial test was carried out using 2×10^{-3} mol monoterpene (3-carene, limonene, α-pinene), 2.5×10^{-3}mol H_2O_2 (35 %), 5×10^{-4} mol octanoic acid, 0.1 g CALB (1670 PLU), 40 °C, 500 rev min^{-1} for 16 h.
   A scale-up was performed using 1×10^{-2} mol monoterpene (3-carene, limonene, α-pinene), 12.5×10^{-3} mol H_2O_2 (35 %), 2.5×10^{-3} mol octanoic acid, 0.1 g CALB (1670 PLU), for a duration of 20 h (45 °C) and 8 h (60 °C) at 500 rev/ min.

2. **U·H_2O_2 as peroxide source:** This test was performed exactly like the aq.H_2O_2 tests mentioned earlier, but for two changes: U·H_2O_2 was used instead of aq.H_2O_2 and the tests were performed at three different temperatures (40 °C, 50 °C and 60 °C) for a fixed reaction time of 20 h.

2.2.1.2 Screening for organic solvents

Following the tests with solvent free epoxidation and before actual optimization of parameters for epoxidation, a preliminary screening round was performed to identify suitable solvents in which chemo-enzymatic epoxidations could be performed. After identification, two of the eight solvents tested were fed into the Taguchi design to be optimized. The tests were performed as explained in section 3.1 [**?**].

2.2.1.3 Optimization of lipase mediated epoxidation of monoterpenes using DoE - Taguchi Method

For the optimization of parameters using the Taguchi method, the reactions were carried out in 1 mL GC vials. A total of 8 parameters that were to be optimized in this work are described in the table below. The parameters and the various levels listed in table 11 were later used in an orthogonal array for process optimization using the Taguchi method.

Table 11: 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 mmol L^{-1}	50 mmol L^{-1}	70 mmol L^{-1}
D	Temperature	20 °C	40 °C	60 °C
E	Monoterpene type	limonene	3-carene	α-pinene
F	Monoterpene concentration	100 mmol L^{-1}	200 mmol L^{-1}	300 mmol L^{-1}
G	H_2O_2 concentration	100 mmol L^{-1}	300 mmol L^{-1}	500 mmol L^{-1}
H	CALB	20 mg	40 mg	60 mg

Once the parameters were optimized, these sets of parameters and levels were validated in another experiment. This experiment was performed in 100 cm^3 round bottomed flasks. The temperature was controlled in an oil bath using Heidolph magnetic stirrers (MR series) fitted with a Pt1000 temperature sensor. The reaction contents were stirred using a magnetic stirrer set at 500 rev min^{-1}.

2.2.1.4 Preliminary tests to scale-down the AQ H_2O_2 synthesis

The industrial AQ autoxidation process to produce H_2O_2 is a well established one. The idea was to scale-down this process to lab-scale levels and then combine it with the lipase mediated epoxidation process for monoterpenes. For a detailed description of the AQ autoxidation process for H_2O_2 synthesis, please refer to section 1.6.3.
The following steps were necessary to determine the new operating conditions for the autoxidation process in the laboratory.

1. Choosing the right solvent: The screening for the most suitable solvents were carried out with toluene, xylene and *n*-heptane as hydrophobic solvents and ethyl acetate, acetonitrile and 2-methyl-2-butanol as the hydrophobic solvents. The best combination were then chosen based on the amount of H_2O_2 produced at the end of the process.

2. Choice of anthraquinone substrates and hydrogenation temperature: Once the solvent system was selected, the next parameter to be fixed was the choice of the anthraquinone substrate. Two substrates were tested for this purpose, *viz.* EAQ and tBAQ. Three temperatures (30 °C, 45 °C, and 60 °C) were chosen to carry out the hydrogenations. Conversion of the EAQ to 2-ethyl anthrahydroquinone (EAHQ) and tBAQ to *tert.*-butyl anthrahydroquinone (tBAHQ) were monitored using the GC-MS method explained in section 2.2.2.

3. Determining the amount of Pd/C to be used for effective hydrogenations: The hydrogenations were performed in a hydrogen atmosphere maintained in the glass vessel in a solution of toluene : ethyl acetate (60 : 40 volumetric ratio (v/v)) in the presence of Pd/C. Four ratios (2.5 mol %, 5 mol %, 7.5 mol % and 10 mol %) of Pd/C with respect to the anthraquinone substrate were tested at a reaction temperature of 45 °C and 1.67 mmol tBAQ

4. Oxidation conditions for the hydrogenated anthraquinones: Prior to the oxidation step, the palladium catalyst needs to be completely removed using a Whatman filter paper of pore size 0.21 µm. The reddish brown solution (hydrogenated anthraquinone solution) was filtered off. The oxidation step was performed in three different ways: (i) by bubbling air through the system, (ii) by maintaining an air atmosphere and (iii) leaving the system open to air. Additionally, four temperatures (25, 30, 45 and 60 °C) were also tested to check for efficient oxidation.

2.2.1.5 One-pot combination of AQ autoxidation H_2O_2 process with the lipase mediated epoxdation process

The following conditions were used to perform the experiments:

- *Hydrogenation of AQ*- 1.67 mmol AQ, 10 mol %,(Pd/C), toluene : ethyl acetate (60 : 40, v/v) total volume of 10 cm^3, hydrogen atmosphere, 45 °C and 6 h reaction time.

- *Combined oxidation and epoxidation reaction* - Performed in an open system with attached reflux using 0.5 mmol monoterpene (α-pinene, 3-carene, and limonene), 0.05 g CALB, reaction time of 16 h, reaction temperature of 25 °C to 30 °C and 100 µL dd.H_2O.

2.2.1.6 Semi-continuous coupling of AQ autoxidation H_2O_2 process with the lipase mediated epoxidation process

The conditions for producing H_2O_2 using the AQ autoxidation process on a lab-scale were researched once again. The following changes were made when compared to the one pot system mentioned earlier (section 2.2.1.5).

- *Optimization of Pd/Al_2O_3 amount for 2-EAQ hydrogenation:* 2×10^{-2} mol 2-EAQ was dissolved in 100 cm^3 working solution. The working solution in this case is a mixture of mesitylene and tributyl phosphate in a volumetric ratio of 3 : 2 as opposed to the toluene : ethyl acetate system used in the one pot process (see section 2.2.1.5). The reaction mixture was maintained under hydrogen atmosphere at 60 °C and mixed at 250 rev min^{-1}. The tests were conducted using 2.5 mol %, 5 mol %, 7.5 mol % and 10 mol % of Pd/Al_2O_3 pellets. After 5 h of hydrogenation, the pellets were removed by filtration using a Whatman filter paper of size 0.21 µm, after which the oxidation step was performed by bubbling air through an aquarium pump at 22 °C for 0.5 h at the maximum operating capacity of the pump. The H_2O_2 was extracted using 10 cm^3 of dd.H_2O.

- *Washing protocol for palladium (Pd) catalysts:* Washing of Pd/Al_2O_3 catalysts was done according to the protocol of Wang *et al.* [**?**]. The procedure was carried out as follows: First, the catalysts were washed with 15 cm^3 of technical grade ethanol. After 1 min, of mixing, the ethanol was discarded and replaced with 15 cm^3 of dd.H_2O. The contents were mixed for 1 min and the water was discarded. This procedure was repeated two times. The wet catalyst was then dried using an inert gas such as argon (alternatively nitrogen can also be used). The Pd pellets were then reused for the hydrogenation of 2-EAQ.

- *Application of the in-house fashioned stainless steel mesh container to enhance catalyst lifetime:* In order to protect the Pd/Al_2O_3 catalysts from mechanical shear forces in the reactor, a stainless-steel mesh (see section 2.1.4) was crafted in-house. The Pd/Al_2O_3 pellets were loaded into this mesh and the mixing rate could be increased from 250 rev min^{-1} to 1000 rev min^{-1}.

- *Lipase mediated terpene epoxidation parameters:* 25 cm^3 ethyl acetate, 5×10^{-3} mole terpene (3-carene, limonene, and α-pinene), 7.5×10^{-3} mole H_2O_2 produced from the AQ process and 0.1 g CALB were used for the epoxidation process.

- *Development of the combined semi-continuous approach for the epoxidation of terpenes:* The detailed description of this process along with a schematic diagram is given in the recently published work of Ranganathan & Sieber [**?**] in section 3.3.

2.2.1.7 Preliminary screening of DES - test for fluidity at 60 °C

In order to prepare a DES, a quaternary ammonium salt such as ChCl and a HBD are to be mixed in a certain mole ratio and heated to 100 °C. Several DES mixtures were prepared based on the list published by Ruß & König [**?**]. Such DES mixtures shall henceforth be referred to as "conventional DES". A detailed description of this procedure can be found in the published work of Ranganathan *et al.* [**?**] in section 3.4.

2.2.1.8 Secondary screening of successful DES mixtures

The successful hits from the preliminary screening round (section 2.2.1.7) were used as the reaction medium to test the lipase mediated epoxidation reaction. A typical screening experiment consisted of the following ingredients: 1×10^{-3} mol 3-carene, 2.5×10^{-4} mol octanoic acid, 3×10^{-3} mol U·H_2O_2, 0.1 g CALB, and the liquefied DES mixture from the first round of screening. The tests were performed at a temperature of 60 °C with a mixing rate of 500 rev min^{-1}.

2.2.1.9 Optimization of the lipase mediated epoxidation of monoterpenes in DES using the Taguchi method of DoE

Similar to the optimization of the epoxidation process in organic solvents (section 2.2.1.3), the reaction in DES needs to be epoxidized as well. Hence, the Taguchi approach of optimization was used. In comparison to the previous case which used the single orthogonal array technique, this time, a crossed orthogonal array technique was used. A total of 4 parameters at 3 levels were tested for two different DES mixtures, *viz.* glycerol:ChCl mixture (GlCh) and sorbitol:ChCl (SoCh) in two L_9 orthogonal arrays. A detailed description of the procedure can be found in section 3.4.

2.2.1.10 Minimal DES mixture

The DES mixture of urea and ChCl in the molar ratio of 2 : 1 was the first DES to be discovered. The peroxide source for synthesizing epoxides in DES mixtures is U·H_2O_2. In order to avoid the addition of an extra compound, the "minimal" DES medium was discovered and implemented. Reaction conditions: ChCl (7.5×10^{-3} mol) and U·H_2O_2 (15×10^{-3} mol) were mixed at room temperature between 0.75 h to 1 h with a magnetic stirrer. The resultant liquid served as both a solvent and a peroxide source for the lipase-mediated epoxidation.

2.2.1.11 Lipase mediated epoxidation in conventional and minimal DES mixtures

The following reaction conditions were used to synthesize epoxides of terpenes.

- *Conventional DES mixture:* GlCh (10×10^{-3} mol glycerol and 5×10^{-3} mol ChCl) and SoCh (5×10^{-3} mol sorbitol and 5×10^{-3} mol ChCl) were liquified at 60 °C, which converted 3-carene completely to its corresponding epoxide. The epoxidation conditions were: GlCh or SoCh, 1×10^{-3} mol terpene, 4×10^{-3} mol U·H_2O_2, 0.1 g CALB (1670 PLU), 500 rev min^{-1} and 50 °C.

- *Minimal DES mixtures:* The minimal DES was prepared as per section 2.2.1.10 and used for epoxidation reactions. The reaction conditions for this reaction were: 5×10^{-3} mol terpene, 0.1 g CALB, 1.25×10^{-3} mol octanoic acid, 500 rev min^{-1} at 50 °C.

2.2.1.12 Purification of monoterpene epoxides

Depending on the method of synthesis, the purification protocol to obtain terpene epoxides differed.

- **Terpene epoxide synthesis in organic solvents:** Following optimization of the chemo-enzymatic epoxidation process using DoE- Taguchi approach (section 2.2.1.3), the epoxides produced were subjected to a purification procedure. Arata & Tanabe [**?**] had reported that the epoxides of terpenes are highly sensitive compounds that undergo rearrangement in a basic medium. If one were to use a strong base such as a 10 mol L^{-1} sodium hydroxide (NaOH) solution, the epoxides immediately open to form diols. To avoid this phenomenon, saturated amounts of sodium bicarbonate ($NaHCO_3$) solution, a weaker base than NaOH, was used for 5 - 7 times to ensure complete neutralization of the residual acid concentration used in the process. The acid in this case was octanoic acid (C8), which was obtained as a sodium salt. A schematic of the whole process is given in section 3.1 [**?**].

- **Terpene epoxide synthesis in DES:** For the epoxides produced in DES, the purification protocol was modified and was performed in two ways. The first way involved the use of *n*-hexane and second way used ethyl acetate as the solvent to extract the products. Both these processes have been described as a schematic in section 3.4.

2.2.2 Analytical Methods

2.2.2.1 GC-MS methods

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^{-1} and finally increasing the temperature to a maximum of 270 °C with a holding time of 3 min at the rate of 70 °C.

- Mass spectrometer: Ion source was maintained at a temperature of 200 °C and the interface temperature was 250 °C.

GC-MS Postrun analysis, a software provided by Shimadzu, was used to analyse the samples' mass to charge ratio (m/Q) for all the compounds used in this work. The mass patterns were compared to the databases of *National Institute of Standard and technology (NIST)* library-version 08 and 14. The samples for analysis were prepared in ethyl acetate of Liquid chromatography-Mass Spectrometry (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.
For the quinones and hydroquinones, the same GC method mentioned above was followed with one small change: the final holding time of 3 min at 270 °C was extended to 7 min. The retention times of the quinones and their hydrogenated products are as follows: 16.55 min for EAQ, 16.45 min for EAHQ, 16.93 min for tBAQ and 16.86 min for tBAHQ. The complete details of the method is given in [**?**].

2.2.2.2 Nuclear Magnetic Resonance (NMR) Methods

The purified epoxides of α-pinene, 3-carene and limonene were analyzed using proton (^{1}H) NMR. This was performed on a Bruker Avance 400 (^{1}H: 400.13 MHz, ^{13}C: 101 MHz, T=300 K apparatus (see section 2.1.4). 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 parts per million (ppm). All resonance multiplicities are designated as singlet (s), doublet (d), triplet (t), multiplet (m) and coupling constants J in Hz[1].

2.2.2.3 Approximate quantification of H_2O_2 concentration

To estimate the amount of the H_2O_2 concentration produced in the autoxidation process or to check for residual concentration of H_2O_2 after reaction, the Quantofix test stripes were used. 5 µL of sample was added to a pre-wetted strip and the amount of peroxide in the system

[1]SI unit of frequency equal to one cycle per second, Hz = s^{-1} (IUPAC goldbook)

was calculated (as an approximate amount) accordingly depending on the intensity of color produced (provided by the supplier as a chart).

2.2.2.4 ABTS assay for H_2O_2 detection

The amount of H_2O_2 produced throughout this work using enzymatic, chemical, or electrochemical means was assayed using the ABTS method [**?**]. Prior to performing the assay, the following reagents were prepared.

- 2×10^{-3} mol L^{-1} ABTS solution (in 0.1 mol L^{-1} potassium phosphate buffer (KP_i), pH 5.0)
- 5×10^{-3}g L^{-1} HRP solution in appropriate amounts. (Note: the HRP solution was freshly prepared on the day of use, prior to the beginning of the experiment)

The typical assay procedure was done as described below:

1. 1 cm^3 ABTS solution was pipetted into a standard cuvette (described in 2.1.3) using a micropipette
2. 100 µL of the sample (typically H_2O_2 or water for blank measurements) was added to the cuvette containing the ABTS solution
3. To this liquid mixture, 100 µL of freshly prepared HRP was added
4. The mixture (purple in color) was mixed well by pipetting the mixture up and down several times to ensure sufficient mixing of the reactants
5. This mixture was left undisturbed at 22 °C for 10 min
6. Following the incubation, the absorbance of the solution (now green due to reaction with H_2O_2) was measured at 405 nm using a Ultra-Violet (UV)-Vis spectrophotometer
7. The concentration of H_2O_2 was determined based on the calibration curves obtained prior to analyses

Chapter 3 Results

3.1 Optimization of the lipase mediated epoxidation process for monoterpenes using the design of experiments - Taguchi method

In this publication, the lab-scale lipase (CALB) mediated epoxidation reaction is introduced in detail. The various reaction parameters that affect the outcome of the process were identified and optimized using the DoE approach to design a process specifically for monoterpenes. A total of 8 parameters (7 parameters at 3 levels and 1 parameter at 2 levels) were shortlisted: reaction medium, carboxylic acid type and concentration, temperature, monoterpene type and concentration, H_2O_2 concentration, and CALB amount.

The manuscript also describes an optimization procedure that was performed using the Taguchi method for robust design in just 18 triplicate runs (54 runs) instead of the OVAT approach. This was beneficial in saving time, resources and the minimization of wastes generated. Of the eight parameters optimized, the H_2O_2 concentration used had the maximum impact on the process, while the type of monoterpene used was of least significance. The optimized process needed 4 h to 6 h to achieve a complete conversion of the starting material. A comparison of the previous works in this field showed that 6 h to 24 h was needed for total conversion, making this process better. Additionally, the optimized process was ideal for a scale-up and could be carried out with relative ease.

The publication also discusses the development of the analytics for the identification of the three monoterpenes tested and their corresponding epoxides. Furthermore, a suitable purification protocol was developed and performed by a simple two phase extraction to complete the production of pure monoterpene epoxide.

The first author designed the whole process, decided on the optimization procedure, performed the calculations and analysis. This author also conducted the experiments in collaboration with the second author. The other co-authors contributed to the content and language of the manuscript.

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

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

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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 h. 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.

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].

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

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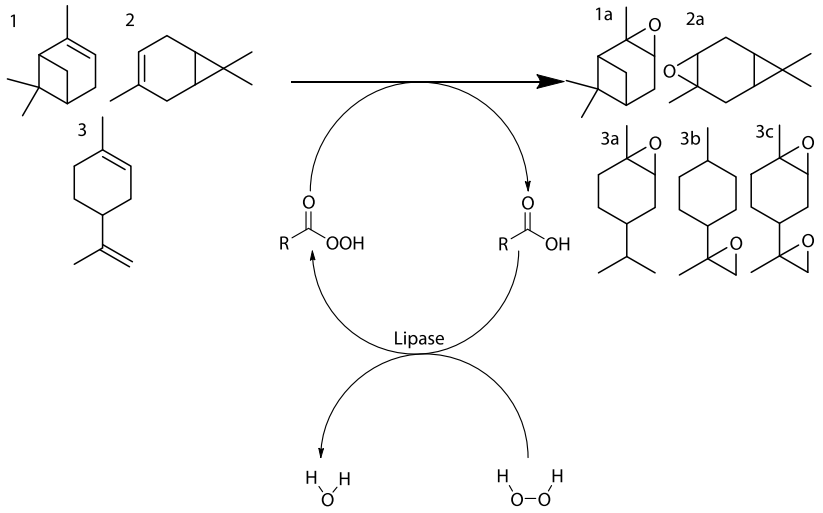

Scheme 1. Lipase mediated epoxidation of monoterpenes using peroxycarboxylic acids according to the method of Björkling et al. [5]. (1-α-pinene, 2–3-carene and 3-limonene; 1a-α-pinene epoxide, 2a-3-carene epoxide, 3a and b-limonene monoepxoide and 3c-limonene diepoxide).

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 and 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

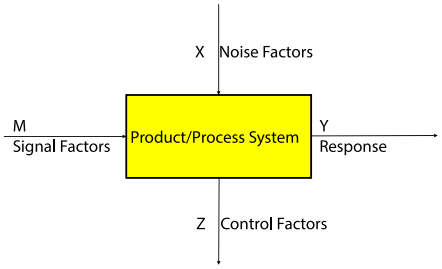

Fig. 1. Parameter diagram for product/process system.

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 (Fig. 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.

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	200 mM	300 mM
G	*Hydrogen peroxide concentration*	100 mM	300 mM	500 mM
H	*Lipase amount*	20 mg	40 mg	60 mg

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.

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

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

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

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].

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 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

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.

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

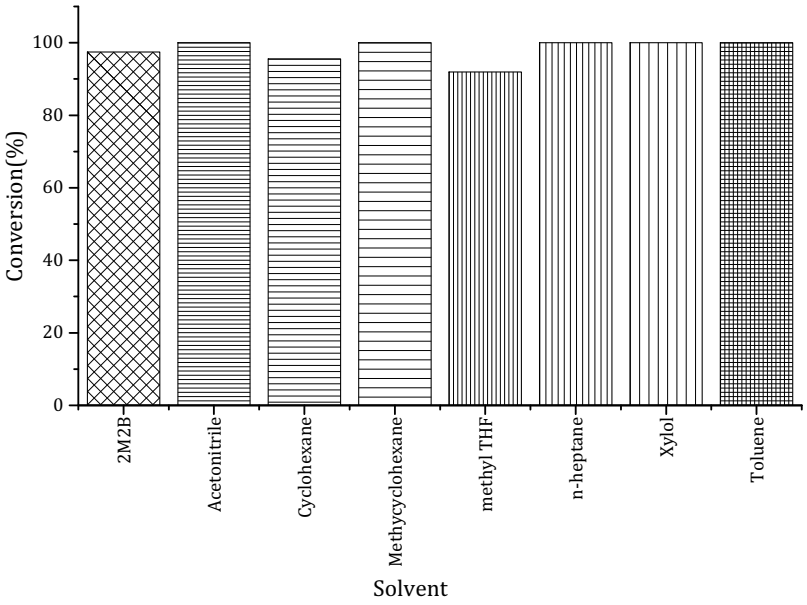

Fig. 2. Results of solvent screening for the conversion of monoterpenes to monoterpene epoxide over a period of 24 h.

multiplicities are designated as s (singlet), d (doublet), t (triplet), m (multiplet) and coupling constants J in Hertz [Hz].

3. Results and 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.

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 (Fig. 2).

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 (Fig. 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.

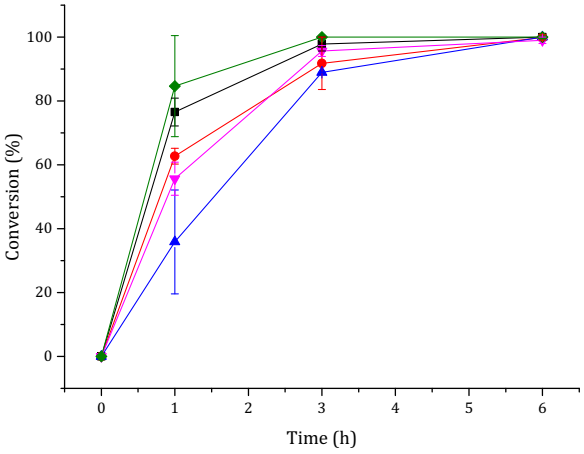

Fig. 3. Kinetics of limonene conversion over a time period of 6 h using acetonitrile (square), methylcyclohexane (circle), *n*-heptane (triangle), xylene (inverted triangle) and toluene (diamond).

Table 3
Different solvents tested and their log P values according to [30].

