EVALUATIONS ON ENZYMATIC EPOXIDATION, EFFICIENCY AND DECAY

by

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Dedicada a mi familia, gracias por el apoyo incondicional.

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TABLE OF CONTENTS

5.
6.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The potential use of enzymes in industrial synthesis of epoxidized soybean oil has been limited through the high cost of the enzyme catalyst, in this work we evaluate the effectiveness of chemo enzymatic epoxidation of high oleic soybean oil (HOSBO) using lipase B from *Candida antarctica* (CALB) on immobilization support Immobead 150 and H_2O_2 in a solvent-free system. Additionally, we evaluated the production decay rates for hydrolytic activity and epoxide product formation over consecutive batches to determine half-life of the enzyme catalyst.

Batch epoxidation of HOSBO using CALB on 4wt% loading shows yields higher than 90% after 12 hrs. of reaction, and with a correlation to the consumption of double bonds suggesting that the reaction is selective and limiting side product reactions. Non-selective hydrolysis of oil was not found beyond the initial hydrolysis degree of raw HOSBO. Evaluations of decay given by epoxide product formation and released free fatty acids shows a half-life of the enzyme catalyst on these activities is of 22 ad 25 hrs. respectively. Finally, we evaluated the physical parameters influencing this decay, and found that H_2O_2 presence is the most important parameter of enzyme inactivation with no significant effect from its slowed addition. We propose a new reactor configuration for the analysis of the specific steps on epoxide formation through peracid intermediates.

1. MOTIVATION

Although public consciousness has enlightened on the problem of global warming and its relation to pollution from single-use plastic and toxic residues, the current times and ongoing COVID-19 pandemic have made it more critical to society to use more of these plastic materials due to health concerns. This has caused a spike in production and waste of single use plastics for food wrapping and personal protective equipment (PPE). In economic terms, the global packaging market is estimated to grow from 909.2 million from 2019 to 1012.6 billion by 2021[1].

Without a doubt, single-use plastics have become essential for our lifestyle and an immediate end of their use is unlikely, so the plastic problematic is far from over. Alternative sources of plastic materials must be produced at larger scale, simply, and economically to fulfill the global demand for these materials.

In the United States alone, nondurable goods are classified by the Environmental Protection Agency (EPA) as any materials for which is lifetime use is of less than six months, such as trash bags, food containers, and utensils. These materials had an annual production in 2017 of over 50.7 million tons, of which more than 75% accumulates in landfills as part of municipal solid waste (MSW)[2]. Additionally, new restrictions or bans on importing solid waste products put in place in countries such as China have made this a more urgent problem for each high waste-producing country like the USA to get rid of this solid waste.

Besides space required for landfills, additional concerns from the continuous and indiscriminate use of plastics are related to human health risks. Although plastics provide a physical barrier to external contaminants, many of these plastics release toxic additives such as orthophtalates and bisphenol A (BPA) monomers to material protected by the plastic barrier.

These two molecules are generally found in poly (vinyl chloride) (PVC). Which is one of the oldest and most common plastics used today because of its low cost, long durability, and high versatility. Nevertheless, without additives, PVC is brittle and stiff. Therefore, a large concentration of additives known as plasticizers are required to enhance the mechanical properties of the material. The most common additives used are not strongly bound to the polymer's chemical backbone and are therefore prone to leaching[3].

This becomes and important problem because PVC is widely present in our lives. It has applications ranging from medical equipment such as hospital tubing and blood bags to food

wrappings, wire and cable insulation, and automobile parts[3].According to the U.S. Department of Health and Human Services, materials such as PVC contain up to 40% of phthalate plasticizers, known as Di(2-ethylhexyl)phthalate (DEHP)[4]. The possibility of releasing DEHP from plastic materials allowed government agencies to express concern and develop guidelines and regulations to decrease and ban phthalates, specifically orthophtalates.

Plasticizers are commonly low molecular weight, high boiling point, organic compounds that reduce the polymer's glass temperature, thus changing a brittle material to a flexible one[5]. As mentioned by Erythropel *et al.* [3], the most important and widespread class of plasticizers are phthalate diesters. Within this class is the previously mentioned DEHP, which interacts with the polar carbon chloride bonds in PVC but it lacks the interaction of the nonpolar parts of DEHP[3]. This lack of chemically binding allows DEHP to migrate, and finally leach out of the polymer. Soft materials are especially prone to this behavior such as liners in canned or bottled food, soft toys for infants and PVC plastic bags.

Other item specially prone to this characteristic, are food containers which makes them the most important route of exposure from phthalates to the general population, with a correlation of risk given by the amounts of exposure[4,6,7]. In the case of DEHP this is more likely in food with a high lipid content due to the hydrophobicity of DEHP[8]. However, this might not be the case for smaller and water-soluble phthalates such as dimethyl phthalate (DMP) and dimethyl terephthalate (DMT), which is fairly easily released from the resin[8].

These additives have been linked to disruption of normal endocrine function in humans[9], as well as cancer and chronic kidney inflammation[10]. In addition, these plasticizers have been shown to be associated with cardiovascular risk factors[11] by possibly through its interference with insulin signaling and increase oxidative stress leading to insulin resistance.

Other types of added plasticizer prone to leaching contamination is bisphenol A (BPA). This is a small-molecule additive used in hard plastic materials, specifically polystyrene and polycarbonate. BPA binds with other molecules to add flexibility and mechanical strength to the polymeric chains and resins. Although the low-dose safety is a matter of debate by regulators, different concentrations of BPA as a leachate have been found in waste disposal sites[8]. Bisphenol A has a wide range of effects on the human body. Initially it was discovered that BPA served as artificial estrogen and was used in poultry to enhance the rapid growth to promote industry profits, similar estrogen effects were employed in women for hormonal treatments until a more effective

replacement was found in the mid 1930s[12]. Recently, research has shown effects like structural and neurochemical modifier throughout the brain with more notable effects in fetal and early childhood changes[12]. This makes its exposure a critical concern, especially in children's toys.

Regulations on the state level have been developed all over the United States since 2008, mainly to prohibit both additives from plastics aimed to children like toys or any pediatric use[12], as well as food contact, and medical devices[5]. Given the omnipresence of both PVC and polystyrene, the exposure to these additives is still a problem, which points out the urgency for plasticizer alternatives.

Low toxicity polymers that can also be environmentally degradable, are of great interest for the future of plastics. One alternative already use widely is epoxides. These materials contain the functional oxirane ring group or epoxy, a 3-member cyclic ether, which allows are capable of cross-linking with or without other substances to form three dimensional infusible networks[13]. The epoxy group allows great versatility as organic synthesis intermediates; they can make cross-linked networks or be modified by the opening of the oxirane oxygen. These materials can react easily with aliphatic, cycloaliphatic and aromatic primary, secondary and tertiary plastic resins[13].

Epoxide resins and multifunctional epoxide materials present high thermal, oxidative, and hydrolytic stability as well as high strength, stiffness and better creep, heat and solvent resistance than most thermoplastics[14]. Epoxides can be derived from synthetic compounds or natural resources and used for the synthesis of bio-bases plasticizers and lubricants. Additionally, with a high demand for their use as thermosetting polymers of high-quality performance in different industrial applications for thermal solidity, mechanical strength, corrosion protection, moisture resistance and strong adhesive qualities. Therefore epoxy resins are expected to have market revenue of more than 8,300 million USD by 2021 with important application in coatings, packaging, adhesives, PVC and arts[15].

As mentioned before, the general awareness on climate change and pollution has trigger a growing interest in the exploration of renewable resources to replace petrochemical-based materials. Natural oils, such as soybean oil, are amongst the most promising sustainable building blocks for the production of polyesters and polyols[16]. The synthesis of epoxide resins and thermosets has already been explored successfully with results depending on the application of the material[17].

2 INTRODUCTION

2.1 Vegetable oils as feedstock for bio-based polymers

Vegetable oils are important raw materials for a variety of chemical reactions to replace fossil fuels. With applications ranging from biodiesel fuels to structural and coating materials in oleochemicals, the chemical structure of vegetable oil has suitable functionalities to undergo several chemical reactions[18].