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

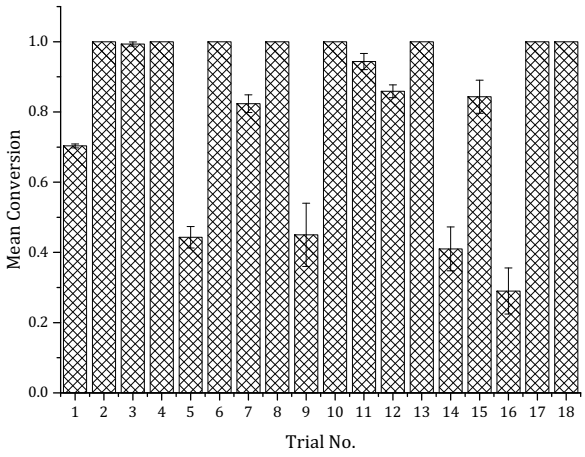

Fig. 4. Conversion obtained by trying out different formulations of parameters and levels as suggested by the orthogonal array design (L_{18}).

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).

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 Fig. 4.

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 Fig. 5.

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.

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

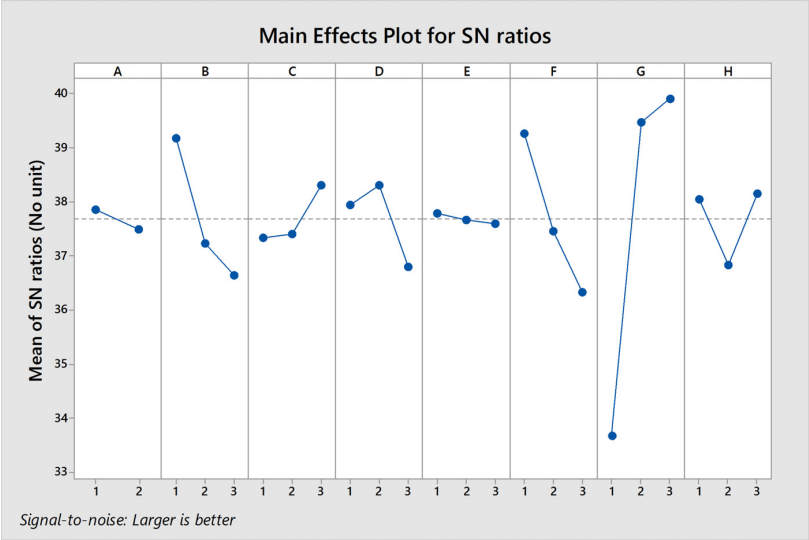

Fig. 5. Signal to noise ratios for various parameters and levels tested using the L18 orthogonal array and "larger the better" characteristic. Dashed line implies the mean signal to noise ratio for all 18 trials. (A–H parameters tested at different levels 1, 2 and 3 (Table 1)).

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*

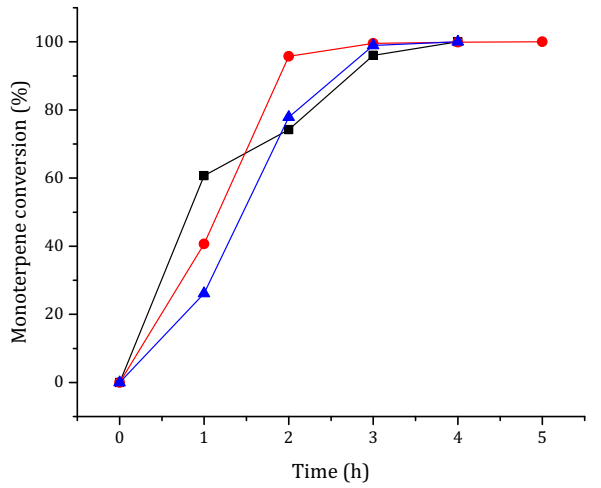

Fig. 6. Conversion profile of limonene-200 mM (triangle), alpha-pinene-200 mM (square) and 3-carene-200 mM (sphere) using optimized parameters from Taguchi method. (Note: Conversion for the limonene reaction refers to the epoxidation of the molecule as such with two olefin bonds being epoxidized.).

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 Fig. 6.

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 5–7 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 Fig. 7.

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.

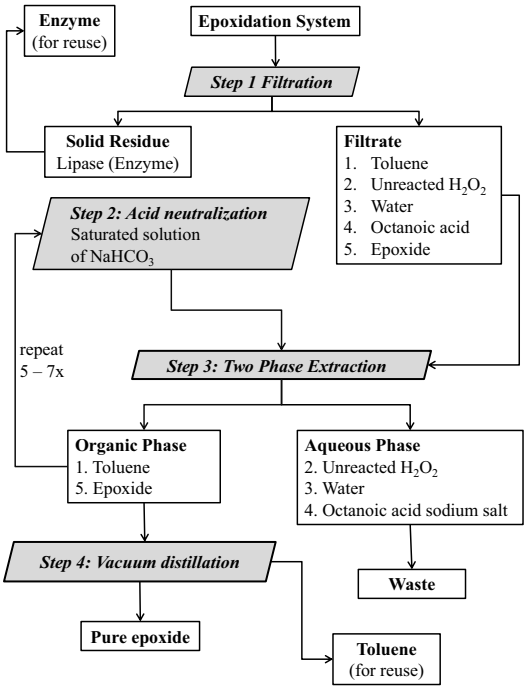

Fig. 7. Epoxide purification process after the use of optimized lipase mediated epoxidation systems.

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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.07.005.

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SUPPLEMENTARY MATERIAL

Conversion (Trial 1-3) rates, Mean (MEAN), standard deviation (STDEV) and signal to noise ratios (SNRA) for the L_{18} orthogonal array

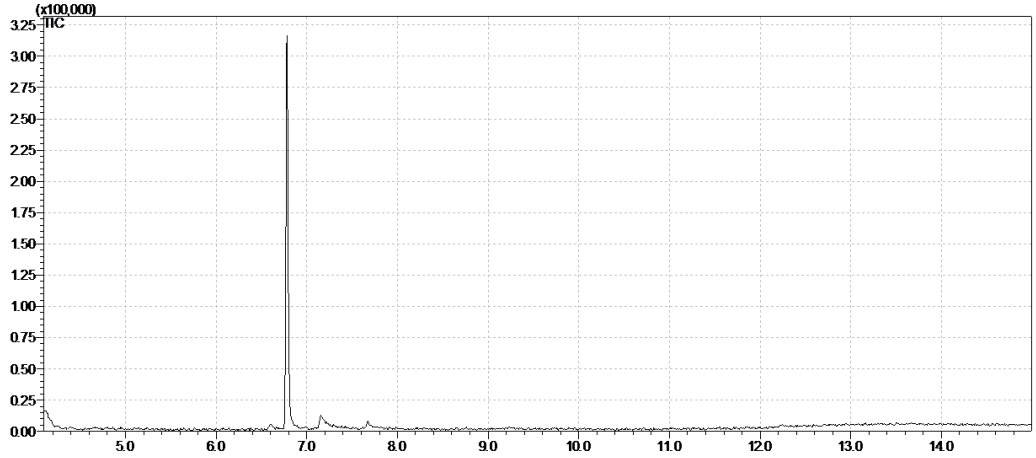
Experiment #	Trial 1	Trial 2	Trial 3	SNRA	STDEV	MEAN
1	71.00	70.00	70.00	36.9426424	0.57735027	70.3333333
2	100.00	100.00	100.00	40	0	100
3	99.00	99.00	100.00	39.9416081	0.57735027	99.3333333
4	100.00	100.00	100.00	40	0	100
5	45.00	47.00	41.00	32.8920458	3.05505046	44.3333333
6	100.00	100.00	100.00	40	0	100
7	85.00	82.00	80.00	38.3034584	2.51661148	82.3333333
8	100.00	100.00	100.00	40	0	100
9	36.00	45.00	54.00	32.7073593	9	45
10	100.00	100.00	100.00	40	0	100
11	97.00	93.00	93.00	39.4881939	2.30940108	94.3333333
12	84.66	85.00	88.00	38.6746193	1.83788215	85.886863
13	100.00	100.00	100.00	40	0	100
14	43.00	46.00	34.00	32.0331593	6.244998	41
15	86.00	88.00	79.00	38.4917074	4.72581563	84.3333333
16	36.00	28.00	23.00	28.8190352	6.55743852	29
17	100.00	100.00	100.00	40	0	100
18	100.00	100.00	100.00	40	0	100

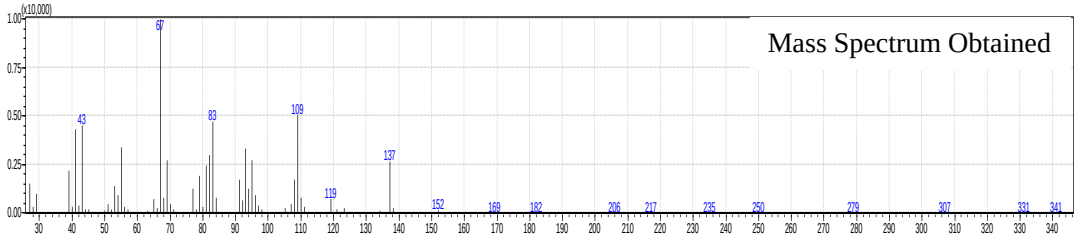
For the actual L18 orthogonal array setting of each of the experiment numbers, please refer to Table 4.

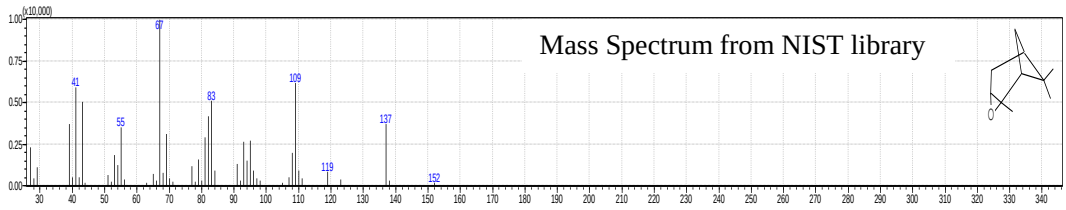
Supplementary material for "Optimization of the lipase mediated process for the epoxidation of monoterpenes using the design of experiments – Taguchi method"

GCMS and ^{1}H-NMRSpectrum

α-pinene epoxide



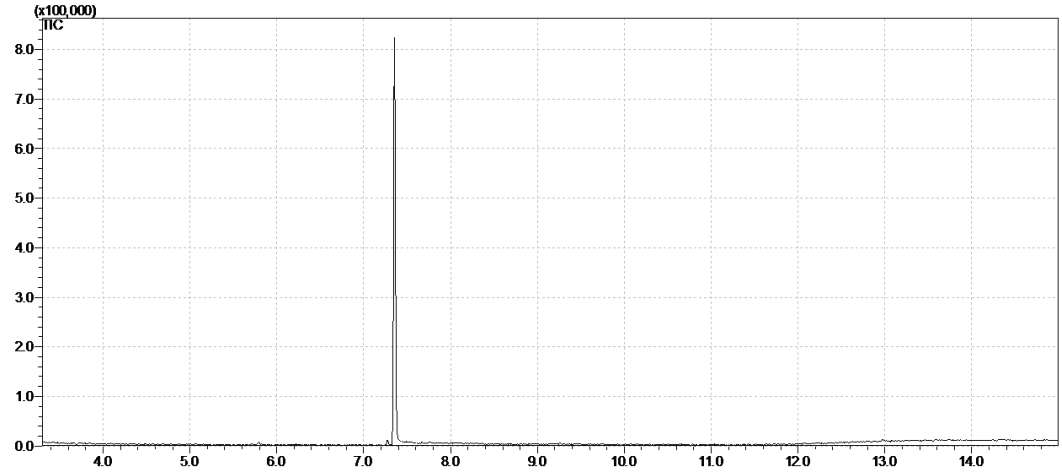


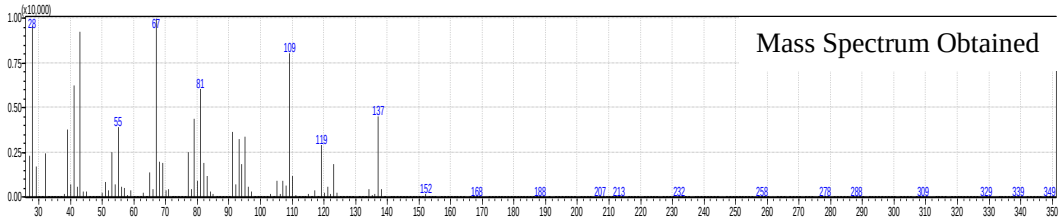


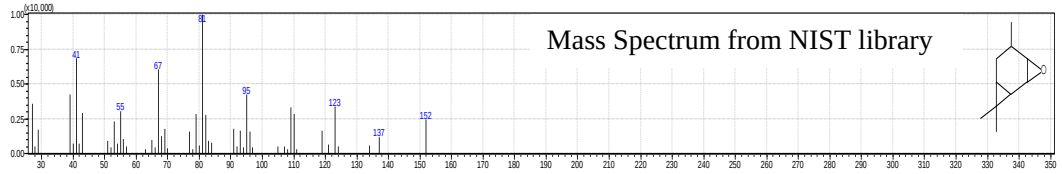
^{1}H NMR (400 MHz, CDCl3): D= 0.94 (s, 3H), 1.29 (s, 3H), 1.35 (s, 3H), 1.63(d, J=9.5Hz, 1H), 1.72 (br m, 1H), 1.87 - 2.04 (m, 4H). 3.08 (d, J = 3.7 Hz)

Supplementary material for "Optimization of the lipase mediated process for the epoxidation of monoterpenes using the design of experiments – Taguchi method"

3-Carene epoxide



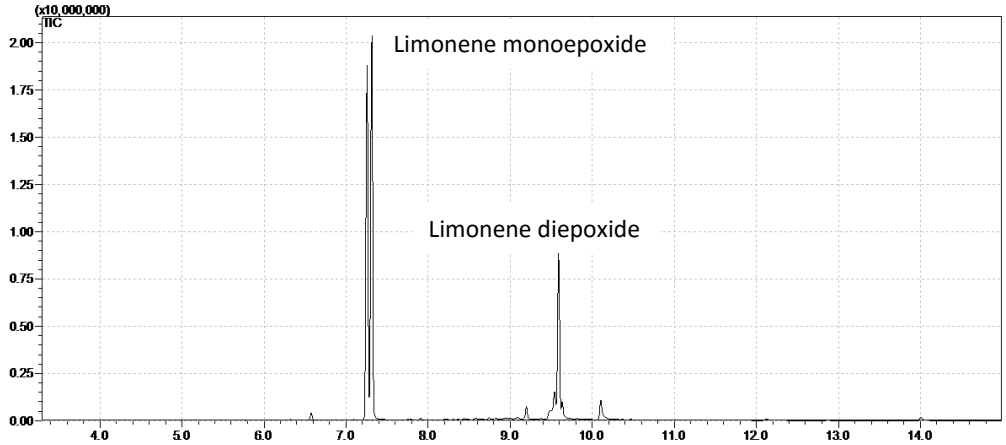


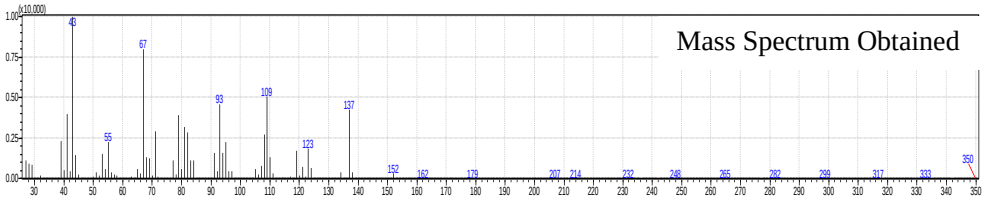


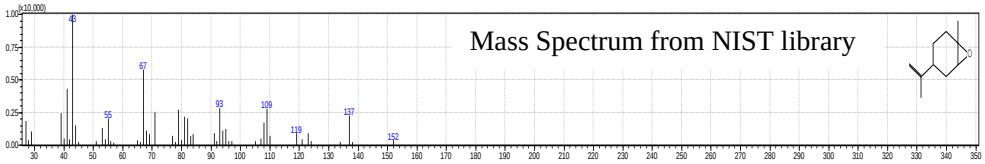
1H NMR (400 MHz, CDCl3): d = 0.39 (td, J = 9.1, 2.2 Hz, 1H), 0.47 (td, J = 9.1, 2.3 Hz, 1H), 0.67 (s, 3H), 0.95 (s, 3H), 1.20 (s, 3H), 1.44 (dd, J= 16.2, 2.3 Hz, 1H), 1.58 (dt, J = 16.5, 2.3 Hz, 1H), 2.09 (dd, J = 16.0, 9.2 Hz, 1H), 2.24 (ddd, J= 16.4, 9.1, 1.9 Hz, 1H), 2.77 (s, 1H)

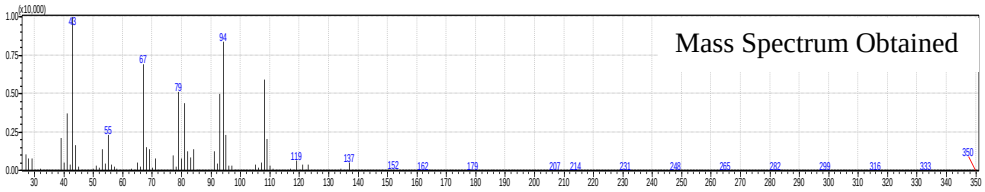
Supplementary material for "Optimization of the lipase mediated process for the epoxidation of monoterpenes using the design of experiments – Taguchi method"

Limonene (mixture of mono and di epoxide 80:20)









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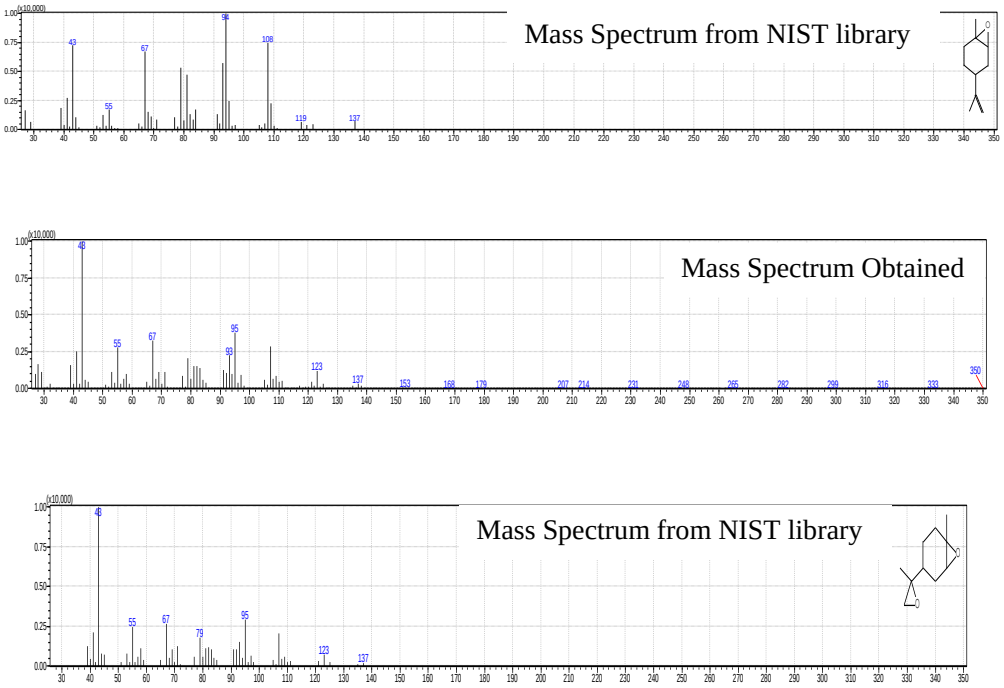
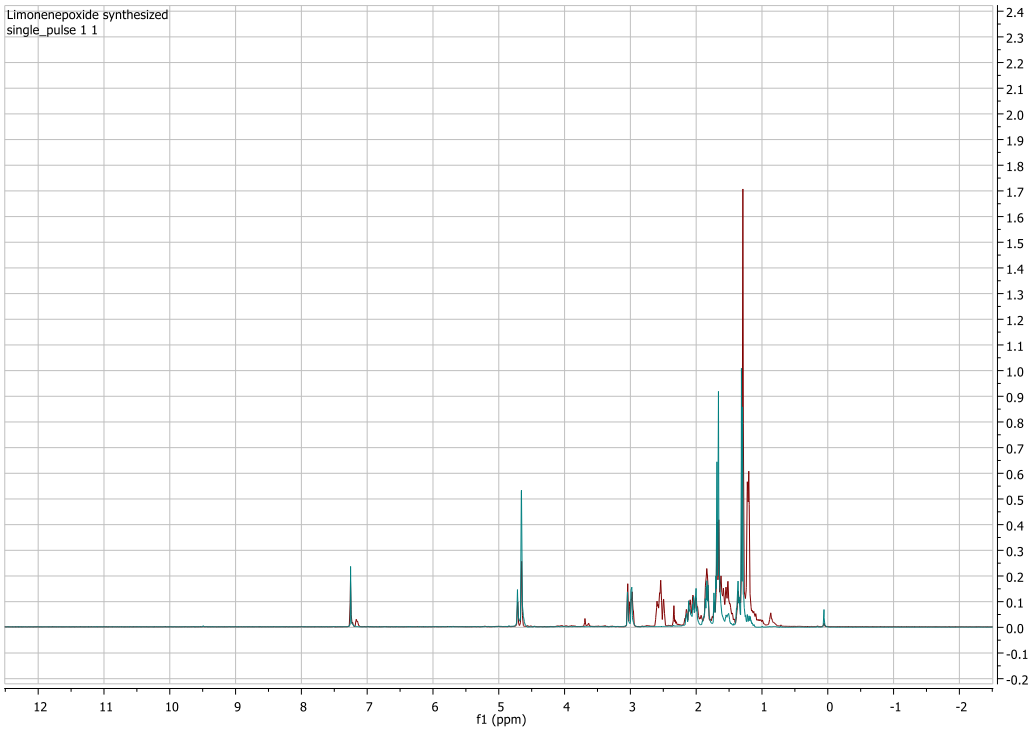


The NMR data of the mixture of mono and diepoxide of limonene was hard to differentiate, hence, the synthesized mixture was compared with a commercial sample of limonene-1,2-oxide (Sigma Aldrich, USA). The samples were analyzed as in the previous case for 3-carene oxide and α-pinene oxide and the spectrum is given below. (Blue represents commercial sample and red represents synthesized mix of limonene mono and diepoxide).

Supplementary material for "Optimization of the lipase mediated process for the epoxidation of monoterpenes using the design of experiments – Taguchi method"



Supplementary material for "Optimization of the lipase mediated process for the epoxidation of monoterpenes using the design of experiments – Taguchi method"

3.2 A one pot reaction cascade of *in situ* hydrogen peroxide production and lipase mediated *in situ* production of peracids for the epoxidation of monoterpenes

In this publication, the AQ autoxidation process for the production of H_2O_2 was combined with the lipase mediated epoxidation process for the very first time. The coupling of the processes was made in a single pot and semi-batch fashion. The well established industrial AQ method was scaled-down and adapted for a laboratory scale synthesis. The path to producing monoterpene epoxide using this procedure consists of the following stages: the reduction of quinone followed by filtration of Pd catalysts and finally the combination of the oxidation step with the lipase mediated epoxidation reaction.

First, the working solution of toluene : ethyl acetate (60 : 40 v/v) was chosen to used to dissolve the quninones. Second, the amount of Pd/C catalyst was optimized using the OVAT approach and found to be 10 mol %. Third, the temperature for hydrogenation was finalized to be at 45 °C. Finally, the oxidation step and epoxidation step were combined at a particular temperature, *i.e.* 30 °C. In addition to determining the operation conditions of the process, the analytic method for following the AQ autoxidation was developed using GC-MS. Epoxidations were performed using ethyl acetate as the substrate/solvent and overall conversions of (82 ± 8) %, (76 ± 8) %, and (83 ± 9) % were achieved for 3-carene, α-pinene, and limonene, respectively.

The first author designed the complete process, conducted the experiments, calculations and analysis. The final version of the manuscript was the result of the contributions made by the co-authors.

A one pot reaction cascade of *in situ* hydrogen peroxide production and lipase mediated *in situ* production of peracids for the epoxidation of monoterpenes

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A one pot reaction cascade of *in situ* hydrogen peroxide production and lipase mediated *in situ* production of peracids for the epoxidation of monoterpenes

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ABSTRACT

In this work, the epoxidation of monoterpenes in the presence of *Candida antartica* lipase B (CALB) by the *in situ* generation of peroxy acid was combined with the industrial anthraquinone (AQ) process of hydrogen peroxide production. The reaction cascade consists of two major steps: reduction of an AQ to its corresponding anthrahydroquinone (AHQ) followed by the reverse auto-oxidation step of AHQ to AQ yielding equimolar amounts of hydrogen peroxide. Temperatures for each of the steps, ratio of substrate to catalyst, possible inhibition of lipases by the AQ and reaction medium (a mixture of hydrophobic and hydrophilic solvents) to be used were investigated. By using this reaction cascade, the addition of large amounts of hydrogen peroxide was avoided and conversions to epoxides of up to 83 (±9)% for limonene, 76 (±8)% for α-pinene and 82 (±8)% for 3-carene were achieved within 16 h.

1. Introduction

Epoxides are important intermediates in the chemical industry due to their reactivity. Generally epoxides can be synthesized by the addition of molecular or chemically bound oxygen such as: peroxy acids, hydrogen peroxide or halohydrins [1]. All these approaches are disadvantageous as unwanted side products such as hydroxy esters and diols are formed in addition to a surplus of acidic wastes [2,3]. Epoxides produced by the Prileschajew reaction involve the use of stoichiometric amounts of peroxy acid to the alkenes used and is generally carried out in organic solvents in the presence of a mineral acid, the general scheme of which is given in Scheme 1.

Different types of peroxy acids have been used to epoxidize alkenes, but most commonly: *meta*-chloroperbenzoic acid (*m*-CPBA) [4]. *m*-CPBA is a strong electrophilic reagent and thus highly reactive in the oxidation of alkenes, sulfides, selenides and amines; but a major drawback is the shock sensitivity and a detonative nature [5]. Hence, the use of high amounts of peroxy acids as starting material for such reactions is avoided; however desensitized or *in situ* generated versions of peroxy acids can be used. Industrially, they are synthesized by reacting hydrogen peroxide with carboxylic acids or carboxylic anhydrides or carboxylic acid chlorides [6]. Nevertheless, these processes require the addition of excess amounts of mineral acids and severe reaction conditions, which is a major disadvantage [7].

1.1. Lipase mediated epoxidation

In 1990, Fredrik Björkling and his co-workers were able to reproduce the Prileschajew reaction using lipases (glycerol ester hydrolases E.C. 3.1.1.3). Carboxylic acids (C_8–C_{16} aliphatic acids) when treated with hydrogen peroxide in organic solvents using lipases as catalysts formed the corresponding peroxy acids, which subsequently formed epoxides from their corresponding alkenes. This process is advantageous due to mild reaction conditions (temperature and pH) and avoidance of mineral acids [8]. Using this method, various alkenes including terpenes have successfully been epoxidized [8,9] and even Baeyer–Villiger oxidations on a range of ketones were achieved [10]. Hydrogen peroxide is needed in equimolar amounts for the epoxidation of alkenes and is consumed during the course of the reaction. On the other hand, adding large amounts of hydrogen peroxide at the start leads to potential inactivation of the lipase [8,9]. Moreover, in harsh environments, terpenes and its epoxides are rapidly rearranged to unwanted side products [11]. It has also been proven that with an excess of hydrogen peroxide, the tendency of the lipase mediated epoxidation system to produce secondary products other than the epoxide have already been documented for limonene [12] and for α-pinene [13].

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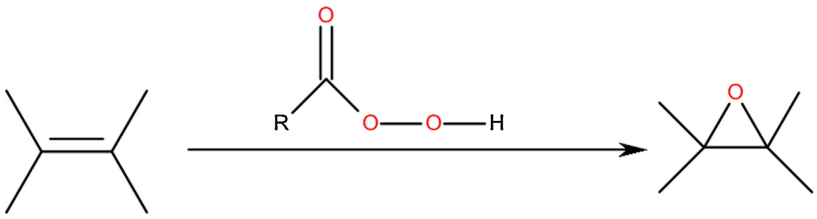


Scheme 1. Alkene epoxidation by Prileschajew method [4].

This can partly be overcome by stepwise addition of hydrogen peroxide to the reaction over a stipulated point of time as has been done previously [8,9] or by *in situ* generation of hydrogen peroxide.

1.2. Hydrogen peroxide production process

Out of the known processes to produce hydrogen peroxide, such as the traditional wet chemical process of L.J. Thenard (treatment of barium peroxide with nitric acid), the electrochemical process of Meidinger (1853) and Berthelot (1878) involving the electrolysis of sulphuric acid and the shell 2-propanol process (oxidation of secondary alcohols at 60 °C), we considered the anthraquinone (AQ) process [14]. The anthraquinone process consists of hydrogenation, oxidation, extraction of hydrogen peroxide and working solution treatment steps. The working solution generally consists of a hydrophobic solvent to dissolve the AQ like xylene, mesitylene or benzene and a hydrophilic one to dissolve the produced anthrahydroquinone (AHQ) in the form of alkylcyclohexanol esters, nonyl alcohols or alkyl phosphates. The AQ used is generally a 2-alkyl anthraquinone, for *e.g.* 2-ethyl anthraquinone, 2-*tert*-butylanthraquinone, or a mixture of any 2-alkyl anthraquinones. The hydrogenation step is carried out in the presence of a catalyst, typically palladium or nickel at a temperature of 40–50 °C, at the end of which, the catalyst has to be removed to prevent the decomposition of the hydrogen peroxide. The oxidation step is carried out at 25–60 °C and involves the supply of air or oxygen to the hydrogenated working solution to form AQ and equimolar amounts of hydrogen peroxide. The hydrogen peroxide is then extracted in a counter-current fashion to produce 30% by weight, further capable of concentration till 70%. The working solution devoid of impurities is then recycled to the hydrogenation tank for the repetition of the process [14,15].

1.3. Combination of hydrogen peroxide production with lipase mediated epoxdiation

There have been previous successful attempts in combining the *in situ* hydrogen peroxide production by photo-catalytic [16] or electrolytic means with enzymatic processes involving chloroperoxidase [17] or horse radish peroxidase [18] or using cofactor analogs [19]. The present work however, focuses on the combination of the classical chemical method of hydrogen peroxide production, *i.e.* the anthraquinone process with the lipase mediated epoxidation system (Scheme 2). In an initial reaction step, AQ is reduced to AHQ in the presence of a palladium catalyst at 45 °C for a time period of 6 h. Then the palladium catalyst is filtered off from the reaction system followed by another step, the oxygenation of AHQ to AQ at 25–30 °C which is then combined with the lipase mediated epoxidation system. In addition to checking the feasibility of such a process, a 323A variety of hydrophobic solvents (toluene, xylene and n-heptane), hydrophilic solvents (ethyl acetate, acetonitrile and 2-methyl 2-butanol), catalyst to substrate ratio (2.5, 5, 7.5 and 10%) and a variety of monoterpenes such as limonene, alpha-pinene and 3-carene were also tested.

2. Materials and procedures

2.1. Chemicals

2-Ethyl anthraquinone (EAQ), palladium on carbon (10% loading, matrix activated carbon support (Pd/C)), 3-carene, α-pinene and 2-methyl-2-butanol were purchased from Sigma–Aldrich Co. LLC. Toluene was purchased from Chem Solute, Germany. Ethyl acetate, acetonitrile and n-heptane were purchased from Carl Roth, Germany. Hydrogen peroxide (35%) was purchased from Avantomaterials, Netherlands. Lipase enzyme (CALB, 7500 TBU/g) was purchased from Chiral Vision, Netherlands.

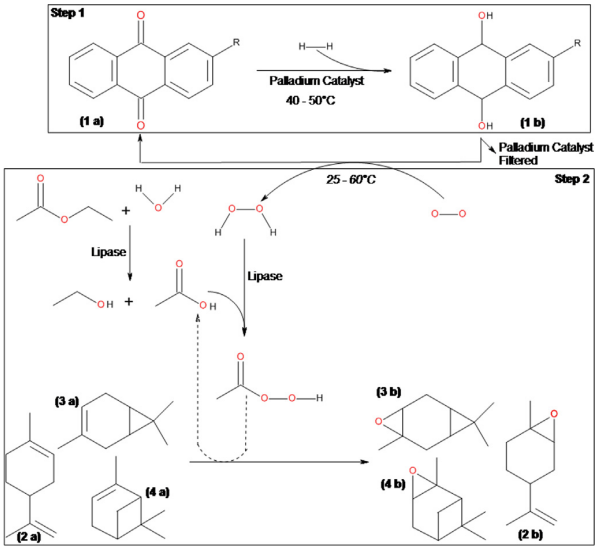
2.2. Typical reaction condition

For the final epoxidation system the following reaction conditions were used and all reactions were performed as duplicates.

Hydrogenation of anthraquinone (AQ): 1.67 mmol AQ, 10 mol% (palladium on carbon), hydrophobic solvent: hydrophilic solvent (60:40, v/v) total volume of 10 mL, hydrogen atmosphere, 45 °C and 6 h reaction time

Filtration of the palladium catalyst: The reactants from the hydrogenation step were filtered twice using a Whatman filter paper of pore size 0.21 μm.

Combined oxygenation and epoxidation reaction: Carried out using an open system under reflux with 0.5 mmol monoterpene



Scheme 2. Combination of the anthraquinone process of hydrogen peroxide production with the lipase mediated epoxidation of monoterpenes. (R: 2-ethyl and/or 2-*tert*-butyl) (1a – anthraquinone (AQ), 1b – anthrahydroquinone (AHQ), 2a – limonene, 2b – limonene oxide, 3a – 3-carene, 3b – 3-carene oxide, 4a – α-pinene, 4b – α-pinene oxide).

(α-pinene, 3-carene or limonene), 50 mg lipase, reaction time of 16 h, reaction temperature of 25–30 °C and 100 μL of distilled water was added to the reaction system.

Standard tests for lipase mediated epoxidation: 0.05 mmol monoterpene, 0.15 mmol hydrogen peroxide (35%), 30 mg CALB, 0.025 mmol carboxylic acid, reaction temperature – 60 °C.*tBAQ inhibition tests*: Varying concentrations of tBAQ (50 mM to 1 M), CALB 30 mg, 0.05 mmol monoterpene, 0.15 mmol hydrogen peroxide (35%), 0.025 mmol carboxylic acid.

Quantification of hydrogen peroxide concentration: The hydrogen peroxide concentration in the system was determined using peroxide quantification strips (Quantofix®). 5 μL of sample was added to a pre wetted Quantofix® strip and the amount of peroxide in the system was calculated accordingly depending on the intensity of color produced (provided by the supplier as a chart).

2.3. Analytics

The analytics of monoterpenes, their corresponding epoxides, AQ and AHQ were carried out on a gas chromatograph (GC-QP 2010, Shimadzu) coupled with an autoinjector (AOC-5000 by Jain, compipal) fitted with a mass spectrometer (GC-MS-QP2010 Plus, Shimadzu). A 30 m long BPX5 column with a diameter of 0.25 mm and thickness of 0.25 μm was used with helium as the carrier gas and a temperature profile as given below:

1. *Gas chromatography*: Start at 60 °C (hold 1 min), increase to 170 °C with 10 °C/min and then increase to 270 °C with 70 °C/min (hold 3 min).
2. *Mass spectrometer:* Ion source temperature of 200 °C, interface temperature of 250 °C.

The analysis was carried out using the software *GC–MS Postrun Analysis* provided by Shimadzu and the mass to charge ratio (*m*/*Q*) of all the compounds used in this study were compared to the database of *National Institute of Standard and Technology* (NIST) library; version 08. In order to avoid huge signals from the ethyl-acetate (solvent), a solvent cut of peak at 3.9 min was implemented with the help of the software.

The retention time for the different substrate and products are: 5.705 min for limonene, 7.282 min for limonene oxide, 4.392 for α-pinene, 6.735 for α-pinene oxide, 5.420 min for 3-carene and 7.217 min for 3-carene oxide. For the EAQ and tBAQ measurements on the GC the same method was followed with one alteration: the final holding time of 3 min at 270 °C was extended to 7 min. The retention times of the quinones and their hydrogenated products are as follows: 16.551 min for EAQ, 16.459 min for 2-ethyl anthrahydroquinone (EAHQ), 16.938 min for tBAQ and 16.861 min for *tert*-butyl anthrahydroquinone (tBAHQ).

3. Results and discussion

3.1. Optimization of the anthraquinone process

3.1.1. Choice of solvents

The first step in this reaction cascade was the hydrogenation of AQ in the presence of a palladium catalyst in a suitable reaction medium. The AQ/AHQ reaction medium should consist of a hydrophobic part to dissolve the AQ and a more hydrophilic part to dissolve the AHQ. Industrially, the use of benzene, methyl naphthalene or trimethyl benzene for the AQ system and alkyl phosphates, nonyl alcohols or tetra alkyl ureas for the AHQ system [14] would be recommended; however, in the interest of combining it with the lipase system, different solvent systems were investigated. The screening for the most suitable solvents were carried out with toluene, xylene and n-heptane as hydrophobic solvents and ethyl acetate, acetonitrile and 2-methyl 2-butanol as the hydrophobic solvents. The best solvent combination was selected based on the maximal amount of hydrogen peroxide produced at the end of the autooxidation process of AQ after 6 h of hydrogenation at 60 °C and 16 h of oxygenation at 50 °C. Results revealed that the initially targeted system containing ethyl acetate as acid donor for the lipase mediated peracid formation (see scheme 2), *i.e.* toluene:ethyl acetate (60:40, v/v) was the best combination for producing a surplus of hydrogen peroxide.

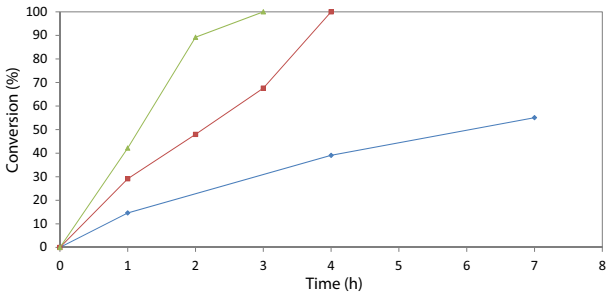


Fig. 1. Reduction of EAQ at different temperatures in 60% toluene and 40% ethyl acetate mixture in the presence of a Pd/C (10% loading) catalyst. (Diamonds – 30 °C, boxes – 45 °C and triangles – 60 °C).

3.1.2. Choice of anthraquinone substrates

Following the choice of the solvent system, the best substrate for the hydrogen peroxide production was to be chosen. 2-Ethyl anthraquinone (EAQ) and 2-*tert*-butyl anthraquinone (tBAQ) were selected [14,20] and their hydrogenation at three different operating temperatures (30, 45 and 60 °C) was tested. These moderate temperatures were chosen because of the fact that at temperatures above 60 °C, unselective hydrogenation of AQ substrates was expected [20], thereby affecting the yield of hydrogen peroxide. The conversion was quantified by GC–MS analysis and the ratio of substrate peak decrease and product peak increase. From Figs. 1 and 2, it can be seen that at low temperatures, the conversion tends to be less than 60% and for 45 °C and 60 °C, 100% conversion is achieved within 4 h in both cases.

Considering the energy input and the reaction time needed for 100% conversion of EAQ and tBAQ, 45 °C was chosen as the reaction temperature for hydrogenation and tBAQ was chosen as the suitable substrate.

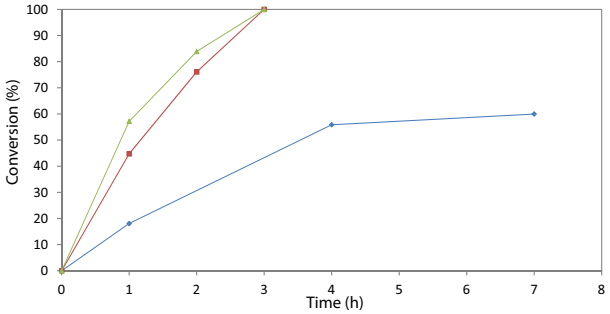


Fig. 2. Reduction of t-BAQ at different temperatures in 60% toluene and 40% ethyl acetate mixture in the presence of a Pd/C (10% loading) catalyst. (Diamonds – 30 °C, boxes – 45 °C and triangles – 60 °C).

3.1.3. Determination of best mol% of Pd-catalyst

The hydrogenations of tBAQ were all performed in a hydrogen atmosphere in the working solution of toluene: ethyl acetate (60:40, v/v) in the presence of a palladium catalyst. Four ratios, 2.5, 5, 7.5 and 10 mol% of Pd/C were tested at a reaction condition of 1.67 mmol tBAQ, (60:40, v/v) toluene:ethyl acetate, reaction temperature of 45 °C. The results were quantified based on the hydrogen peroxide produced at the end of oxygenation for 16 h at a temperature of 30 °C. Of the four ratios tested, 10 mol% yielded the maximum amount of hydrogen peroxide (more than 200 mg/L of peroxide) at the end of reaction time, while the other ratios yielded less hydrogen peroxide.

3.1.4. Oxidation step of the hydrogenated tBAQ

Before the oxidation step could take place it was mandatory to completely remove the palladium catalyst via filtration because trace amounts of the catalyst lead to quick decomposition of hydrogen peroxide in the oxygenation step [14]. This was evident from initial experiments where traces of palladium were still in the reaction solution upon oxygenation and hydrogen peroxide yields were very poor. The Pd/C was carefully filtered using Whatman filter papers of pore size of 0.21 μm and when all of the palladium catalyst was filtered off, the solution was reddish orange to dark brown in color which was then subjected to a non-catalytic oxidation step.

The oxygenation step is the penultimate step in the anthraquinone process where the hydrogenated tBAQ is converted to tBAQ and equimolar amounts of hydrogen peroxide. It was carried out in three different ways in the present work: by bubbling air through the system, maintaining the system under air atmosphere and by leaving the system open to air. Of all the three systems tested, the bubbling of air was the most successful way to produce surplus amounts of hydrogen peroxide. Leaving the system open to air was also successful, but with considerable loss of solvents as well. The air atmosphere (achieved by balloon filled with air) was considered unsuitable as solely trace amounts of hydrogen peroxide were produced and this was not feasible for the combination with the lipase system. Hence a combination of bubbling air through the system was chosen as the best possible way to carry out the oxygenation step of the AQ process. Of all the temperatures tested for the oxidation (25, 30, 45 and 60 °C), best results were obtained with 25–30 °C.

3.2. Combination of AQ process with lipase mediated epoxidation

The combined system was setup as mentioned in Section 2.2. Three different monoterpenes, limonene, 3-carene and α-pinene were tested for possible integration into the new combined system. Of all the substrates tested, limonene yielded a maximum conversion of 83 (±9%), 3-carene 82 (±8%) and α-pinene, a maximum conversion of 76 (±8%) (Fig. 3).

No side reactions such as rearrangement or oxirane ring opening were observed under these rather mild reaction conditions. Tests with these different monoterpenes and similar reaction conditions with commercial hydrogen peroxide (35%) yielded 100% conversion within 6 h at 60 °C (data not shown). In a previous work we showed that limonene was converted to diepoxides as well when using hydrogen peroxide in excess, with the ratio of monoepoxide to diepoxide in the range of 76.4:26.6%. However, in this work, no diepoxide formation was observed [12]. A possible inhibition of CALB by the tBAQ was suspected for the low conversion of the monoterpenes to their corresponding epoxides and hence, tests were done with varying amounts of tBAQ to check for inhibition and it was discovered that up to a concentration of 1 M tBAQ, there was no inhibition. Reaction time of 16 h was suspected to be too short since residual hydrogen peroxide was still present in the system (checked with hydrogen peroxide strips, Quantofix). However

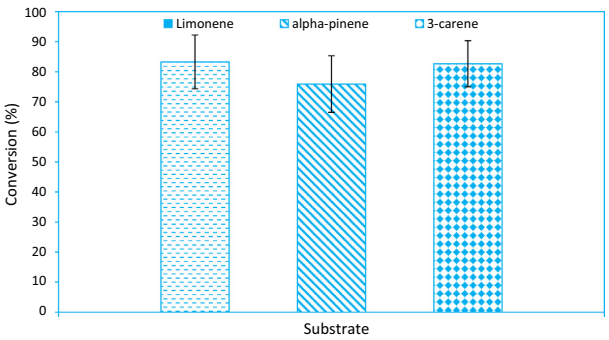


Fig. 3. Conversion of the various monoterpenes using the combined AQ process for hydrogen peroxide production and lipase mediated epoxidation.

extended reaction times up to 40 h, showed no higher conversion of the monoterpenes compared to 16 h. Most likely, the H_2O_2-peroxide concentration was below the critical minimum needed to exhibit lipase activity for the peroxy acid generation. The effect of acetic acid and ethanol set free from the enzymatic cleavage of ethyl acetate and water by CALB could also be a contributing factor for the incomplete conversion of the monoterpenes.

4. Conclusions and developments to be implemented

The industrial method of hydrogen peroxide production using the AQ process could be scaled down to be combined with the lipase mediated epoxidation system making this the first of its kind to the best of our knowledge. Safe operating conditions in terms of temperature (45 °C for hydrogenation and 25–30 °C for oxygenation) and prevention of harmful chemicals such as strong mineral acids and explosive peroxy acids is considered a huge advantage of this process. Moreover, no side product formation of terpenes and corresponding epoxides were observed. The drawback of enzyme grinding poses a great threat to the reusability of the enzymes which can be overcome by the use of our in-house designed "tea-bag" to hold the enzymes and avoid grinding effects and also making the downstream operations simple. However, the major drawback that needs to be solved is the problem of the incomplete conversion of monoterpenes – most likely by further increasing the H_2O_2 concentration, which should be easily accomplished by further optimization.