Vegetable oils are nontoxic, renewable, and domestically abundant in the U.S.[18,19]. According to the United States Department of Agriculture, the global production of vegetable oils is around 187 million tons per year, the majority being soybean and palm oil[20]. Therefore, their use as feedstock represents one of the cheapest and abundant feedstocks. The materials produced from them are capable of competing and possibly surpassing the properties of petroleum based polymers[21].

Vegetable oils has the potential to be converted into basic oleochemicals, which later serve as building blocks for more complex molecules in value-added products[21]. Conversion to these materials can be represented in terms of the functional group reacting, in the case of vegetable oils the triglycerides are composed of three, usually different, fatty acid groups linked together through ester bonds to a glycerol backbone[22].

This characteristic chemical configuration, of long fatty acid chains and polar end groups make the structure of vegetable oils amphiphilic in nature[19]. Additionally, the high viscosity, high lubricity, high flash point and low volatility make them of great industrial importance[23]. The fatty acids can be unsaturated containing at least one alkene group within its chain, or saturated when it does not. It has been reported that 90% of the oleochemical reactions occur in the carboxylic group and 10% represents reactions involving the alkyl chain[24]. The types of potential reactions are shown in figure 1, taken from the extensive review made by Zhu *et al.* The process exemplified in figure 1, follows the chemical transformation of soybean oil in which the triglycerides are broken apart into the individual fatty esters with double bonds are reacted for polymerization.

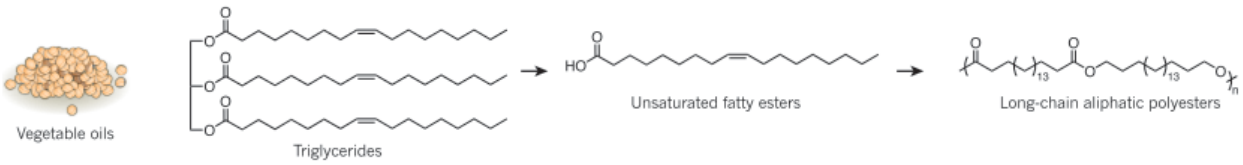

Figure 1: Conversion of vegetable oil. Taken from Zhu *et al. "Sustainable polymers from renewable resources"*.

Vegetable oil-based oleochemicals have been reported in the chemical industry for years with applications as coatings[25,26], paints[13,22], lubricants[19,27,28], soaps, inks, and agrochemicals[29]. These materials are derived from the reactivity of the fatty acids in oil and their unsaturation bonds. Therefore, the reactivity of oil depends on the degree of unsaturation of the fatty acids. This is the most important characteristic of an oil because it affects the physical and chemical properties made possible through modifying the stereochemistry of the double bonds. Additionally, the unsaturation dictates how the finishing material can be conjugated or polymerized to have properties raging from soft rubbers to hard, tough, or brittle plastics[30].

Biobased plastics and plasticizers can be made from vegetable oils, modified epoxidized oils, or trans esterified oil, and they represent a feasible alternative to traditional petroleum-based plastics and have been extensively studied for over 20 years[31]. These modifications are made through different methods in which the focus is high efficacy of the process in order to reduce costs. The main reactive species, alkenes in the unsaturated fatty acids, participate in a common set of polymerization methods such as: thiol-ene reactions, acyclic diene metathesis, epoxidation, and radical crosslinking reactions[32].

The most frequent studied modification involving plant oils and their derivatives has been epoxidation for the synthesis of epoxy resins, which are substances containing at least two epoxide groups and are capable of cross linking with or without other substances to form three-dimensional infusible networks[13]. Thus these are an important raw materials for polyesters or polyurethanes[33], as well as for the synthesis of non-phthalate plasticizers alternatives[34].

Vegetable oil plasticizers are the most used biological substrate for epoxides[35], and are often called bio-based plasticizers or "green plasticizers." The name is related to green-chemistry validation in terms of carbon footprint. Bio-based materials obtain carbon from a renewable feedstock or biomass such as corn or soybean and result in a reduced lifecycle greenhouse gases emission[36].