Acknowledgement

The technical assistance of Ms. Manuela Kaiser is greatly acknowledged.

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3.3 Development of Semi-Continuous Enzymatic Terpene Epoxidation: Combination of Anthraquinone and the Lipase Mediated Epoxidation Process

This publication explains the development of a semi-continuous process for the epoxidation of monoterpenes. The developed process consists of two parts: a H_2O_2 manufacturing part based on the industrial AQ process and the lipase mediated epoxidation part that utilizes the synthesized H_2O_2. The combination was made possible by adding a H_2O_2 reservoir in between the two processes.

Initially, the AQ autoxidation method of H_2O_2 was scaled-down to the laboratory scale (maximum working volume: 150 cm^3). As the first step in the development, the amount of Pd/Al_2O_3 was optimized using the OVAT approach. Following this, a stainless steel mesh container was designed in-house to retain the Pd/Al_2O_3 pellets within the container. This prevented the catalytic pellets from shear and grinding forces that accompanies a high mixing rate, thereby enhancing good contact of the reactants on the catalyst surface. Additionally, the catalysts were capable of reuse for upto five times due to the container. Moreover, this so-called "hybrid" reactor could combine the effects of a continuous stirred tank reactor (CSTR) and a packed bed reactor (PBR) in one. This process was able to produce H_2O_2 (up to 50 % weight by volume (w/v)) at high isolated yields and was stored in a reservoir at 4 °C. Following the successful scaling down of the AQ process, the H_2O_2 was used to epoxidize monoterpenes, *viz.*, 3-carene, limonene, and α-pinene. One run of this novel semi-continuous process lasted 8 h in total (5 h for H_2O_2 production and 2 h to 3 h for the epoxidation). Epoxides of 3-carene and α-pinene were obtained at isolated yields of (88.8 $\pm$ 2.8) % and (76 $\pm$ 8) %. Limonene oxides were obtained as mono (70 %) and di-epoxide (30 %) limonene mixture at an isolated yield of (71.5 $\pm$ 3.1) %. The new process has the potential to be scaled-up to industrial standards, which is the next step.

The complete process design, experiments, calculations, and analyses were designed by the first author. The second author helped in fine-tuning the final version and making the manuscript fit for publication.

Development of Semi-Continuous Enzymatic Terpene Epoxidation: Combination of Anthraquinone and the Lipase-Mediated Epoxidation Process

Sumanth Ranganathan and Volker Sieber

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Development of semi-continuous chemo-enzymatic terpene epoxidation: combination of anthraquinone autooxidation and the lipase-mediated epoxidation process†

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Lipase has been used for epoxidizing olefins such as monoterpenes for more than two decades. This epoxidation is accomplished by adding hydrogen peroxide (H_2O_2) to a carboxylic acid in the presence of a lipase such as *Candida antartica* lipase B (CALB) to produce percarboxylic acid, which then epoxidizes monoterpenes according to the Prilezhaev mechanism. One drawback of this process is the need for continuous addition of hydrogen peroxide to maintain maximum productivity. To overcome this hurdle, the industrial anthraquinone autooxidation process for hydrogen peroxide production was scaled down and coupled with lipase-mediated epoxidation in a semi-continuous manner. Palladium on alumina pellets (5% loading) was used as the catalyst for obtaining high yields of high-concentration hydrogen peroxide (50% weight by volume), followed by epoxidation of 3-carene, (+) limonene, and α-pinene. A total reaction time of 5 h was used for hydrogen peroxide production and 2–3 h for the epoxidation reactions. Pure 3-carene epoxide and α-pinene epoxide were obtained in isolated yields of 88.8 ± 2.8% and 83.8 ± 2.6%, respectively. Limonene epoxide was obtained as a mixture of mono- and di-epoxides in a ratio of 70% and 30%, respectively, with an isolated yield of 71.5 ± 3.1%.

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1 Introduction

Epoxides, also known as oxiranes, are cyclic ethers that are industrially significant owing to their high reactivity to form intermediates, which in turn form products of high value. The process is straightforward and involves the addition of a free or substituted oxygen atom to an olefin.[1] Epoxides can be produced using pure oxygen or air,[2–4] hydroperoxides,[5,6] hydrogen peroxide,[7,8] and peroxy compounds.[9] Industrially, oxygen- or ozone-based epoxidation is practiced as a gas-phase reaction in the presence of a metal catalyst for ethene,[10] propene,[11] and butene;[12,13] however, other olefins in the liquid state are seldom epoxidized in this manner. An alternative to this method is the Prilezhaev reaction, which uses peroxycarboxylic acid in stoichiometric amounts to perform epoxidations. Note that *meta*-chloroperbenzoic acid (*m*-CPBA) is the most commonly used peroxycarboxylic acid in these syntheses.[14] Generally, handling and cleaning issues coupled with the possibility of an explosion hazard make this process dangerous at industrial levels of production; therefore, *in situ* generation of peroxycarboxylic acid or slow addition of the compound is recommended. However, slow addition of peroxycarboxylic acid produces huge amounts of waste (equimolar to the amount of product); hence, *in situ* generation is preferred.[15]

Peroxycarboxylic acid can be generated *in situ* either chemically or enzymatically. Harsh reaction conditions, such as a strong mineral acid catalyst with carboxylic acid and hydrogen peroxide, are required to produce peroxycarboxylic acid. This leads to waste neutralization issues and unwanted side reactions that contribute to polluting processes, *e.g.*, formation of performic acid. Therefore, the enzymatic method reported by Björkling *et al.* is preferred.[16] The reaction schemes for both chemical and enzymatic means of epoxidation are depicted in Scheme 1. Ever since this report was published in 1992, Prilezhaev-based epoxidation that uses lipases has been the most preferred route for epoxidations[17–26] owing to its safer and simpler synthetic conditions.

However, as evident from the scheme above, lipase-mediated epoxidation has one major drawback, *i.e.*, the exhaustion of hydrogen peroxide, which limits the synthesis to a batch

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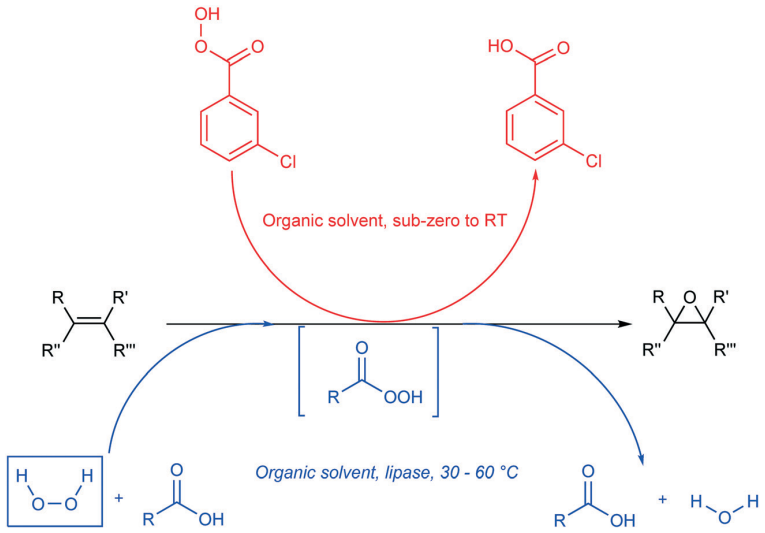


Scheme 1 Epoxidation of alkene (black) using the procedure of Prilezhaev (red) and Björkling *et al.* (blue).[16] The rectangular, dashed box implies that the compound was generated *in situ*. (R, R′, R″, and R‴ are functional groups present on the alkene).

process. This implies the need to add fresh hydrogen peroxide continuously or *in situ* generation using enzymatic,[27–30] electrochemical,[31–33] photocatalytic,[34–36] or chemical means (Schemes 3 and 4).[37–39] Ni *et al.* reported the use of an enzymatic cascade for producing H_2O_2 and CO_2 from methanol in combination with a peroxidase enzyme for the production of halogenated thymols.[40] Holtmann *et al.* used an electrochemical approach to generate H_2O_2 from the same reaction.[41] Churakova *et al.* used EDTA in the presence of light to efficiently generate moderate amounts of H_2O_2 from an aromatic peroxygenase in order to obtain aromatic phenols from their corresponding precursors.[42] In addition to these specific examples, several other works have used the combination of *in situ* H_2O_2 generation methods with reactions requiring H_2O_2.[43,44] One common trend in these processes is that they are excellent innovations for lab-scale applications when only low concentrations of hydrogen peroxide are required. The feasibility of the industrial use of these methods has not been reported so far or is pending investigation. In industrial applications, hydrogen peroxide is produced chemically using autooxidation[45] or direct H_2/O_2 (ref. 46) processes as well as using the 2-propanol oxidation process for a brief period.[47]

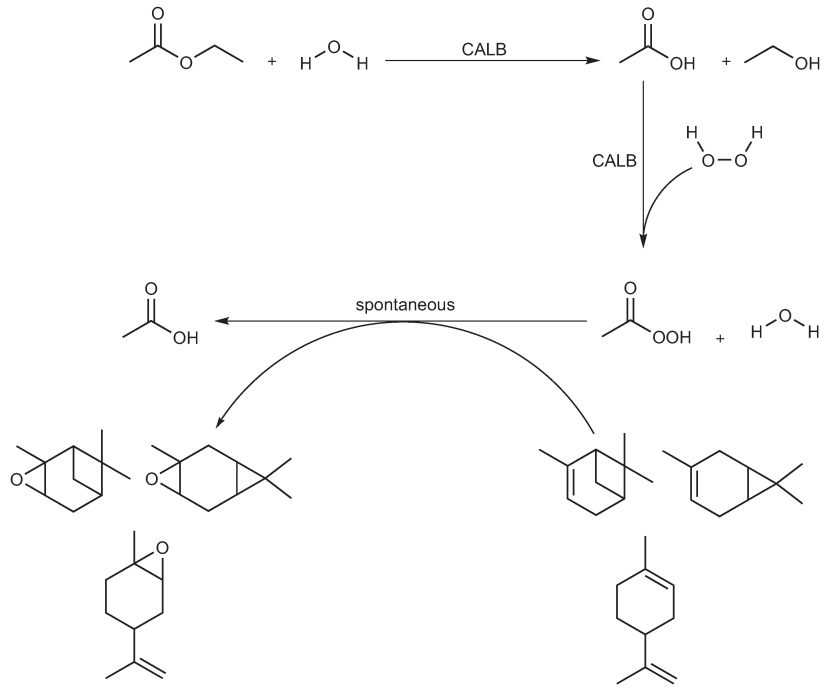
The focus of this work is the functionalization of renewable resources for the production of value-added fine chemicals. Terpenes are one such resource as they are naturally occurring hydrocarbons predominantly obtained from plants as ethereal oils. Terpenes comprise repeating isoprene units, which are susceptible to biological degradation and can be obtained as waste products from the paper and pulp industry.[48–50] Currently, these hydrocarbon reserves are combusted for energy production or used in paints and varnishes; however, functionalizing these terpenes would be beneficial for the fragrance, flavor, fine chemical, and of late, the polymer industry.[51–54] Recently, our research group reported a combination of the chemical anthraquinone process and lipase-mediated epoxidation,[55] the very first report on using such a combination for producing epoxides. Although innovative, the process could only be used for a single run, *i.e.*, a batch reaction. In this study, terpenes were chosen as the olefins to be epoxidized. This study also focuses on designing a semi-continuous epoxidation method for terpenes and developing a prototype process for the industry. We combined the processes of H_2O_2 production and epoxidation, which provided us better control on production as well as room for other reactions that require H_2O_2. H_2O_2 production was increased by optimizing the catalyst loading. The transfer of gaseous hydrogen to dissolved hydrogen in the working solution was enhanced using high mixing rates. Moreover, a stainless steel mesh container for the palladium catalyst was used to protect the palladium catalysts from shear forces associated with the high mixing rates. The combined effect of both should yield higher H_2O_2 yields. For epoxidation, ethyl acetate was used as the reaction solvent owing to its greenness and capability as a peroxy acid generator (Scheme 2).

2 Results & discussion

First, the anthraquinone-based autooxidation process was optimized to obtain maximum H_2O_2 production. Lipase-mediated epoxidation has already been optimized in our previous studies[24,25] and will be used herein with a single change in the reaction medium. Ethyl acetate was used in this work as opposed to toluene and deep eutectic solvents, which were used previously.

2.1 Optimization of palladium loading

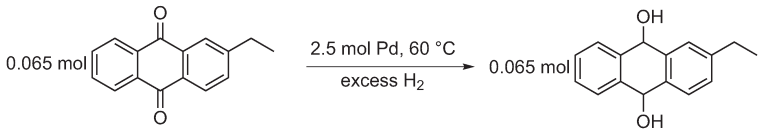
The first part of the developmental stage of the H_2O_2 production process was determination of the amount of palladium



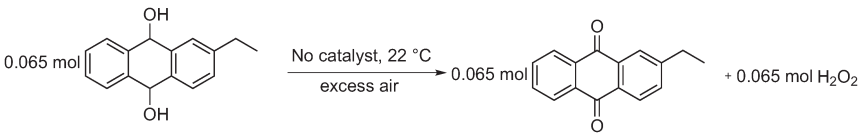
Scheme 2 Lipase-mediated (CALB) epoxidation of 3-carene, limonene and α-pinene in ethyl acetate.

required for the hydrogenation reaction in the anthraquinone process. Previously, we had determined that 10 mol% was necessary to successfully convert 2-ethyl anthraquinone (2-EAQ) to 2-ethyl anthrahydroquinone (2-EAH$_2$Q). The catalyst used previously was a fine commercial powder of Pd/C as opposed to Pd/Al$_2$O$_3$, which was used in this study.[55] Hence, optimization was performed using the one variable at a time (OVAT) strategy, wherein one of the parameters is varied whilst keeping the others constant. Here, the parameter of interest was the percentage of palladium used with respect to the amount of 2-EAQ used. Tests were performed by mixing 2 × 10^{-2} mol 2-EAQ with a 100 cm^3 working solution (60% mesitlene and 40% tributyl phosphate) at 250 rpm, as explained in section 4.2.1. The H_2O_2 concentration obtained at the end of the reaction was calculated using the ABTS assay, which has been described in section 4.3.1. The isolated yields obtained at the end of the run for various palladium-to-2-EAQ ratios are given in Table 1, which shows that 2.5 mol% yielded the best results for hydrogen peroxide production.

According to Table 1, a loading of 0.5 mol% did not produce any 2-EAH$_2$Q; as a result, no H_2O_2 was produced. Using 1.25 and 2.5 mol% resulted in yields of 94.3% and 97.2%, respectively; however, when using 5 and 10 mol%, the yields were less than 97%. This behavior could be the result of nonspecific hydrogenation of the aromatic rings of the 2-EAQ molecule, reduction of a single keto function of 2-EAQ, or dimerization of nonspecific products.[38]



Scheme 3 Reduction of 2-ethyl anthraquinone (2-EAQ) in the presence of 2.5 mol% palladium at 60 °C to produce 2-ethyl anthraqhydroquinone (2-EAH$_2$Q).



Scheme 4 Auto oxidation of 2-EAH2Q to 2-EAQ and hydrogen peroxide (H_2O_2) production at 22 °C in the presence of air.

Table 1 Isolated yields of H_2O_2 (50%) after one cycle of the anthraquinone process upon using 2×10^{-2} mol 2-EAQ with 60 cm^3 of mesitylene and 40 cm^3 of tributyl phosphate

S. no.	% Pd/Al_2O_3 : 2-EAQ (mol : mol)	Isolated yield (%)
1	0.5	—
2	1.25	94.3
3	2.5	97.2
4	5	93.8
5	10	90

Interestingly, when using scaled-up conditions, *i.e.*, 1.25 mol% Pd/Al_2O_3, 6.5×10^{-2} mol 2-EAQ, 60% mesitylene, 40% tributyl phosphate (working-solution volume of 150 cm^3), 60 °C temperature, and mixing at 250 rpm, there was a substantial reduction in the isolated yield of H_2O_2 (~75%). The reason for the decrease in the H_2O_2 yield was poisoning of the catalyst, as confirmed when the catalysts were transferred from the working solution to a glass beaker and left overnight in the fume hood to dry after washing with 2-propanol. The following day, yellowish crystals were visible on both the beaker and the catalyst, which we hypothesize was 2-EAQ. Increasing the washing steps with 2-propanol did not help with the removal of this impurity; however, such a behavior was not observed when using 2.5 mol% Pd/Al_2O_3 under scaled-up conditions. We presume that this is because of an appropriate ratio of Pd-to-2-EAQ was being used for the hydrogenation reaction. At the end of the reaction, a similar H_2O_2 yield was obtained. Hence, in the interest of scaling up, the decision to use 2.5 mol% of Pd/Al_2O_3 with respect to 2-EAQ was made.

2.2 Reusability tests for palladium

Once the palladium loading for H_2O_2 production was finalized, the next step was to check for the number of cycles for which the catalyst could be used. All tests were performed in triplicate. The first tests were performed without washing between runs. The H_2O_2 concentration was tested using the ABTS assay (section 4.3.1), and the results are given in Table 2. The reaction conditions used in this test are given in section 4.2.1.

According to Table 2, the amount of H_2O_2 decreased drastically over three cycles possibly because of catalyst inactivation, leaching of the palladium on account of the high shear and grinding forces associated with mixing, or residual reactants sticking to the catalyst. First, the catalyst was washed in an attempt to increase reusability. For this purpose, several solvents were screened, such as acetone, acetone : water (1 : 1 v/v), water, 2-propanol, and *n*-hexane : acetone (1 : 1 v/v). None of the solvents improved the H_2O_2 yield and are therefore not discussed here. A literature survey was conducted, and, finally, the washing protocol reported by Wang *et al.* in 2004 (ref. 56) was selected. The palladium on alumina catalyst was first washed with 15 cm^3 of ethanol and then with 15 cm^3 water; this process was repeated two times. A detailed account of the procedure can be found in section 4.2.2.

Table 2 Isolated yields of 50% H_2O_2 when performing the reusability test (in triplicate)

% Isolated H_2O_2 yield		
Run 1	Run 2	Run 3
87.5 ± 2.2	58.7 ± 4.3	18.5 ± 3.8

2.3 Use of a stainless steel mesh container to enhance Pd/Al_2O_3 reusability

Once the washing protocol was finalized, the washing process was performed again; consequently, the reusability of the catalyst was slightly higher (Fig. 2) than the previous tests performed (Table 2) without washing. However, the loss of activity over four cycles was still high. Catalyst pellets were recovered *via* filtration. During this step, the filter paper showed black spots of palladium particles, possibly resulting from leaching of the catalyst caused by shear forces or grinding operation of the magnetic stirrer.

To overcome this drawback, the hydrogenation step was repeated using a stainless-steel mesh container for Pd (Fig. 1). In this step, the catalyst was loaded into a stainless steel mesh designed in the form of a pouch. Upon performing hydrogenation using this setup, the loss of activity was minimal (Fig. 2). As shown in the figure, the experiment with the container that houses the catalyst yielded better results than the reaction without it. Unfortunately, there was a considerable loss in the activity of the palladium catalyst despite the washing and protection from shear and grinding forces; the reason for this could be due to the leaching of the catalyst from the support or the formation of intermediates during hydrogenations that could not produce H_2O_2. The exact reason needs to be investigated further.

2.4 Comparison of H_2O_2 production in the industry with that achieved this work

H_2O_2 is produced worldwide on an industrial scale exclusively *via* the anthraquinone autooxidation process.[57] One cycle of the optimized anthraquinone process comprises hydrogenation, catalyst separation, oxidation, and extraction/concentration steps.[37–39] Traditionally, a slurry-type reactor is used for the hydrogenation step,[57] as in the case of our previous study.[55] This leads to leaching of the palladium catalyst into the

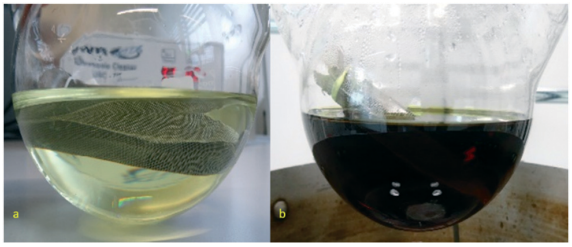


Fig. 1 Use of a stainless-steel container to reduce catalyst leaching and to increase the miscibility of hydrogen with 2-EAQ. a – 2-EAQ in the working solution before hydrogenation. b – 2-EAH2Q, the product of hydrogenation of 2-EAQ.

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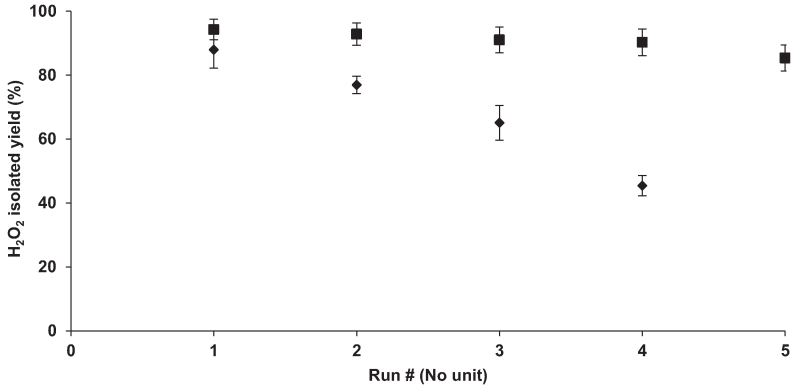

Fig. 2 Isolated H_2O_2 yields obtained using a stainless-steel container (squares) and that obtained without it (diamonds). Reaction conditions: 0.02 mol 2-EAQ, 65 cm^3 mesitylene, 35 cm^3 tributyl phosphate, and 2.5 mol% Pd/Al_2O_3 (5% loading).

working solution, thereby catalyzing the decomposition of H_2O_2. As a result, fixed-bed reactors are now used in the industry to avoid the tedious unit operation of filtration.[58] This study combines the advantages of both reactor types and presents an initial case of a "hybrid reactor" for this purpose. Pd/Al_2O_3 pellets are placed in a self-assembled stainless-steel container with a mesh size of 0.2 mm, which is advantageous for preventing any shear loss due to mixing whilst ensuring proper diffusion of reactants to the catalytic surface. The mixing of reactants in this work was performed at 1000 rpm, which ensures maximum mass transfer.

Step 1: reduction and inert gas sparging. Herein, 6.5×10^{-2} mol 2-EAQ was dissolved in 150 cm^3 of the working solvent in a 250 cm^3 round-bottom flask (hydrogenation chamber). The working solvent was yellow in color at this point. Then, 2.5 mol% Pd/Al_2O_3 (5%) pellets enclosed in a stainless-steel mesh container were added to this mixture. The reaction vessel was sparged with an inert gas to remove traces of air locked within the reaction vessel. Hence, argon was used for 2–4 min; alternatively, nitrogen gas could be used. Post sparging, a hydrogen atmosphere was maintained and the reactants were mixed at 1000 rpm and 60 °C in an oil bath for 5 h. In this study, a hydrogen balloon was used for this purpose; however, at a pilot-plant scale, a hydrogen feed line with a maximum pressure of 1–2 bar is required to avoid any nonselective hydrogenation of 2-EAQ.