Additionally, epoxidized plasticizers are highly desired because they can neutralize radicals released by PVC in thermal and light degradation. Thus increasing the stability of the polymer chain[34]. Other benefits from the use of renewable sources for bio-based plasticizers is expressed in terms of toxicity and health hazards from finished materials. The use of vegetable oils as a starting material offers considerably less toxicity, inherent biodegradability and a high purity, thus allowing for high quality polyols[29]. This reduced toxicity can be explain by the elimination of phthalate leachates, which makes the material useful for applications sensitive to migration and toxicity such as in the cable manufacturing industry, food packaging, children's toys and medical devices[37].

The characteristics and benefits of epoxidized vegetable oil (EVO) materials have been showed for years. In applications like cable manufacturing, Brostow *et al.*[37] evaluated the effectiveness of EVO as alternative to petroleum based material and they concluded that in combination with a fully acetylated hydrogen castor oil, the material has comparable characteristics to traditional petroleum based cable plasticizer.

In lubricant applications, Borugadda *et al.*[23] evaluated the physicochemical and rheological characteristics of epoxidized waste cooking oil and found that the removal of unsaturation increases the thermal oxidative stability of the oil and allows a better polarity and intermolecular forces making it a potential alternative lubricant.

As coating materials for stabilization and food applications, Alam *et al.*[18] reviewed the benefits of vegetable oil epoxides that undergo curing reactions with acids, anhydride, amines, and carboxylic acids to produce chemically resistant coatings. This could potentially reduce the costs of the finished material without compromising on their performance and service life[18]. Similar effects were shown by Varganici *et al.*[38], in which epoxide resins from vegetable oils decrease UV degradation in wood materials.

Similar benefits are also reported in the synthesis of composites and hard plastics such as thermosets. Jian *et al.* found an increase in tensile strength, Young's modulus, and elongation at break when epoxidized soybean oil was cure with dicarboxyl-terminated polyamide1010 (NYL). The resulting thermoset had a good durability and excellent thermal stability, which open the possibilities for more structural applications like castings in the electrical industry, adhesives and glass fiber laminates[13]. All of these characteristics have enable the vegetable oil-based chemicals market to increase about 3% per year[39] with a market size valued at USD 20.1 billion in 2019. This

market is expected to grow at a compound annual growth rate of 5.8% from 2020 to 2027 globally[40] as an ideal substitute for petroleum-based products.

This growing demand highlights the need to optimize the current production methods for epoxidation of vegetable oils, such as soybean oil. The first point to address is related to an important drawback from the use of vegetable oils to synthesize polymeric materials and additives, which is related to the diversity in the degree of unsaturation (C=C), and stereochemistry of the double bond position in fatty acids of the oil. As mentioned before, the reactivity of the oil is given by the amount and location of the carbon double bonds within the fatty acid, because they act as functional sites for the introduction of acrylic polymers and copolymers[18]. Therefore, the composition of fatty acids in a vegetable oil becomes critical because it affects the yields of epoxide and the required time of reaction. These changes are given by variation in the reaction rates for epoxidation between the various unsaturated fatty acids found in vegetable oils.[41].

The second challenge is related to the initial modification of oil. Direct polymerization of the carbon double bonds is difficult because they are not very reactive to direct radical polymerization[42]. Therefore, the modification of double bonds, to an epoxide for example, is necessary for further conjugation and polymerization of vegetable oils.

Several reaction configurations and catalyst have been explored to optimize the epoxidation of vegetable oils in terms of yield and overall production costs as well as scale up for industrial applications. Modifications in the reaction process potentially impact the stereochemistry of the epoxide, as well as the physical properties such as viscosity and crystallinity. For example, non-radical epoxidation will form *cis*-epoxides whereas radical epoxidation forms both *cis*- and *trans*-epoxides[21]. We will discuss the epoxidation process in higher detail further.

2.1.1 Composition of oil and its effect on synthesis of plasticizers

As mentioned above, one of the major challenges of using vegetable oils is related to its composition. The degree of unsaturation is one of the major characteristics for vegetable oil that influences its effectiveness as an oleochemical. The higher the percentage of unsaturation, the higher degree of configurations that can be achieve from the chemical modification of oil. The chemical composition of the fatty acids, and thus the carbon chain length and degree of unsaturation depends which plant crop and genetic variation as well as the climate of cultivation[19].

Soybean and linseed oil have been reported as the best candidates for epoxidation due to their high content of unsaturation, usually expressed as iodine values from the method of chemical analysis of unsaturation[14].