Step 2: catalyst separation. After completion of hydrogenation, the palladium catalyst was separated from the reaction mixture by removing the stainless-steel mesh container containing the catalyst. The catalyst, along with the stainless-steel container, was washed according to the process explained in section 4.2.2. The working solution, which was dark red to brown in color at this stage, was transferred to a fresh 250 cm^3 round-bottom flask (sparge chamber). The liquid contents were sparged once again with argon for 2–4 min and then transferred into a fresh 250 cm^3 round-bottom flask (oxygenation chamber).

Step 3: oxidation. The oxidation step was performed by pumping air (maximum amount of 250 cm^3) through the dark red to brown working solution 15–30 min using a commercially available aquarium pump. The color of the solution was inspected visually. The oxidation step was prolonged until a yellow color was observed, after which the solution was transferred to a separating funnel. No catalyst was needed for the oxidation step.

Step 4: extraction with water. Following the oxidation step, H_2O_2 was extracted from the organic phase using a separating funnel with 4.4 cm^3 water to obtain a *ca.* 50% (weight by volume) mixture of H_2O_2. The organic phase was then pumped into the first vessel for the second round of reduction. The aqueous phase with hydrogen peroxide was stored in a reservoir vessel at 4 °C prior to use in the epoxidation process.

2.5 Development of the combined semi-continuous approach for the epoxidation of terpenes

A semi-continuous approach based on the combined anthraquinone process and a lipase process was used to epoxidize monoterpenes. The scheme of this setup is shown in Fig. 3.

First, the anthraquinone process was performed for hydrogen peroxide formation. It was followed by lipase-mediated epoxidation of monoterpenes. After 5 h, a membrane pump was used to transfer the working solution (dark red to brown) from the hydrogenation chamber to a sparging chamber. A flow rate of 100 $cm^3\ min^{-1}$ (maximum capacity of the pump) was used throughout this process for transferring liquids. The reduced working solution was then transferred from the sparging chamber to the oxygenation chamber, and, after oxidation for 15–30 min, the working solution (oxidized, yellow) was transferred to a separating funnel to which water was added for extraction. The working solution was then transferred to the hydrogenation chamber and the reaction was repeated.

The hydrogen peroxide solution produced using the anthraquinone autooxidation process (50% w/v) was collected in a reservoir until further use (Fig. 3). The epoxidation chamber comprised two parts: a reaction chamber and a purification chamber. The reaction chamber contained ethyl acetate, terpene, and CALB. To this mixture, an appropriate

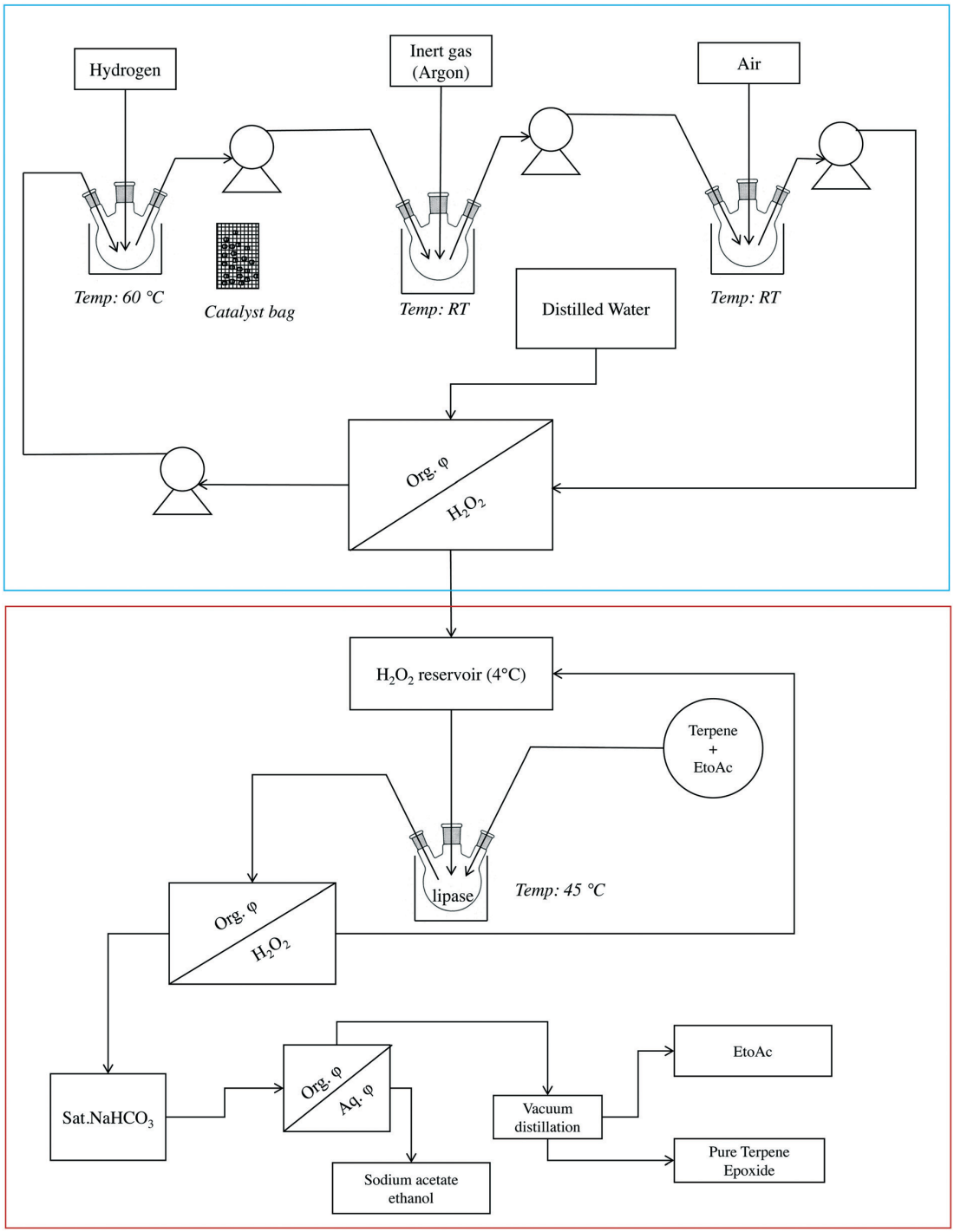

Fig. 3 Semi-continuous approach for combining the industrial anthraquinone process (blue-box contents) for hydrogen peroxide production and lipase-mediated epoxidation (red-box contents) of monoterpenes.

amount (with respect to terpene) of H_2O_2 was added to start the reaction, which was monitored by sampling at regular intervals and subjecting the samples to chromatography-mass spectrometry (GC-MS) analysis. After confirming 100% conversion of the starting material, the reaction components were transferred to a purification chamber and pure epoxide was obtained according to a protocol published previously.[24]

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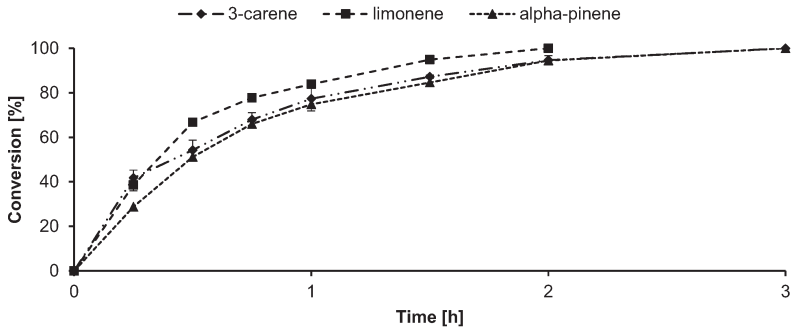

Fig. 4 Conversion profile of 5×10^{-3} mol reactant (3-carene – diamond; limonene – square; α-pinene – triangle), 7.5×10^{-3} mol H_2O_2 (50% w/v), and 0.1 g CALB in 25×10^{-3} L ethyl acetate at 45 °C.

2.6 Epoxidation results

Lipase-mediated epoxidation of monoterpenes, namely 3-carene, limonene, and α-pinene, was performed according to the procedure mentioned in section 4.2.4. Samples were withdrawn at regular intervals, and the conversion profile was followed using gas GC-MS, as reported previously.[55] The kinetics of the epoxidation of the three reactants is shown in the Fig. 4.

From the figure, it can be inferred that limonene (square) converted to its corresponding mono- and di-epoxide within 2 h and that 3-carene (diamond) and α-pinene (triangle) underwent full conversion within 3 h. It is to be noted that the limonene reaction was *ca.* 67% selective for the mono epoxide and 30% selective for the di-epoxide. Additionally, all three reactions yielded diols (≤3%) because of epoxide ring opening (ESI;† GC chromatograms and mass spectra). Comparing this result with that obtained using our previous system with toluene,[24] the reaction time reduced by a factor of 4 for limonene and 3.33 for 3-carene and α-pinene. The GC chromatograms and mass spectra are attached in the supplementary information. Comparing the results of the epoxidation in this study with the choline chloride : urea·H_2O_2 DES system[25] demonstrates that the reaction shows similar results of total turnover of reactants within 3 h.[25] The epoxides produced were then purified using a procedure developed previously.[24] Isolated yields of 88.8 ± 2.8, 71.5 ± 3.1, and 83.8 ± 2.6% were obtained for 3-carene, limonene, and α-pinene, respectively.

A prototype of this process was recently published by our research group.[55] Previously, a working solution of toluene and ethyl acetate (3 : 2 v/v) was used with a catalyst loading of 10 mol%. Additionally, a fine powder of commercially available palladium on carbon (Pd/C) was used as the catalyst. The use of Pd/C was problematic during the filtration step as fine particles passed through the filter, leading to multiple filtration steps. The oxidation step was coupled with epoxidation using CALB as a catalyst. Lipase converted ethyl acetate into ethanol and acetic acid, thereby restricting the process to be operated strictly in the batch mode. The process used in the present study overcomes all the challenges discussed above and is capable of being operated in a semi-continuous mode. The working solution was replaced by mesitylene : tributyl in a ratio of 1 : 1 (v/v). Although not essential, this was done to facilitate better solubility of 2-EAQ, 2-EAH_2Q, and hydrogen gas. Moreover, this liquid combination is one of the preferred solvents in the industry.[39] To overcome the issues related with filtration, palladium on alumina pellets was used so that the filtration step could be avoided; however, there were issues regarding catalyst leaching that need to be solved. To this end, a stainless-steel mesh container designed in-house was used. A minor change was made in the epoxidation part of the process by using an increased amount of terpene compared with the previous work.[55]

3 Conclusion

This study describes the coupling of chemical and enzymatic processes, *i.e.*, the anthraquinone autooxidation synthesis of hydrogen peroxide and lipase-mediated epoxidation. To the best of our knowledge, such a design is the first of its kind. We used a stainless-steel mesh container to prevent the shear and mechanical grinding forces, thereby enhancing hydrogenation reactions by employing high mixing rates. In other words, this setup is a combination of the continuous stirred tank reactor (CSTR) and a fixed-bed reactor, making it a hybrid reactor that incorporates the advantages of both. High mixing rates ensure maximum mass transfer, catalyst reuse of up to five cycles with minimal loss of activity, and low-temperature operation, which are innovations in hydrogen peroxide production. Owing to the combination of the two processes, there is the opportunity to use the hydrogen peroxide reservoir as feed for other reactions that require H_2O_2. Additionally, this combination gives the option of diluting H_2O_2 according to demand. Since lipase-mediated epoxidation has been studied exclusively for a variety of reactants, the range of this combined process is broad. Moreover, the conversion profiles of the three tested compounds suggest that compared with toluene, the time taken to achieve complete conversion in ethyl acetate was 4 times lower for limonene and 3.33 times lower for 3-carene and α-pinene. To

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summarize, we believe that this new semi-continuous approach can be scaled up to industrial standards with relative ease and extended to other olefins as well.

4 Materials and methods

4.1 Materials

2-Ethyl anthraquinone (2-EAQ) (Lot#: E12206-100G, 97% purity), mesitylene (Lot#: M7200-500ML, 98% purity), tributyl phosphate (Lot#: 158615-1L, 97% purity), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonsäure diammonium-salz (ABTS) (Lot#: A1888-2G, 98% purity), peroxidase from horseradish (HRP) (Lot#: P6782-50MG), 950–2000 units per mg by ABTS assay), α-pinene (Lot#: 147524-250ML, 98% purity), and (+) 3-carene (Lot#: 115576-1L, 90% purity) were purchased from Sigma-Aldrich. Palladium on alumina (Pd/Al_2O_3) pellets were obtained from VWR Chemicals (Lot#: 41825.06, 5% loading). (+) Limonene (Lot#: 179395000, 96% purity) was purchased from Fischer Scientific. Potassium phosphate buffer (KPi) (1×10^{-4} mol L^{-1}, pH 5.0) was prepared in the laboratory according to standard buffering procedure and used as such. Ethanol (Lot#: T171.2-25L, 96% purity) was purchased from Carl Roth. *Candida antartica* lipase B (CALB) was obtained from c-LEcta (Lot#: 20606-4, 17 000 PLU g^{-1}). LC-MS grade ethyl acetate (EtoAc) was obtained from Th. Geyer (Lot: 2278-1L, 99.95% purity). Disposable cuvettes (Ref. #: 67.742, polystyrene material, $10 \times 4 \times 45$ mm^3) were bought from Sarstedt. Membrane pumps (KNF SIMDOS®10) fitted with a PTFE membrane to specifically handle organic solvents were used. Tygon® F-4040-A tubing (Lot#: 224-0525, inner diameter 3.2×10^{-3} m, outer diameter 6.4×10^{-3} m, thickness 1.6×10^{-3} m) was purchased from VWR Chemicals and used for liquid transfer. A UV-Vis spectrophotometer from Shimadzu (UV-1800) was used to measure the absorbance during the ABTS assay. A stainless-steel mesh (wire diameter 0.12 mm; mesh size 0.20 mm) was purchased from Metallwaren-Riffert, Austria.

4.2 Synthetic methods

4.2.1 Optimization of the palladium catalyst for hydrogenation of 2-EAQ. First, the amount of palladium for the reduction of 2-EAQ was determined. For this purpose, the following parameters were kept constant: 2×10^{-2} mol 2-EAQ, 0.1 L yellow working solution (three volume equivalents mesitylene and two volume equivalents tributyl phosphate), hydrogen atmosphere, 60 °C temperature, and mixing at 250 rpm. For the palladium catalyst, prior experience suggested using 10 mol% of 2-EAQ for optimum results.[55] Nevertheless, tests were conducted using 0.5, 1.25, 2.5, 5, and 10 mol%. The reaction was run for 5 h, after which the catalyst was removed *via* filtration. The working solution, which was dark red to brown at this stage, was oxidized using air from an aquarium pump at maximum capacity for 15–30 min at 20–22 °C; 10 cm^3 double deionized distilled water was used to extract H_2O_2. For scaled-up reactions, water was added accordingly to obtain a 50% (w/v) solution of hydrogen peroxide. The H_2O_2 obtained was quantified using the ABTS assay.

4.2.2 Washing protocol for palladium catalysts. The catalysts were washed according to the procedure reported by Wang *et al.* in 2004.[56] That is, 15 cm^3 of ethanol was added to the catalysts in a freshly washed and cleaned beaker. Then, the catalysts were immersed in this beaker and mixed for 30–60 s. There should be no spillage of contents during this time. Ethanol was then discarded and replaced with 15 cm^3 of double deionized distilled water, and the solution was remixed for 30–60 s. The water was then discarded. This procedure was repeated two times. The wet catalyst was then dried using an inert gas (argon). Alternatively, nitrogen gas could be used. The dry and clean catalyst was then used for the hydrogenation of 2-EAQ. The same procedure was followed for the stainless-steel mesh container.

4.2.3 Application of the in-house-designed stainless-steel mesh container to enhance catalyst lifetime. After the optimization step, the reusability of the Pd/Al2O3 catalyst was tested. To establish the reusability, two tests were conducted. The first test involved adding the catalyst directly to the working solution (*i.e.*, 60% mesitylene, 40% tributyl phosphate, and 2-EAQ). In the second test, a stainless steel mesh container designed in-house was used to shield the catalysts from any shear or grinding forces associated with mixing. The reaction was run until hydrogen peroxide was produced and quantified. The catalysts were then washed properly, as mentioned in the previous section (washing protocol). The washed catalysts were then used for a second time and the process was repeated. The peroxide content was measured and compared with that obtained in the previous run for the reactions with and without the stainless-steel mesh container. Reaction conditions: 0.02 mol 2-EAQ, 100 cm3 working solution (60% mesitylene and 40% tributyl phosphate), 60 °C reduction temperature, and 22–23 °C oxidation temperature. A hydrogen atmosphere was maintained in the vessel using a balloon filled with hydrogen gas. The working solution was oxidized using an aquarium pump.

4.2.4 Lipase-mediated terpene epoxidation. Lipase-mediated epoxidation of monoterpenes was performed using 25 cm^3 EtAc. 5×10^{-3} mol monoterpene (3-carene, limonene, and α-pinene), 7.5×10^{-3} mol H_2O_2 (50%) from the reservoir, and 0.1 g CALB for the reaction. The reaction temperature was set at 45 °C, and mixing was controlled at 250 rpm using a magnetic stirrer. Sampling (0.002 cm^3 of the reaction mixture was dissolved in 0.998 cm^3 EtoAc) was performed regularly at 15, 30, 45, 60, 90, 120, and 180 min. Shortly before hydrogen peroxide was added, a sample was taken. Conversion was performed using GC-MS, as explained previously.[24,55] The products were purified using a procedure described previously;[24] subsequently, isolated yields were calculated.

4.3 Analytical methods

4.3.1 ABTS assay for H_2O_2 detection. The amount of H_2O_2 produced by the process was determined using the ABTS

assay.[59] For this purpose, the reagents needed for the assay were first prepared; 2×10^{-3} mol L^{-1} ABTS (in 0.1 mol L^{-1} potassium phosphate buffer, KPi, pH 5.0) and 5×10^{-3} g L^{-1} HRP was prepared fresh in appropriate amounts prior to use. The ABTS assay was performed as follows: a 1×10^{-3} L ABTS (colorless) solution was pipetted into a standard cuvette, followed by the addition of 0.1 cm^3 of the sample (typically H_2O_2; water for blank) and 0.1 cm^3 of the HRP enzyme. This mixture was pipetted up and down several times to ensure sufficient mixing of reactants. This mixture was left undisturbed at 22 °C for 10 min. The absorbance of this (green) solution was measured at 405 nm using a UV-Vis spectrophotometer. The concentration of H_2O_2 was determined based on the calibration curves obtained prior to the analyses.

4.3.2 Analysis of terpenes and terpene epoxides by gas chromatography-mass spectrometry (GC-MS). For the detection of the target compounds, *i.e.* terpenes and their corresponding epoxides, a GC-MS fitted with an autoinjector was used. Details of the equipment are:

- GC: QP 2010, Shimadzu.
- Autoinjector: AOC-5000 by Jain, Compipal.
- MS: GC-MS QP2010 Plus, Shimadzu).

A 30 m long BPX5 with dimensions of 0.25 mm diameter and 0.25 μm thickness was used as the GC column. Helium was used as the carrier gas at a flowrate of 13.2 ml min^{-1}. Temperature profile used for GC and MS for optimal separation of compounds was:

i. Gas chromatograph: start at 60 °C and hold the temperature for 1 minute. Increase the temperature at the rate of 10 °C min^{-1} until 170 °C, after which the temperature was further increased to 270 °C at the rate of 70 °C min^{-1}. This temperature was then held for 3 minutes.

ii. Mass spectrometer: the ion source temperature was 200 °C and the interface temperature was maintained at 250 °C.

The software program "GC-MS Postrun Analysis" from Shimadzu was used to analyze the reaction components and the mass to charge ratio (m/Q) ratio were compared to the database of National Institute of Standard and Technology (NIST) library; *version 14*. Ethyl acetate was used as the solvent and to avoid huge signals form this compound, a solvent cut was introduced at 3.9 min with the help of the software. All GC-MS chromatograms obtained for 3-carene/epoxide, limonene/mono- and di-epoxide, α-pinene/epoxide and octanoic acid are given in the supplemental information.

Conflicts of interest

There are no conflicts to declare.

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3.4 Development of a lipase-mediated epoxidation process for monoterpenes in choline chloride based deep eutectic solvents

The following publication is the first account of a lipase mediated epoxidation process carried out exclusively in DES as the reaction medium. This process was developed to fit into the guidelines of green chemistry, as the original process used toluene that is harmful to the environment and the operator alike. The synthesis of DES requires a quaternary ammonium salt such as choline chloride (ChCl) and a suitable HBD. After two screening rounds, glycerol : choline chloride (GlCh) in the molar ratio of 1 : 2 and sorbitol:choline chloride (SoCh) in the molar ratio of 1 : 1 yielded liquid mixtures that could epoxidize 3-carene as a model substance. To further develop the process in these two reaction media, a DoE using the Taguchi design of crossed arrays was used to optimize 4 parameters (inner array) and 1 parameter (outer array).
Both the GlCh and SoCh yielded complete turnover of the educts within 8 h. A purification procedure was designed to obtain pure epoxides using two different ways– using *n*-hexane and ethyl acetate/water. An impurity in the form of caprylate esters of glycerol and sorbitol (1 - 2.5 %) was identified during the analysis of the purified product; this prompted the utilization of a novel "minimal" DES as the reaction solvent.
This minimal DES system consisted of ChCl : U·H_2O_2, which could be used as the co-substrate and HBD at the same time, hence the name. This system was more efficient than the GlCh and SoCh systems as a reaction time of 2 h was required to completely convert 3-carene and 3 h was required to epoxidize limonene and α-pinene. Owing to the impurity in the GlCh and SoCh systems, isolated yields were not determined. However, the final isolated yields obtained using the minimal DES system were (87.2 ± 2.4) %, (77.0 ± 5.0) %, and (84.6 ± 3.7) % for 3-carene, limonene, and α-pinene, respectively.
The first author designed the whole process, decided on the optimization procedure, performed the calculations, and analyses. The first author also conducted experiments in collaboration with the second author. The other co-author contributed to the content and language of the manuscript.