Additionally, the major crops cultivated in the United States are corn, soybean, wheat and sorghum[43]. The U.S. is the world's leading soybean producer, which makes this feedstock readily available and relatively inexpensive. Additionally, soybean is the second-largest source of vegetable oil and comprises 90% of the country's oilseed production. This contrasts to the global production of plant oils, 25% of which is soybean oil[33].

The fatty acid composition has been described[44–48] for the most common oils used for oleochemistry and other industries and summarized in table 1. It can be observed that there is a high variability in amount of double bonds present in oil from different feedstocks. Although different applications require a defined number of carbon-carbon double bonds, typically a value greater than 2.5 is acceptable for synthesis of polyols[49]

Table 1 Fatty acid composition of commonly used vegetable oils.

Oil	Double bonds	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Others	Reference
Corn	4.5	16.1	0.1	2.6	28.2	50.5	1.2	1.3	[49,50]
Palm	1.7	39.32		4.36	42.52	11.35	-	1.9	[44,49]
Sunflower	4.7	7.1		4.7	25.5	62.4	-	0.3	[44,49]
Linseed	6.6	6.5		6.2	25	14.1	46.5	1.7	[48,49]
Canola	3.9	6.9	0.1	2.9	54.4	19.0	13.0	3.7	[49,50]
Soybean	4.6	11.4		4.4	25.5	62.4	-	0.3	[49,51]
High Oleic Soybean	3.0	11	-	4	76	7	2		[46,52]

In terms of epoxidation, the average number of double bonds defines the application of the epoxidized product. A larger number could provide a larger network of oxirane rings that could help in crosslink reactions for lubricant and thick coating applications[51] whereas a lower number could be more suitable for fuel applications[44] due to the lower viscosity and volatility.

Additionally, the presence of polyunsaturated fatty acids also affects the stability of the oil. High unsaturation makes the oil prone to oxidative, thermal, and hydrolytic degradation[53]. This degradation makes the lifetime considerably shorter than oils with lower degree of unsaturation like monounsaturated fatty acids. In general, higher the degree of unsaturation of the fatty acids

the more susceptible they are to oxidative deterioration[45]. Since chemical modifications are performed on the double bonds, it can also be expected that a more unsaturated oil is also more reactive to chemical modification like epoxidation, hydroxylation, and acylation[53,54].

In terms of economic feasibility, soybean oil varieties represent a good source of oleochemicals, with a market utility of 224 million metric tons in 2004[55]. Its use as an industrial or automobile lubricants on a large scale is effectively employed, as well as biodiesel, and modified grease and lubricants to resist oxidation [19,39,56].

In terms of fatty acid composition, common soybean oil contains two dominant fatty acids, linoleic acid (50% w/w) which has two double bonds in its 18-carbon chain (C18:2), and oleic acid (35% w/w) which has a single double bond in its 18-carbon chain (C18:1)[57]. This results in an average of 4.6 double bonds per molecule in soybean oil[49].

There are several methods to modify the fatty acid composition of oils. Recently, genetic engineering methods have facilitated the modification of fatty acid composition from plant varieties in order to obtain high oleic acid in the oil extracted. One example is novel varieties of high oleic soybean oil, where the content of oleic acid surpasses 75% in the oil weight composition. The reduced concentration of polyunsaturated fatty acids in the oil increases the oil stability index without requiring the addition of antioxidants[58]. Additionally, the reduced concentrations of polyunsaturated fatty acids still retains an average 3 double bonds per mole compared to traditional soybean oil in which the average number of double bonds is 4.6[42], thus high oleic soybean oil varieties are excellent candidate for applications as epoxidized vegetable oil.

The use of high oleic soybean oil or any high oleic oil also modifies the kinetics of the reaction. Chua *et al.*[59] describe the general influence of substrate properties on the kinetics of epoxidation. First is the length of the substituents which correlates to slower reaction rates, as well as the degree of conjugation effects. Additionally, Kamalakar *et al.*[53] reported that an increased concentration of oleic acid allows for better physicochemical properties like low pour point, high viscosity, good thermal and oxidative stabilities in the epoxidized product[53]. Steric hindrance reduces reactivity of carbon-carbon double bones as it gets closer to the glycerol backbone[59], thus increasing the reaction times. The use of high oleic soybean oil allows for more stable production of epoxides and therefore a more robust system control in potential industrial applications.