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Development of a lipase-mediated epoxidation process for monoterpenes in choline chloride-based deep eutectic solvents†

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Chemical syntheses in contemporary process industries today are predominantly conducted using organic solvents, which are potentially hazardous to humans and the environment alike. Green chemistry was developed as a means to overcome this hazard and it also holds enormous potential for designing clean, safe and sustainable processes. The present work incorporates the concepts of green chemistry in its design of a lipase-mediated epoxidation process for monoterpenes; the process uses alternative reaction media, namely deep eutectic solvents (DESs), which have not been reported for such an application before. Choline chloride (ChCl), in combination with a variety of hydrogen bond donors (HBD) at certain molar ratios, was screened and tested for this purpose. The process was optimized through the design of experiments (DoE) using the Taguchi method for four controllable parameters (temperature, enzyme amount, peroxide amount and type of substrate) and one uncontrollable parameter (DES reaction media) in a crossed-array design. Two distinct DESs, namely glycerol : choline chloride (GlCh) and sorbitol : choline chloride (SoCh), were found to be the best systems and they resulted in a complete conversion of the substrates within 8 h. Impurities (esters) were found to form in both the DESs, which was a concern; as such, we developed a novel minimal DES system that incorporated a co-substrate into the DES so that this issue could be overcome. The minimal DES consisted of urea·H_2O_2 (U·H_2O_2) and ChCl and exhibited better results than both the GlCh and SoCh systems; complete conversions were achieved within 2 h for 3-carene and within 3 h for both limonene and α-pinene. Product isolation with a simple water/ethyl acetate based procedure gave isolated yields of 87.2 ± 2.4%, 77.0 ± 5.0% and 84.6 ± 3.7% for 3-carene, limonene and α-pinene respectively.

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1 Introduction

The utilization of renewable resources is one of the twelve principles of green chemistry[1–3] and in addition to the improved sustainability afforded by using renewables, the molecules obtained offer novel functionalities. Recent examples of this include the synthesis of new bio-based materials such as polycarbonates from terpenes[4] or furanic polyesters from sugars.[5] Mere utilization of renewable feedstock, however, is not necessarily a more sustainable practice than using non renewable ones as it is still important that also other rules of green chemistry are followed; for example, green chemistry requires toxic and harmful chemicals to be used only sparingly, if at all, in chemical processes.[1–3] To achieve this, solvent-free synthesis[6–8] or "green" reaction media such as supercritical (SC) fluids[9,10] or ionic liquids (IL)[11–13] can be used. However, these systems often are impractical for chemical synthesis.[7,9,13] An alternative approach is the use of deep eutectic solvents (DESs).[14–16] A variety of chemical reactions that use DESs as reaction solvents have already been reported with subject areas ranging from electrochemistry[17,18] and organic syntheses[19–21] to enzymatic reactions.[22–25]

A good source of renewable feedstock is the secondary plant metabolite called terpenes that are accumulated in large quantities as by-products in the pulp, paper and fruit industries.[26,27] Terpenes are excellent precursors for the flavor, fragrance and fine chemical industries in either a functionalized or a non-functionalized form. A specific functionalization,

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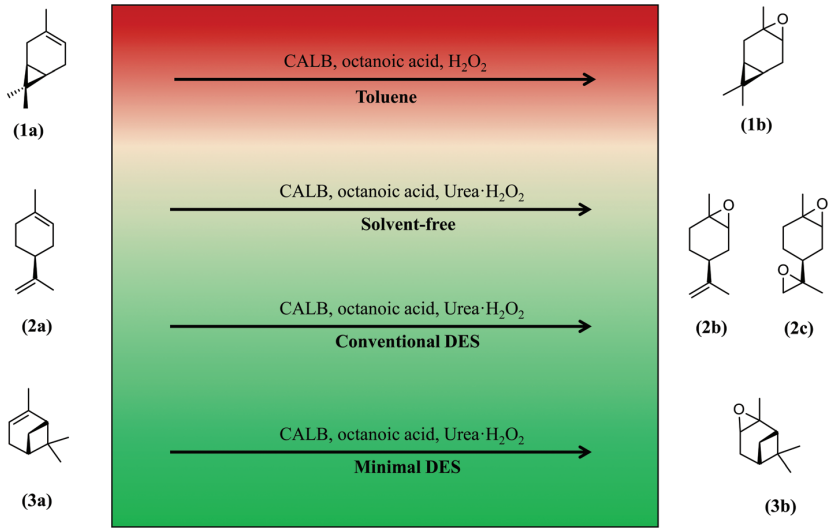


Scheme 1 Development of the lipase-mediated epoxidation process for various reactants (**1a–3a**) and their corresponding epoxides (**1b–3b**), starting with the "ungreen" process utilizing toluene and moving on to greener processes utilizing deep eutectic solvent (DESs) and solvent free conditions.

namely epoxidation, is instrumental in making terpene epoxides useful precursors for the production of diols, alcohols, ketones and as of late, monomers for polymers.[4,28–32] Epoxides can be produced by different chemical means, including enzymatic approaches.[33–41] Björkling *et al.* pioneered work on enzyme-mediated epoxidations in 1992 [41] when they used a lipase (*Candida antartica* lipase B (CALB)) in the presence of an organic solvent, carboxylic acid and aqueous hydrogen peroxide (H_2O_2) to form peroxycarboxylic acid, which was able to epoxidize alkenes through the Prilezahev reaction.[39] It is important to note that the enzyme does not catalyze the epoxidation itself but provides efficient *in situ* formation of the oxidizing species, *i.e.* the peroxycarboxylic acid.

Previously, we had used this technique[41] to develop and optimize a lipase mediated epoxidation process for monoterpenes.[42] Although this process adhered to some principles of green chemistry, in that it utilized renewable reactants and enzymes as catalysts; we also used toluene as the reaction medium; which means the process cannot be considered as "green".

This paper focuses on the development of a more sustainable or "greener" monoterpene epoxidation process that adheres to the principles of green chemistry. To the best of our knowledge, this is the first account that uses DES as solvent for lipase mediated epoxidation of monoterpenes. To begin with, we tested an enzyme-mediated process both under solvent-free conditions and then in DESs as the reaction medium. After comparing these two approaches, we developed the process and optimized it for the DES system. The process development stages consisted of two screenings, an optimization by design of experiments (DoE) – Taguchi method, purification stage and a final scale-up phase. We also wanted to examine how using a DES as the reaction solvent affected the outcome (*i.e.*, yield of the epoxide) of the process as well as how other reaction parameters (*i.e.*, substrate type, enzyme amount, temperature of reaction and hydrogen peroxide) affected the outcome. We were subsequently able to develop a novel DES mixture that could act as both the solvent and the co-substrate source in fast and efficient epoxidations (Scheme 1).

2 Results and discussion

2.1 Solvent free epoxidation system

The initial test of the solvent-free synthesis was performed using only terpene (3-carene (**1a**), limonene (**2a**) and α-pinene (**3a**)), octanoic acid, a peroxide source (aqueous (aq.) or urea (U)·H_2O_2) and CALB, as specified in section 4.2.1.1. There were two distinct phases: a top organic phase that contained both the monoterpene and octanoic acid and a bottom phase containing H_2O_2. A single point measurement at the end of 16 h revealed that 0.1 mmol 3-carene (**1a**) was totally converted to its corresponding epoxide (result not shown). Based on this result, we scaled up the process using a greater amount of the reactants at 45 °C and 60 °C. The results are shown in Fig. 1.

At 45 °C (Fig. 1(a)), conversions of 53.0 ± 0.8%, 46.6 ± 1.8% and 13.8 ± 0.1% were achieved after 20 h for **1a**, **2a** and **3a** respectively. A second reaction was carried out using identical conditions, but the reaction temperature was increased to 60 °C. At this temperature (Fig. 1(b)), a conversion of 100% was seen for **1a**, whereas **2a** and **3a** yielded 82.8 ± 2.2% and 5.5 ± 1.2%, respectively. When the reaction time was extended to

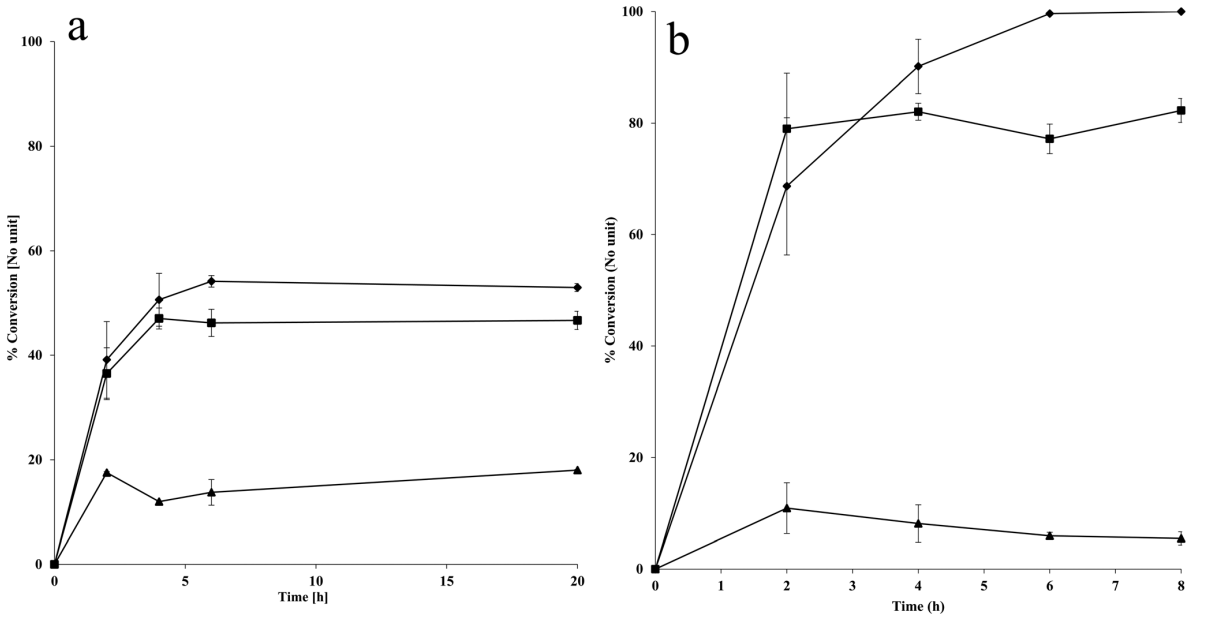


Fig. 1 Conversion profile of 3-carene (diamond), limonene (square) and α-pinene (triangle) at 45 °C (a) and 60 °C (b). (Reaction conditions: 10 mmol monoterpene, 12.5 mmol aq.H_2O_2, 2.5 mmol octanoic acid, 100 mg CALB (1670 PLU), 500 rpm.)

20 h, no increase in the conversion of **2a** could be achieved. Only when adding fresh enzyme after 6 h an increase in conversion was observed (data not shown), implying that inactivation of the enzyme, possibly due to the high amount of octanoic acid in the reaction medium was limiting.

More interestingly, the results of this experiment are different from those obtained in our previous work, which had implied that the best results for the lipase epoxidation of monoterpenes in toluene were to be achieved at a reaction temperature of 45 °C.[42] Our present results also indicate that the sequence in which the three substrates were oxidized, at both temperatures, was different from that reported by Bakhvalov *et al.* in 2008.[43] The present work suggests that the oxidation follows the order **1a** > **2a** > **3a** rather than **1a** > **3a** > **2a**. However, the published findings are for solvent-based oxidation reactions, which have better heat and mass transfer conditions than solvent-free reactions. Additionally, each substrate behaves differently as a solvent, which could have caused the variation in the oxidation pattern. Furthermore, the results in both of the previously published studies[42,43] were obtained using optimized conditions as opposed to the single variable change technique used in the present study.

As mentioned earlier, two distinct phases were observed when aq. H_2O_2 was used. Suspecting that the water content may have interfered with the reaction, we conducted a third test using three different temperatures and U·H_2O_2. On mixing all the reactants together, a single solid phase was obtained. After 20 h, a single point measurement was made and conversion of the monoterpenes was calculated using gas chromatography-mass spectrometry (GC-MS), as shown in Fig. 2.

Conversions of 85.3 ± 10.4%, 73.4 ± 7.9% and 84.5 ± 14.4% were obtained for **1a** and 86.2 ± 4.8%, 62.2 ± 1.8% and 75.8 ± 7.2% for were obtained for **2a** at 40, 50 and 60 °C, respectively after 20 h. There was no conversion of **3a** at any of the three tested temperatures. On a closer observation of Fig. 2, it can be seen that the results of the epoxidations at 40 and 60 °C were identical for **1a**, but not for **2a**. However, an interesting phenomenon can be seen at 50 °C, for which the conversion was the lowest. This may have occurred due to the difference in the solubility of the octanoic acid in each of the substrates (**1a–3a**).

Both the systems, *i.e.* aq. H_2O_2 and U·H_2O_2, exhibited incomplete conversions of the starting materials (**1a–3a**) with the exception of the reaction at 60 °C using aq. H_2O_2 for **1a**. If a process has to be developed so that maximum conversion is obtained for all three substrates, optimization using, for example, the DoE approach can be carried out. However, each substrate would act as its own solvent and any optimization will be useful only for that particular substrate. Additionally, both the cases (*i.e.* aq. H_2O_2 and U·H_2O_2) had issues in terms of both handling and reproducibility (as evidenced by the high error percentage of the tests); therefore, we shifted our focus from a solvent free system towards utilizing green reaction media, namely DESs.

2.2 Conventional DES, first screening round

Because of the drawbacks experienced on using the solvent-free system (section 2.1), DESs were chosen as a "green" alternative to carry out the lipase mediated epoxidation reactions. To determine the best DES candidates, a two-step

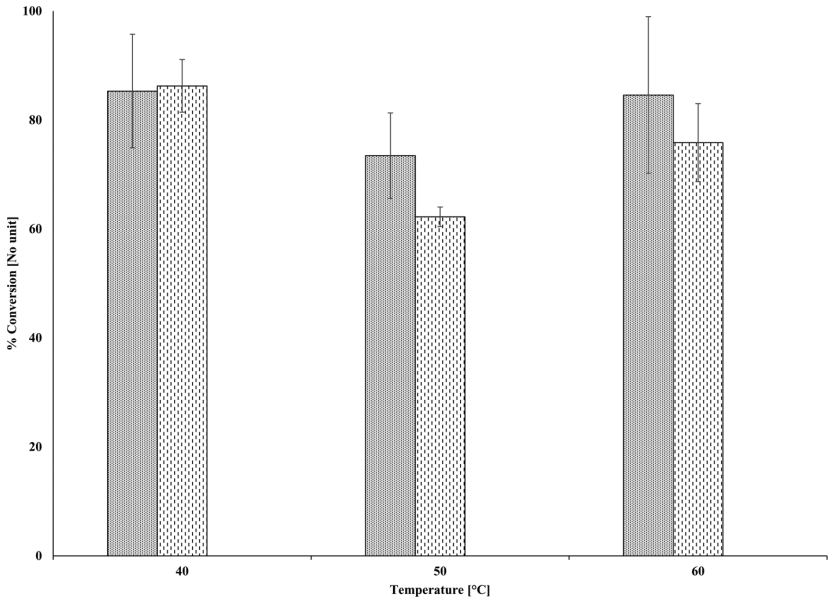


Fig. 2 Conversion of 3-carene (dotted) and limonene (vertical dashes) obtained using U·H_2O_2 under solvent-free conditions at 40, 50 and 60 °C after 20 h. (The reaction conditions were 2 mmol monoterpene, 2.5 mmol U·H_2O_2, 0.5 mmol octanoic acid, 100 mg CALB (1670 PLU), 500 rpm.)

screening approach was used: an initial screening step to evaluate the fluidity of the selected DES reactants and a second step was performed to evaluate their epoxidation of **1a**. The mixtures that were assumed to be suitable for the epoxidation process were chosen from the list published by Russ and Koenig.[15] The DES mixtures were prepared in ratios described in Table 2 according to the procedure described in section 4.3. All the mixtures in Table 2 had been previously described by Russ and Koenig[15] apart from the 4-hydroxy phenyl acetic acid (HPA) and ChCl mixture (Table 2, #3). This particular chemical (HPA) was chosen because phenylacetic acid was described in the same work as having a melting point of 25 °C in the same molecular ratio. Because both the chemicals are similar in structure but for the presence of an additional OH group, this mixture was tested to see if a new DES mixture could be formed in a similar temperature range.

From previous experience it has already been established that 45–60 °C is the ideal temperature for performing lipase based epoxidation reactions.[41,42,44] Hence, only those DESs that were liquids at 60 °C were selected for the second round of screening. The DES mixtures from Table 2 were heated to 100 °C and cooled to 60 °C before the samples were visually examined for fluidity; the results are given in Table 2. HPA, L-(+)-tartaric acid, L-glutamic acid and D-glucose in combination with ChCl did not yield a liquid at 60 °C and malonic acid with ChCl (1 : 1) only yielded a very viscous liquid that could not be stirred. As a result, these mixtures were not used for the epoxidation reaction.

Literature[15] suggested that the selected mixtures are supposed to yield liquids at temperatures much lower than those tested in this work. Meng *et al.* suggested that the presence of moisture can interfere with the hydrogen bonding between DES components (urea and ChCl), which would lead to increased melting temperatures.[45] Working on the assumption that this phenomenon could be extended to other DES mixtures, the individual DES components (that were not liquid) were dried under vacuum and tested again; no changes in their behaviors were observed. Since the DES mixtures mentioned above (HPA, tartaric acid, glutamic acid and D-glucose in combination with ChCl) did not form liquids and this step was a mere screening round, they were omitted from the second round of screenings and were not investigated further.

2.3 Conventional DES, second screening round

The eight successful liquid DES mixtures from the previous screening round were screened for epoxidation activity, as described in section 4.4. The conversions after 24 h of reaction time are given in Table 2. It can be inferred that the sugar and sugar alcohol systems were the ones that performed best. For the carboxylic acid systems (Table 2, #1 & 2), no additional peroxy acid generator, *i.e.* octanoic acid, was added. These two reactions yielded minimal conversion, which may have been due to the polar nature of both these acids, as the polarity of a carboxylic acid increases with a decrease in its aliphatic chain. However, the melting temperature required to produce a DES mixture also increases;[15] as a result, these mixtures were not tested. In the case of urea : ChCl, it could be inferred that the combined effect of urea as the HBD and the additional U·H_2O_2 could have led to the inactivation of the lipase after a certain amount of time. Because urea at a concentration of 6 M is known to be a denaturant of enzymes,[46] we assumed that this could be a reason for the reduced conversion. In order to test

this, the lipases were washed three times with water to remove the residual urea and then with ethanol to remove any terpene or terpenoid impurity present. The reactions were then repeated and the conversion was either less than the previous occasion or there was no conversion observed at all.

Considering the alcohol HBDs in combination with ChCl, we found the glycerol system (81.1%) yielding a better conversion than ethylene glycol (57.5%). A suitable explanation for this behavior could be obtained from the work of Rengtsl *et al.*[47] that described the fluidity of a DES system to be directly proportional to the number of hydrogen bonds on offer from a HBD. As the conversion obtained for the ethylene glycol DES mixture was below 80%, it was not used for the optimization round. The fructose based DES yielded a dark brown mixture and was not as stable as the sugar alcohols, hence it was also not included in the optimization step. Lastly, because sorbitol is a cheaper resource than xylitol, it was preferred for use in the optimization phase. As a result, only glycerol (GlCh) and sorbitol (SoCh) DES mixtures were considered further.

2.4 Conventional DES-optimization using the Taguchi method

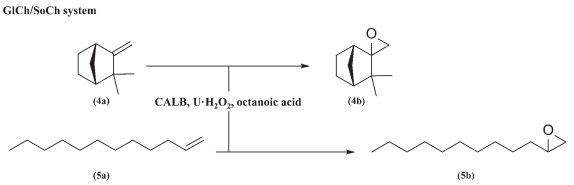
The lipase-mediated epoxidations were optimized for the GlCh and SoCh systems using the Taguchi crossed array method. A detailed explanation of the choice of parameters for this optimization, the theory behind the Taguchi method and the signal to noise ratio can be obtained from literature.[48–53] All reactions were performed in the order as described in the ESI† (once for each of the systems, *i.e.* GlCh and SoCh) in triplicate. Minitab (version 17) software was used to analyze the results. The response variable used was the conversion of the monoterpenes (**1a–3a**) to their corresponding epoxides (**1b–3b**). The results of the optimizations are given in detail in the ESI.†

The optimized set of parameters for maximum conversion of 1 mmol of terpene was similar for both DES systems used GlCh (5 mmol ChCl, 10 mmol glycerol) and SoCh (5 mmol ChCl, 5 mmol sorbitol): 4 mmol U·H_2O_2, 100 mg lipase at 40° C to 50 °C. Independent of the DES used, the conversion is more efficient for **1a** and **2a**. Decreasing the amount of enzyme to 75 mg only slightly decreased the amount of conversion. SoCh was found to work slightly better at lower temperatures (40 °C). Interestingly, a strong dependence on the amount of U·H_2O_2 was found for the conversion amounts of the two DES systems.

2.5 Evaluation of the substrate range in the GlCh and SoCh systems

After the optimal conditions for the processes, *i.e.* the GlCh and SoCh systems, were identified, two additional substrates, camphene (**4a**) and 1-dodecene (**5a**), were tested to verify the range of the DESs. **5a** was tested because it is a monoterpene and **4a** was tested to verify if the process could be extended to the terminal double bond of linear olefins as well (Scheme 2).

All reactions were performed with the optimized set of conditions described in section 2.4. The results for the new substrates in addition to those tested in the GlCh system are shown below in Fig. 3.



Scheme 2 Lipase-mediated epoxidation of camphene (**4a**) and 1-dodecence (**5a**) to their corresponding epoxides (**4b** and **5b**) using the optimized set of parameters for the GlCh and SoCh systems.

After 8 h, **1a** and **2a** were almost fully converted to their corresponding epoxides (**1b** and **2b**), as predicted by the DoE. **3a** and **4a** were approximately 83–88% converted and only 35% of **5a** was converted to 1-dodecene epoxide (**5b**) after 8 h with 69% being converted after 24 h. Although increased reaction times may ultimately improve the conversion, we did not test for this.

Similar tests were also performed for the SoCh system and the results are shown in Fig. 4; similar findings were obtained. The conversions of **1a** and **2a** were 100% and that of **3a** was approximately 63–70% after 8 h. However, **4a** had a slightly lower conversion of 75–83% in the SoCh system than in the GlCh system. Sampling proved to be more difficult for the SoCh system than for the GlCh system. This was because of the separation of phases (in the reaction vessel), which took longer for the SoCh system compared to the GlCh system. **5a** had a conversion of 55–65%, which was surprising given that GlCh system had a conversion of 25–35% for this substrate. We assume that the viscosity of the SoCh system played a major role in this difference. A possible explanation is that sorbitol and ChCl may have formed a dynamic DES system, wherein U·H_2O_2 and octanoic acid might have been dissolved better (than the GlCh system) leading to a faster peroxycarboxylic acid formation, resulting in a faster epoxidation process.

One major drawback of both the systems was the formation of caprylate esters of both glycerol and sorbitol, which we detected using GC-MS (ESI†). To produce pure epoxides (**1b–5b**) and avoid the formation of esters, we decided to shift the search toward DES mixtures that did not contain any alcohol groups. This led to the development of the "minimal" DES system which consisted of ChCl : U·H_2O_2 that was to be used as both the peroxide source and the solvent.

2.6 Minimal DES results

We already demonstrated that the urea : ChCl (Table 2, # 9) system was liquid at the desired temperature, *i.e.* 60 °C and yielded a conversion of 67%. We therefore elected to use this system, albeit with a small modification: U·H_2O_2 was used instead of urea for a novel DES to be formed. Doing so meant that additional amounts of U·H_2O_2 did not need to be added, as the compound already contains urea for DES formation and

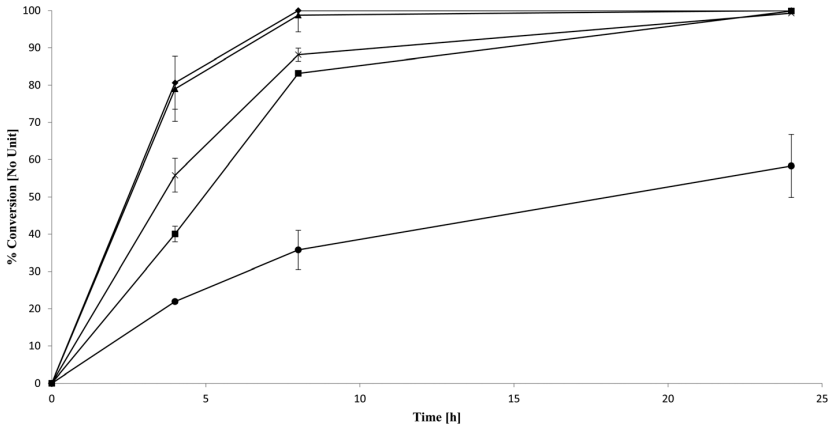


Fig. 3 Conversions obtained for 1-dodecene (circle), α-pinene (square), camphene (×), limonene (triangle) and 3-carene (diamond) over time using the optimized GlCh system.

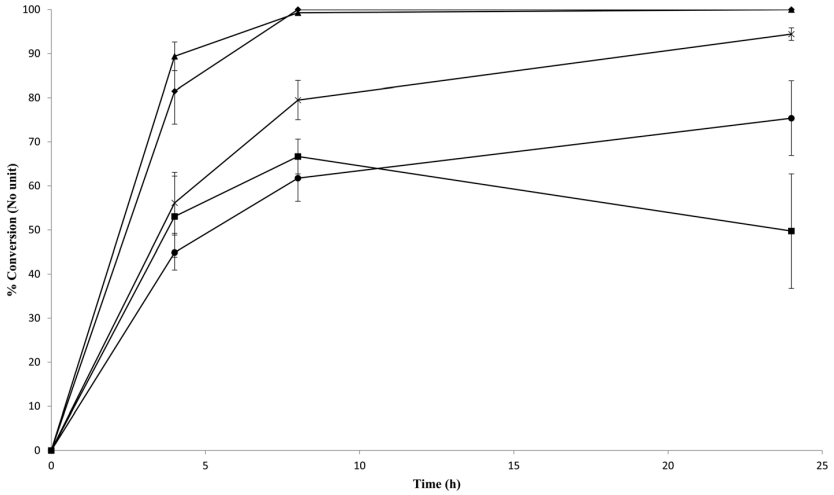


Fig. 4 Conversion obtained for 1-dodecene (circle), α-pinene (square), camphene (×), limonene (triangle) and 3-carene (diamond) over time using the optimized SoCh system.

the H_2O_2 needed for epoxidation. This method was used along with the same reaction conditions outlined in section 4.6 and the epoxidation was successful within 2 h for **1a** and **2a**, whereas it took 3 h for **3a**; this is shown both in Fig. 5 and also in Table 5 of the ESI.† It can be seen that after 2 h, **1a** was completely converted to **1b**, **2a** was 99 ± 1% and **3a** was converted to 92 ± 6%. After 3 h, all the samples were completely converted to their epoxides.

This surprisingly good result could have been due to the urea and the ChCl forming a proper DES, with the remaining H_2O_2 being dissolved in the DES. This resulted in the educts and the peroxy acid generator having better solubility, which led to faster reaction kinetics. This makes this reaction medium, which was the simplest of all of the ones tested, the most effective one as well. In fact, it performed even better than the toluene system that we had previously developed.[42] It should be noted that this process itself was not optimized using the Taguchi method, but the results of the previous optimizations were used here. The epoxides produced were then purified according to the procedure described in section 4.8.

To analyze the purity of epoxide **1b**, we carried out GC-MS and nuclear magnetic resonance (NMR) analyses. As described above, in the samples from the GlCh and SoCh systems, esters formed between octanoic acid and the alcohol groups of the DES was detected as impurity peaks in the GC-MS. No such peak was present when the synthesis was performed in U·H_2O_2 (ESI†). In theory the cholinium species could also lead to side product formation as it also contains an alcohol group. Since the GC-MS exhibited no impurities (ESI,† Fig. 6), we performed

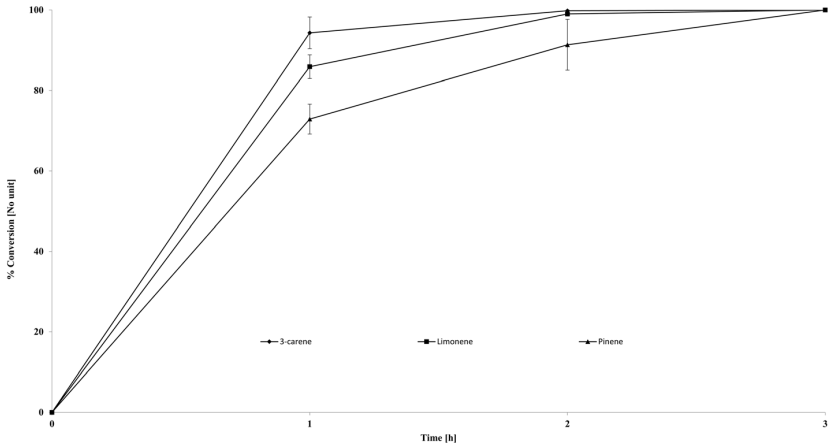


Fig. 5 Conversion profile of 3-carene (diamond), limonene (square) and α-pinene (triangle) using the ChCl : U·H_2O_2 DES mixture.

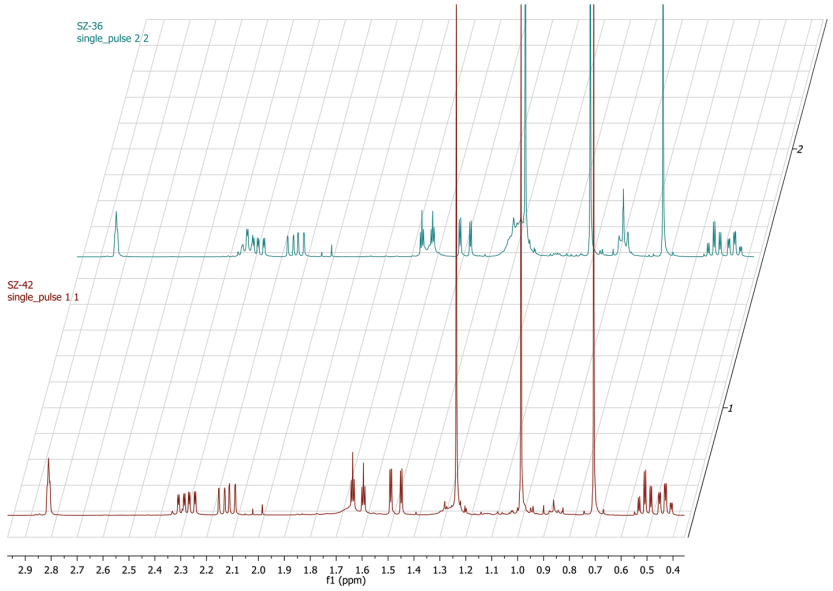


Fig. 6 NMR spectrum of 3-carene epoxide produced by GlCh (top) and ChCl : U·H_2O_2 (bottom) systems.

an additional NMR analysis of purified **1b** and compared the spectra of the samples from the two different systems. The chemical shift presents the spectrum for **1b** from the GlCh system (cyan) and ChCl : U·H_2O_2 system (red) (Fig. 6). The NMR shifts of **1b** match to the ones reported in literature.[42,44] It can be seen, however, that peaks that correspond to octanoic acid esters at around 0.9, 1.3 and 2.3 ppm, are present in **1b** from the GlCh system, while these peaks are absent in **1b** from the ChCl : U·H_2O_2 system. Apparently, lipase based esterification of choline with CALB is less efficient, possibly due to the positive charge of the molecule, leading to decreased side product formation. We can therefore conclude that the ChCl : U·H_2O_2 system is much more efficient in producing epoxides in a purer form than the GlCh or SoCh systems.

2.7 Product purification and isolated yields

The utilization of DES has implications for product purification. The low solubility of DES in organic solvents can be exploited for a simple extraction process. Accordingly, *n*-hexane was first used as the extraction solvent as described in detail in section 4.8.1. Isolated yields close to 90% could be obtained (Table 1). However, the utilization of *n*-hexane counteracts the green principles of the process as it is considered a harmful organic solvent.[54] An effective replacement for the extraction solvent was

Table 1 Comparison of isolated yields (%) of terpene epoxides (**1b–3b**) obtained on using the *n*-hexane and water + ethyl acetate processes

S. no.	Product	Isolated yield (%) obtained on using *n*-hexane	Isolated yield (%) obtained on using water/ethyl acetate
1	**1b**	89.8 ± 5.9	87.2 ± 2.4
2	**2b** (70%), **2c** (30%)	74.0 ± 4.5	77.0 ± 5.0
3	**3b**	80.4 ± 7.0	84.6 ± 3.7

to be investigated. The high water solubility of the DES constituents combined with the low water solubility of the products actually might allow a water based extraction process. Hence, a new water based purification scheme was developed. Water indeed dissolves the DES and three phases appear – the upper organic phase with the product and octanoic acid, the middle phase with lipase beads and a lower DES phase that can be discarded. The octanoic acid in the organic phase could then be deprotonated and transferred to the aqueous phase yielding pure epoxide as an upper phase. However, this led to the loss of terpene epoxide on the walls of the separating funnel, due to the work in small scale. To solve this issue, we used ethyl acetate in combination with water in order to facilitate better separation of the DES and the organic phases. On using the protocol described in 4.8.2, we were able to isolate the products with relative ease and the results obtained are shown above in Table 1. It can be seen that the water and ethyl acetate purification procedure is equal to or even slightly better than the *n*-hexane process.

3 Conclusions

This work presents the epoxidation of monoterpenes under solvent-free conditions. Owing to the incomplete conversion of reactants, with the exception of **1a**, it can be inferred that individually tailored optimizations are necessary for each monoterpene. To overcome these issues, DES, which is considered a green reaction medium, was used to epoxidize monoterpenes. Two of these systems, *i.e.* GlCh and SoCh, were successful in yielding complete conversions of the starting material within 6–8 h. However, both these systems produced ester impurities. To avoid this, a novel "minimal DES" consisting of ChCl and U·H_2O_2 was developed, which achieved a total conversion of the reactants within 2–3 h. We were able to reduce the reaction time by a quarter using this new DES system. In addition to this, we developed a purification procedure using water and ethyl acetate that enables a good recovery of terpene epoxides whilst maintaining the green aspect. To summarize, we believe that this new system could inspire future works in this field not just at the laboratory scale but also at the industrial scale.

4 Materials and methods

4.1 Materials

All the materials in this study were used as purchased without further modification or purification steps. **1b** was produced in house[42] and was used as an analytical standard. (+)-Limonene (96%) was purchased from Acros Organics, Germany. Toluene (≥99.9%) was purchased from Merck KGaA, Germany. Gylcerol (≥99.5%) was purchased from Roth chemicals, Germany. D-Sorbitol (min. 99%) and sodium hydroxide (min. 99%) were obtained from Applichem GmBH. 3-Carene (≥90%), α-pinene (98%), 1-dodecene (95%), camphene (95%), choline chloride (≥98%), D(−)-fructose (≥99%), potassium carbonate(≥99%), L-(+)-tartaric acid (≥99%), laevulinic acid (99% FG), malonic acid (99%), octanoic acid (98%), urea–hydrogen peroxide (U·H_2O_2) (97%), urea (molecular biology grade), xylitol (99%) and zinc bromide (98%) were bought from Sigma Aldrich, Germany. Ethyl acetate (LC-MS grade, min. 99.95%), aqueous hydrogen peroxide (aq. H_2O_2) (35%) and *n*-hexane (>95%) were obtained from Th.Geyer GmBH, Germany. Ethylene glycol (≥99.5%) was purchased from VWR chemicals, Germany. The enzyme *Candida antartica* lipase B (CALB) was procured from two suppliers – Chiral Vision (IMMCALB-T2-TXL, 15 000 PLU g^{-1}) was used for optimization reactions and c-LEcta (CALB Immo plus, 16 700 PLU g^{-1}) was used for all the other reactions. Both the commercial CALB preparations used in this work were immobilized covalently on to identical hydrophobic supports with a similar enzyme loading. Moreover, previous tests performed showed no characteristic difference in reactivity (results not shown).

4.2 Methods

4.2.1 Solvent free epoxidation systems. The tests for the solvent-free epoxidation systems were carried out using two different peroxide sources: aqueous (aq.) H_2O_2 and urea (U)·H_2O_2.

4.2.1.1 Aq. H_2O_2. An initial test was carried out to determine whether a solvent-free epoxidation was even possible for monoterpenes; this was done using 2 mmol **1a**, 2.5 mmol aq. H_2O_2 (35%), 0.5 mmol octanoic acid, 100 mg (1670 PLU) CALB, 40 °C and 500 rpm for a duration of 16 h.

The scaled-up version was carried out using 10 mmol monoterpene (**1a**, **2a** and **3a**), 12.5 mmol of aq. H_2O_2 (35%), 2.5 mmol of octanoic acid, 100 mg (1670 PLU) CALB, 45 and 60 °C and 500 rpm for a duration of 20 h (45 °C) and 8 h (60 °C).

4.2.1.2 U·H_2O_2. The test was performed using 2 mmol monoterpene (**1a–3a**), 2.5 mmol U·H_2O_2, 100 mg (1670 PLU) CALB, 0.5 mmol octanoic acid, 40, 50 and 60 °C and 500 rpm for a reaction time of 20 h.

4.3 Conventional DES, first screening round

Several DES mixtures described by Russ and Koenig[15] were prepared with the assumption that they would be appropriate reaction media for the lipase-mediated epoxidation reaction. ChCl was used as the halide salt and different HBDs at certain ratios (described in detail in Table 2) were used to form the DES mixtures. For the preparation of the DES, ChCl and the corresponding HBD were carefully weighed into an empty 20 ml reaction vessel. The vessel was then heated to 100 °C for 120 minutes, after which the samples were cooled to 60 °C.

Table 2 List of HBDs and ChCl screened as DES for the first round of screening. ChCl : HBD are given in molar ratios. *T* °C refers to the melting point of the mixtures and RT refers to room temperature as described by Russ & Koenig.[15] Conversion refers to the amount of 3-carene converted to its respective epoxide during the second round of screening

S. no.	HBD	Type	ChCl : HBD	*T* °C	Fluidity at 60 °C	Conversion (%)
1	Valeric acid	Carboxylic acid	1 : 2	RT	Yes	No conversion
2	Laevulinic acid	Carboxylic acid	1 : 2	RT	Yes	17.4
3	4-Hydroxy phenyl acetic acid	Carboxylic acid	1 : 2	No data	No	NA
4	Malonic acid	Dicarboxylic acid	1 : 1	10	Yes[a]	NA
5	L-(+)-Tartaric acid	Dicarboxylic acid	2 : 1	47	No	NA
6	L-Glutamic acid	Amino acid	1 : 2	13	No	NA
7	Glycerol	Alcohol	1 : 2	−40	Yes	81.1
8	Ethylene glycol	Alcohol	1 : 2	−20	Yes	57.5
9	Urea	Amide	1 : 2	12	Yes	66.9
10	D-Fructose	Sugar	1 : 2	5	Yes	100
11	D-Glucose	Sugar	1 : 2	14	No	NA
12	D-Xylitol	Sugar alcohol	1 : 1	RT	Yes	100
13	D-Sorbitol	Sugar alcohol	1 : 1	RT	Yes	100

[a] Was liquid, but highly viscous; NA – not applicable.

The fluidity of the DES mixture was visually examined and noted. The samples that were liquid at 60 °C were then used as the reaction media in the second round of the screening process.

4.4 Conventional DES, second screening round

All the DES mixtures that were liquids at 60 °C (Table 2) from the previous screening round were used as the reaction media for the lipase mediated epoxidation of **1a**. A typical lipase reaction screening experiment consisted of 1 mmol **1a**, 0.25 mmol octanoic acid, 3 mmol U·H_2O_2, 100 mg (1670 PLU) CALB and the liquefied DES mixtures from the first screening round. The reaction was carried out at 60 °C and 500 rpm in an oil/sand bath. In order to have sufficient reaction medium for the epoxidation reactions, the DES mixtures were prepared at an increased factor of five whilst maintaining the same molecular ratio (for example: 5 mmol ChCl with 10 mmol glycerol). A single point measurement was taken at the end of 24 h to determine the conversion of **1a** to **1b**, and the measurement is described in Table 2.

4.5 Optimization

The lipase-mediated epoxidation process was optimized using the DoE Taguchi method. The theory behind this method has already been described in detail in our previous work[42] as well as in various other studies,[48–52] as such, it will not be discussed in the present study. Although this method was used in the present study, DESs were used instead of organic solvents. The parameters chosen and the levels used are given in ESI.† The optimizations were performed using two L_9 orthogonal arrays (ESI†) and the performance criterion used was "larger is better". Each row in an array corresponds to the combination of parameters at their respective levels. The constant parameters used in the process were: 500 rpm mixing and the source of lipase (CALB from Chiral Vision with a loading of 15 000 PLU g^{-1}). The reactants were mixed in the order described in the ESI.† The arrays for the trials and the analysis of the results were generated using Minitab (version 17) software.

4.6 Minimal DES mixture

ChCl (7.5 mmol) and U·H_2O_2 (15 mmol) were mixed at room temperature between 45 min to 1 h with a magnetic stirrer. The resultant fluid mixture was then used as both a solvent and a peroxide source for the lipase-mediated epoxidation reaction. The following reaction conditions were used: 5 mmol monoterpene (**1a–3a**), 100 mg (1670 PLU) CALB, 1.25 mmol octanoic acid, 50 °C and 500 rpm.

4.7 Analytics and sampling

The GC-MS and NMR settings, heating profile of gas chromatography and mass spectrometry details, in addition to the retention times of the reactants and products have already been described in our previous works.[42,44] The sampling was performed as follows (as DESs do not follow the traditional rules of solvents, this study utilized different sampling techniques to analyze the compounds):

- Up to 1 mmol of the starting material: 2 μl of organic phase (DES and enzyme free) was mixed with 198 μl *n*-hexane. 10 μl of this sample was then transferred to 990 μl ethyl acetate
- Up to 10 mmol starting material: 2 μl of organic phase was added to 998 μl ethyl acetate
- Up to 100 mmol of starting material: 1 μl of organic phase was mixed with 999 μl ethyl acetate

The samples were then subjected to GC-MS measurements. For the NMR measurements, 20 μl of pure epoxide was mixed with 600 μl deuterated chloroform ($CDCl_3$) and the sample was measured with ^{1}H proton NMR.

4.8 Purification procedure

Terpene epoxides (**1b**, **2b**, **2c** and **3b**) were produced in the minimal DES setup as mentioned in 4.6 and was purified using an adapted version of our previous work.[42] The purification

procedure was developed after an initial screening step (detailed description of the development is described in the ESI†). Two purification processes were tried for the effective recovery of the terpene epoxides (**1b–3b**) in triplicates.

4.8.1 Purification using *n*-hexane. The first method that was tried used *n*-hexane as the extraction solvent to extract the nonpolar fractions (epoxides (**1a–3b**) and octanoic acid). This mixture was vortexed for 30 seconds to 1 minute. This mixture was then cooled down to −20 °C for a time period of 1–2 h, which yielded three phases: a top organic phase; a middle phase containing the lipase and a bottom DES phase (ESI Fig. 3†). The top organic phase was decanted and if necessary filtered – when lipase beads were found in the organic phase. The organic phase was washed 3–5 times with 5 ml saturated sodium bicarbonate solution ($NaHCO_3$) for complete removal of octanoic acid. The organic phase was then dried using anhydrous sodium sulphate (Na_2SO_4). The *n*-hexane was then removed using vacuum distillation, following which, the acid free epoxide was weighed and the isolated yield of the process was calculated.

4.8.2 Purification using water and ethyl acetate combination. Owing to the harmful nature of the *n*-hexane and in the interest of making the process greener, the following protocol was adapted. First, 10 mass equivalents of distilled water was added to the DES mixture. This mixture was then vortexed at maximum speed for 30 s to 1 min. To this mixture, 10 ml of ethyl acetate was added and vortexed for 30 s to 1 min. This mixture was then transferred to a separating funnel and 20 ml of saturated sodium bicarbonate ($NaHCO_3$) solution was added to this mixture. The organic phase was retained while the aqueous phase was discarded. This was repeated till the octanoic acid was completely neutralized. The organic phase was then dried using anhydrous sodium sulphate (Na_2SO_4) as before. The excess ethyl acetate was then removed using vacuum distillation, the terpene epoxide (**1b–3b**) weighed and the isolated yield of the process calculated.

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Electronic Supplementary Material (ESI) for Green Chemistry.

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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

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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)

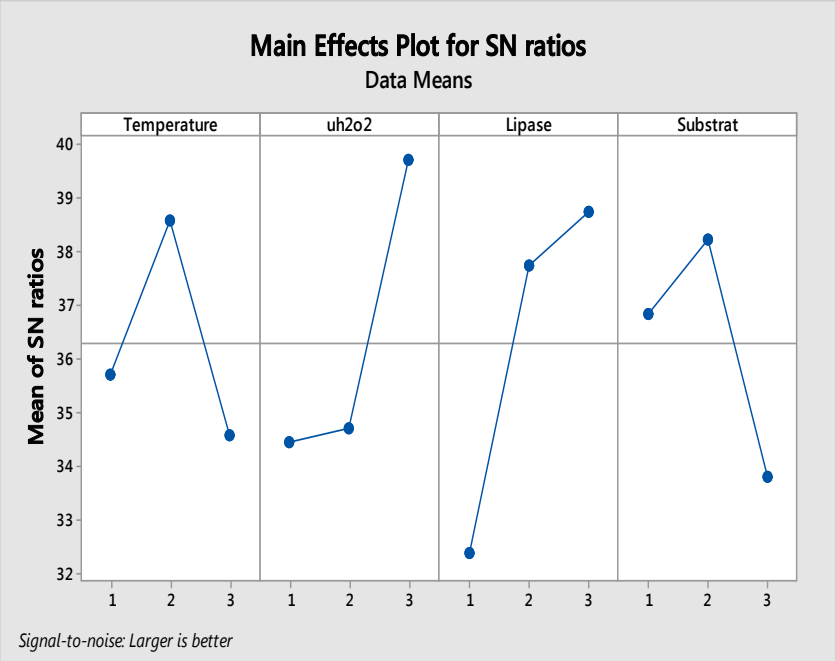

Figure 1: Main effects plot for the signal to noise (s/n) ratios of the GlCh system tested.

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.

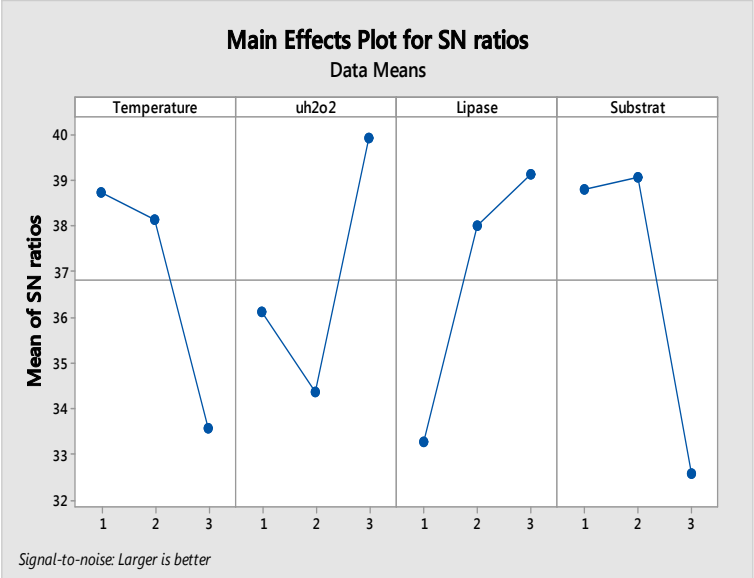

Figure 2: Main effects plot for the signal to noise (s/n) ratios of the SoCh system tested.

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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 %.

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- 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

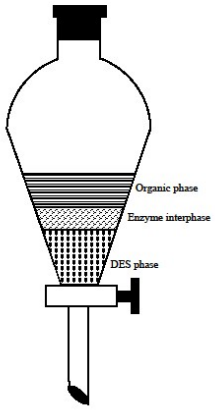

Figure 3: Separating funnel with DES (bottom most layer, diamonds), lipase enzyme (interphase, slanted bricks) and the ethyl acetate phase (top-most, horizontal lines) consisting of terpene epoxide and octanoic acid.

DETECTION OF SORBITOL AND GLYCEROL ESTER IMPURITIES

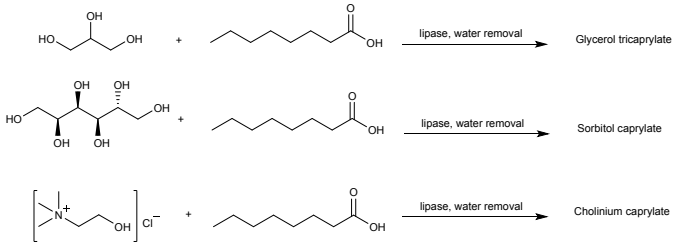

Figure 4: Formation of caprylate esters using lipases and DES mixes as the reaction medium for the epoxidation of terpenes

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.

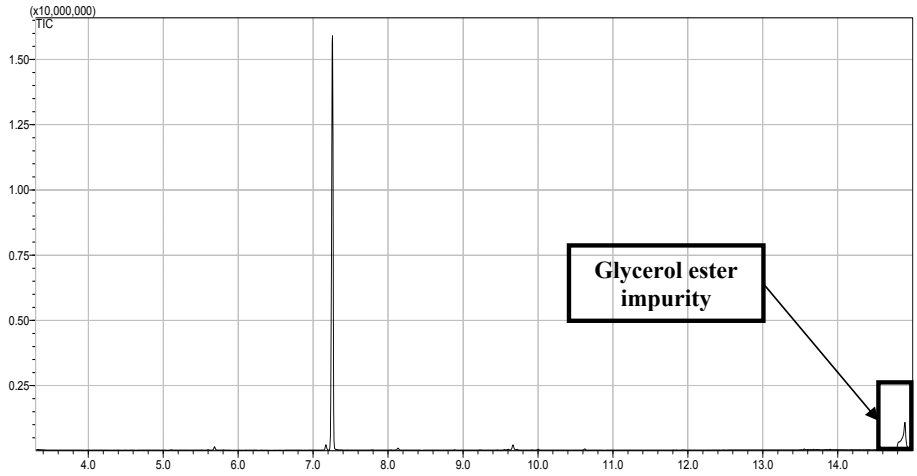

Figure 5: GC-MS chromatogram of the purified 3-carene oxide with the impurity towards the far end of the chromatogram

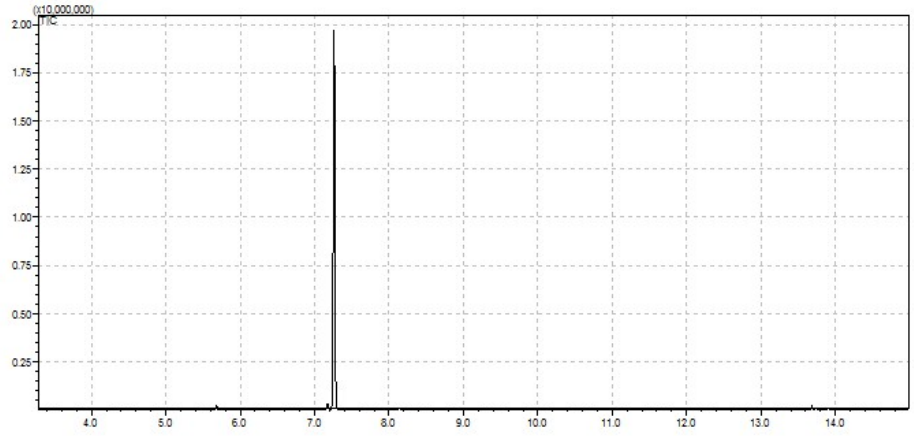

Figure 6: GC-MS chromatogram of 3-carene epoxide on using the ChCl:U·H_2O_2 DES mixture as the reaction solvent for epoxidation

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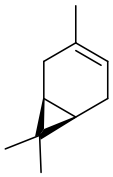
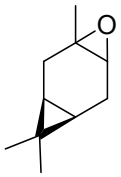
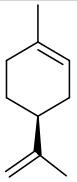
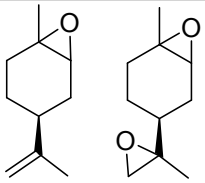
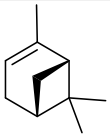
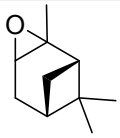
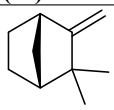
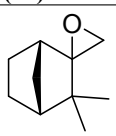
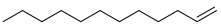
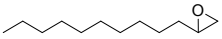
Summary of conversion on using different reaction media for various reactants

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

Chapter 4 Discussion

4.1 Development of a robust lipase mediated epoxidation process for terpenes

The lipase-mediated epoxidation process was developed by Björkling *et al.* for alkenes in 1992 [**?**]. The method uses the Prilezhaev mechanism of epoxidation [**?**], wherein a peroxycarboxylic acid is used for the epoxidation of the double bond. The Prilezhaev method produces equimolar waste (as the product), is operated under harsh reaction conditions, and poses a risk to the experimenter, which is why this method is unfavourable. These drawbacks are overcome by F. Björkling's method, which produces the peroxycarboxylic acid *in situ* by using carboxylic acid and H_2O_2 in an organic solvent, in the presence of a lipase (section 1.5.1). This method can be used to epoxidize alkenes at the laboratory scale, as it is a quick and easy method [**?**, **?**].
The first account of using lipases to epoxidize terpenes was reported by V. Skouridou's team in 2003. The team had used α-pinene ($C_{10}H_{16}$), a monoterpene, as their model compound for epoxidations [**?**, **?**]. Following the works of Skouridou *et al.*, Moreira *et al.* used 3-carene, a structural isomer of α-pinene as their model compound in 2007 [**?**]. All the research groups came to the conclusion that the lipase-mediated epoxidation process was dependent on several factors such as the concentration of the H_2O_2 and carboxylic acid used along with the amount of lipase enzyme used to catalyse the reaction that needed optimization.
Several accounts of the optimization have already been published. The first attempt was made by Moreira *et al.* in 2005. They used the OVAT approach to optimize the process [**?**]. A similar approach was used by Abdulmalek *et al.* in 2012 to epoxidize 1-nonene [**?**]. In 2011, Sun *et al.* used the response surface methodology (RSM) of process optimization to optimize the epoxidation of an unsaturated oil using CALB [**?**]. In 2014, Abdullah *et al.* used the D-optimal design of process optimization to optimize the lipase mediated epoxidation of linoleic acid [**?**]. All these methods were very informative and successful in implementation. However, these methods were specific for one particular educt.
The OVAT technique of process optimization could not be used for this purpose, *i.e.* the lipase mediated epoxidation of monoterpenes, due to the following reasons:

1. the necessity to run approximately $20,000$ runs to arrive at an optimum
2. generation of wastes
3. costs
4. time consuming and strenuous effort.

Additionally, the RSM and the D-optimal design were not ideal, owing to the aforementioned issues and the complexity of designing the experiments. To overcome these issues and achieve the goal of a robust lipase-mediated epoxidation process, the Taguchi method of optimization was chosen. The motivation of the present work was to develop a single robust process that could produce various epoxides from their corresponding educts at maximum efficiency. 8 parameters namely, reaction medium, carboxylic acid type and concentration, monoterpene type and concentration, reaction temperature, H_2O_2 concentration and the amount of lipase used were identified to affect the final outcome of the process. The results of the optimization revealed that the H_2O_2 concentration used was the factor the affected the process the most, while the type of monoterpene used affected it the least. Additionally, the optimized process was scaled up from 1 cm^3 to 100 cm^3. The results of the scaled up experiment comply with the findings of the small scale optimizations. Comparing this work with previous works [**?**,**?**,**?**,**?**,**?**], a total reaction time of 6 h to 8 h was required to obtain complete conversion, as opposed to the 8 h to 24 h. Besides, an easy-to-construct downstream unit was also designed for isolating pure epoxides, which was not reported in the previous cases. These results point to a better epoxidation process that could be controlled to produce specific epoxides at desired isolated yields. Moreover, the previous accounts of optimization mentioned before, *i.e.* the OVAT, the RSM, and the D-optimal design were for a single substrate.

Table 12: List of substrates epoxidized after optimization

S.No.	**Olefin**	**Type**	**No. of double bonds**
1.	1-dodecene		
2.	1-octene		
3.	cyclohexene	alkene	one
4.	styrene		
5.	α-methylstyrene		
6.	3-carene		
7.	camphene	unsaturated terpene	one
8.	α-pinene		
9.	limonene		
10.	α-phellandrene		
11.	β-phellandrene	unsaturated terpene	two
12.	α-terpinene		
13.	γ-terpinene		
14.	myrtenol		
15.	nopol	unsaturated terpene alcohol	one
16.	terpineol		

17.	carvone		
18.	α-ionone	unsaturated terpene ketone	two
19.	β-ionone		
20.	oleic acid	unsaturated carboxylic acid	one

By using the Taguchi design, as many as 20 substrates were optimized as mentioned in table 12. Despite the process being successfully optimized and robust, there is still room for improvement. The use of toluene as the reaction medium is not ideal as this would bring down the green quotient of the process. To overcome this, a greener alternative needs to be used such as:

- using ethyl acetate as solvent and acyl donor to produce peroxycarboxylic acid *in situ*
- using alternate reaction media as reported in section 3.4.

4.2 Combining hydrogen peroxide production with the lipase-mediated epoxidation process

As mentioned in section 1.6.2, the H_2O_2 used in the process is the exhaustible resource in the lipase-mediated epoxidation process. In order to ensure a continuous production of epoxides, the H_2O_2 needs to be replenished during the course of the reaction. This can be achieved by either adding H_2O_2 at regular intervals or regenerating the H_2O_2 *in situ*. Adding H_2O_2 at regular intervals is the most ideal and simplest solution to this problem. However, over time, the H_2O_2 is known to decay, which will subsequently affect the final outcome of the process. To overcome this and to ensure high productivity, the H_2O_2 needs to be regenerated.
H_2O_2 can be produced by *(i)* chemical, *(ii)* electrochemical, *(iii)* enzymatic, or *(iv)* photocatalytic means [**?**, **?**, **?**]. Tests revealed that a minimum amount of $1 : 1.2$ molar ratio of substrate to H_2O_2 was needed to achieve complete conversion of monoterpenes (results not shown). Photocatalytic means of H_2O_2 production is incapable of producing such high amounts of H_2O_2 [**?**, **?**] and was not investigated. The maximum H_2O_2 concentration obtained when using the electrochemical method of Peralta *et al.* [**?**] was $0.5\,\mathrm{mmol\,L^{-1}\,h^{-1}}$, which is way below the required amount and was not investigated either. Alcohol oxidase (E.C. 1.1.3.13) and glucose oxidase (E.C.1.1.3.4) were chosen to convert methanol and glucose respectively to H_2O_2, which could then be integrated with the lipase-mediated epoxidation process. Both enzymes were water soluble and incapable of operating in an organic solvent such as toluene. Hence a two-phase system resulted. Even though the amount of H_2O_2 required to completely convert the monoterpene to its epoxide was achieved, no conversion was observed. The reasons for the same were that the H_2O_2 remained in the polar phase and there was no contact of the H_2O_2 with the lipase enzyme for catalysis to occur. Hence, this method was also rejected and the

focus was shifted towards integrating the chemical means of H_2O_2 process, especially, the AQ autoxidation process[**?**] with the lipase mediated epoxidation process.
The very first account of such a coupling in one pot was reported by Ranganathan *et al.* in 2015 [**?**]. The process consisted of two steps: the autoxidation H_2O_2 production process and the lipase mediated epoxidation of monoterpenes. The autoxidation process normally has a working solution that consists of hydrophobic and hydrophilic solvents to dissolve the anthraquinone and the hydrogenated version. Normally, the working solution is a mixture of mesitylene and trialkyl phosphate [**?**, **?**]. This was replaced by toluene and ethyl acetate to favour the lipase-mediated epoxidation part. As in the Riedl & Pfleiderer process [**?**], the four steps of H_2O_2 manufacture *i.e.* (hydrogenation, filtration, oxidation, and extraction) as explained in section 1.6.3 were followed in this work as well. The amount of Pd/C to be used for the hydrogenation reaction was optimized using the OVAT approach. Thus, a one-pot batch process was designed that was capable of converting α-pinene, 3-carene, and limonene to their corresponding epoxide(s)(section 3.2).
The process was the first of its kind and is innovative. However, the process needed major improvements. Primarily, the process could be operated only once and the solvents needs to be replaced with every batch, leading to the generation of wastes, which have to be avoided. Secondly, the process yielded incomplete conversion probably due to inadequate amounts of H_2O_2 produced. Both these drawbacks were solved by an alteration in the process design as explained in section 3.3.

4.3 The semi-continuous combination of H_2O_2 production with the lipase-mediated epoxidation process

To overcome the drawbacks of the one-pot batch process, the semi-continuous combination was designed [**?**]. The following changes were made to the existing design:

- Pd/Al_2O_3 pellets were used instead of Pd/C
- a stainless steel mesh to house the Pd/Al_2O_3 pellets
- separating the H_2O_2 production and the epoxidation step by using a H_2O_2 reservoir

The amount of Pd/Al_2O_3 pellets for the hydrogenation step was optimized (5 mol % with respect to substrate) and H_2O_2 was produced at 50 % (w/v) at high isolated yields. The mass transfer of hydrogen in the liquid phase is governed by the stirring rate, in other words, mixing. When using a magnetic stirrer to achieve adequate mixing, shear and grinding effects set in and the solid Pd pellets loose activity over time. This means a filtration step would be required after the hydrogenation stage, which is the reason for choosing the Pd/Al_2O_3 pellets instead of the Pd/C powder. To avoid the use of this filtration step and overcome the shear effect, a

"hybrid" reactor was designed that combined the positives of a continuous stirred tank reactor and a packed bed reactor. The catalysts were protected in a stainless steel mesh and the stirring was maintained at maximum ensuring better mass-transfer and protection of the catalyst. To ensure the same productivity of H_2O_2 over subsequent cycles, a washing protocol was used. Moreover, the use of a reservoir at 4 °C to store the excess H_2O_2 ensures better process control, as the amount of H_2O_2 needed can be diluted according to demand. Next, the low temperature of the reservoir minimizes the risk of H_2O_2 decay over time, ensuring uniform yields of epoxide. Additionally, this semi-continuous combination can be used to run other reactions that require H_2O_2. Finally, this prototype combination of the autoxidation process and lipase-mediated epoxidation could be scaled up to industrial scale with the appropriate measures and a possibility of using different olefins as well.
There are no previous accounts of such a combination and therefore, this process cannot be compared with any other report. However, there is still room for improvement in terms of production volume of this process. Presently, this process has been designed to be operated only in the 0.15 L scale, which needs to be extended to a minimum of 1 L.

4.4 Lipase-mediated epoxidation in DES

As mentioned in section 4.1, the use of toluene as the reaction solvent makes the optimized lipase-mediated epoxidation process *"ungreen"*. This is because volatile solvents such as toluene are harmful to both, the environment and the experimenter alike. The reports of Denis Prat *et al.* from 2013 until today have listed toluene as a solvent that needs to be avoided or replaced [**?**, **?**, **?**]. Moreover, the practice of green chemistry suggests that any chemical synthesis should try to avoid the use of organic solvents and be performed in water or in the absence of any solvent [**?**, **?**].
In accordance with these guidelines, the first tests were done without any solvents and performed directly in monoterpenes, which served as both solvent and substrate. There were complications in the process development phase because of incomplete conversion of starting material to product. It can be argued that on optimization using the DoE, the solvent free lipase-mediated epoxidation process would yield complete conversion. However, it must be considered that each substrate would behave as a solvent with varying properties. This implies that the lipase-mediated epoxidation process cannot be optimized for one substrate and generalized for others. Additionally, there were issues with handling that led to the rejection of the lipase-mediated epoxidation process under solvent free conditions for terpenes.
The focus was then shifted to DES as the reaction medium. Besides, the use of lipases in DES is not new and has already been documented for esterification reactions [**?**, **?**, **?**, **?**]. Conversely, the use of DES as reaction medium for lipase-mediated epoxidation reactions was not reported until recently. The first report of lipase-mediated epoxidation in DES was published by Zhou *et*

al. in the year 2017 for linear olefins focussing on the stability of the lipase in the DES system [**?**]. The work described the use of a DES system consisting of ChCl and sorbitol (SoCh) as the reaction medium, with a terminal double-bond containing olefin as the substrate and aq.H_2O_2 as the co-substrate.

The work of Ranganathan *et al.* on the other hand describes the overall process development for the epoxidation of monoterpenes [**?**]. In this work, monoterpene and U·H_2O_2 were used as the substrate and co-substrate instead of the linear alkene and aq.H_2O_2. This was because of the fact that on using aq.H_2O_2 as the co-substrate for epoxidation reactions, there were diols being formed due to the acidic nature of the DES, water, and a reaction temperature of 50 °C (results not shown). The process was optimized using the Taguchi method of DoE for two DES systems: GlCh and SoCh. Both systems yielded complete conversion of α-pinene, 3-carene, and limonene within 6 h to 8 h. Despite a purification unit to produce pure epoxide, there were impurities (2 % to 3 %) in the form of glycerol and sorbitol esters for GlCh and SoCh DES systems. To circumvent this problem, a "minimal" DES was developed consisting of ChCl and U·H_2O_2 in equimolar amounts. By using this minimal DES, the time taken to achieve complete conversion was 2 h to 3 h instead of 6 h to 8 h for the DES and the process with toluene as the reaction medium. In the near future, the minimal DES system ChCl and U·H_2O_2 can be used to produce epoxides at the industrial level. However, the scale up procedures are to be investigated in detail and the process needs to be extended for other terpenes as well.

4.5 Future perspectives

The lipase mediated epoxidation was successfully optimized for monoterpenes to yield a robust process capable of epoxidizing 20 different substrates. Despite being robust, the process needs to be further developed and tested for individual substrates other than α-pinene, 3-carene, and limonene. If the anthraquinone process of H_2O_2 production were coupled with this process in a semi-continuous manner, the process can be scaled up to industrial levels. However, tests for the scale-up and safety issues need to be done and designed accordingly before actual implementation. The tests with ethyl acetate as the reaction solvent and acyl donor were done in order to bring in a green quotient to the process, since toluene is considered to be harmful to both the environment and the operator. One drawback of using the ethyl acetate process would be that the solvent be replaced after each cycle. To overcome this issue, DES needs to be used as the reaction solvent. The minimal DES system consisting of ChCl and U·H_2O_2 is an ideal case to perform epoxidations. Tests at the laboratory scale have been successful, but the pilot-plant and industrial scale syntheses needs to be designed and tested.

List of Abbreviations

ABTS 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

AQ anthraquinone

aq.H_2O_2 aqueous H_2O_2

C Celsius

CALB *Candida antartica* lipase B

cm^3 cubic centimetre

C/min Celsius/min

ChCl choline chloride

cm centimetre

CO_2 Carbon dioxide

CSTR continuous stirred tank reactor

dd.H_2O double deionized distilled water

DES Deep Eutectic Solvents

DoE design of experiments

EAHQ 2-ethyl anthrahydroquinone

EAQ 2-ethyl anthraquinone

e.g. example

g gram

GC-MS Gas chromatography-Mass spectrometry

GlCh glycerol:ChCl mixture

h hour

H_2O_2 hydrogen peroxide

HBA hydrogen bond acceptor

HBD hydrogen bond donor

HRP peroxidase from horseradish

ILs Ionic Liquids

KP_i potassium phosphate buffer

KU KiloUnit

l litre

LC-MS Liquid chromatography-Mass Spectrometry

m metre

***m*-CPBA** *meta*-choro perbenzoic acid

mg milligram

mM millimolar

min minute

ml millilitre

mm millimetre

M Molar

MSDS Materials and Safety Data Sheet

$NaHCO_3$ sodium bicarbonate

NaOH sodium hydroxide

nm nanometre

NMR Nuclear Magnetic Resonance

OVAT one variable at a time

PBR packed bed reactor

Pd palladium

Pd/Al_2O_3 Palladium on alumina

Pd/C palladium on activated carbon

PLU Proply Laurate Unit

PLU g^{-1} Propyl Laurate Unit per gram

ppm parts per million

PTFE Polytetraflouroethylene

RPM revolutions per minute

RSM response surface methodology

SCFs Super Critical Fluids

S/N signal-to-noise ratio

SoCh sorbitol:ChCl

tBAHQ *tert.*-butyl anthrahydroquinone

tBAQ *tert.*-butyl anthraquinone

TBHP *tert-* butyl hydroperoxide

TBU g^{-1} Tributyrin Unit per gram

TBU ml^{-1} Tributyrin Unit per millilitre

U g^{-1} units per gram

$U{\cdot}H_2O_2$ Urea · hydrogen peroxide

μl microlitre

μm micrometre

U mg^{-1} units per milligram

UNO United Nations Organization

USD US Dollar

UV Ultra-Violet

VOCs volatile organic compounds

v/v volumetric ratio

w/v weight by volume

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Curriculum Vitae

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