2.2 Epoxidation of high oleic soybean oil

The epoxidation of soybean oil among other oils serves as platform chemicals as it allows us to synthesize environmentally friendly lubricants, plasticizers, polymer stabilizers, paint and coating components, as well as important intermediates for production of alcohols, alkanolamines, glycols among other polyols[14,29,60].

As mentioned above, epoxidation is the process to incorporate an oxirane ring into the unsaturation sites of vegetable oil, and epoxides are highly reactive, versatile, polyfunctional intermediates that are used for the production of chiral compounds[59]. An oxirane ring is three membered cyclic ether ring[61], arguably the most synthetically useful small ring because of its ease of synthesis and wide-ranging ring opening reactions[62] at moderate reaction conditions[63]. The addition of oxirane rings into the oil composition will increase the viscosity, activation energy and melting point properties[46] thus making it a good industrial material for polymerization and conjugation.

Traditionally, the epoxidation process carried out industrially is performed by acid catalysis of peroxides, also called the Prileschajew or Prilezhaev reaction. This process has been performed since the late 1940s. It consists of the transfer of a terminal oxygen of a peracid to an olefin[64]. In other words, the carbon-carbon double bonds in oil is oxidized using a highly reactive per-carboxilic acid or peracid of free fatty acids (PFFA) this is synthesized *in situ*[65] to form the oxirane ring of epoxide. The preliminary step of peracid formation happens in the aqueous phase. It requires a short chain alkyl or carboxylic acid as substrate, such as acetic or formic acid, to react with hydrogen peroxide as oxidant with mineral acids such as sulfuric acid to catalyze the reaction. When formed, the peracid migrates into the organic phase of the system and spontaneously reacts with the double bond of the oil, thus forming the oxirane ring of the epoxide[66].

This reaction is highly exothermic, and control of the temperatures in the reactor is monitored by limiting the addition of the hydrogen peroxide oxidant to form the peracid. The best temperature of reaction is usually between 60-75°C, with reaction times between 8 and 12 hours to achieve epoxide yields of 80% and double bond conversion of 98%[66].

This shows some of the drawbacks in the Prileschajew epoxidation process. First, the process is non-selective which leads to low yields and is prone to side reactions that open the epoxide ring[65]. Second, the peracid formation in the presence of mineral acid is strongly corrosive, highly exothermic. Furthermore, the accumulation of peracids can be unstable and explosive,

therefore it is critical that heat removal controls be employed[63]. Epoxidation under these harsh conditions leads to low productivity and yields due to oxirane ring-opening reactions, as well as corrosion of the equipment and highly hazardous operating conditions[67,68].

As described above, this reaction is carried in heterogeneous conditions with an organic phase of the oil substrate and an aqueous phase of the hydrogen peroxide and acid solutions. Often solvent like toluene is added to the reaction mixture to increase miscibility between the phases. To understand the specifics of the process it's important to see the simultaneous reactions happening, such as the formation of the percarboxylic acid (also called perhydrolysis), the decomposition of the percarboxylic acid, the epoxidation step and the degradation of the oxirane ring (also called ring-opening)[69].

Conversion and efficiency of the process are measured by three methods. First, the degree of unsaturation or available carbon double bonds is measured by the iodine value method. This method can measure the conversion of double bonds and can be expressed as a percent conversion to oxirane.

$$Relative\ conversion\ to\ oxirane = \frac{OO_{experimental}}{OO_{theoretica}} * 100\%$$

The content of oxirane oxygen found in the epoxide product is measure as the progress from the theoretical amount:

$$OO_{theoretical} = \left[\frac{IV_0/2Ai}{100 + (IV_0/2A_i) * A_0}\right] * A_o * 100\%$$

where A_i and A_0 are the atomic weights of iodine and oxygen respectively and *IV0* is the initial iodine value of oil. The total conversion of double bonds dictates some of the oxidative behavior of the epoxide[70]. Finally, quantifying the acidity in the medium is used to calculate the total hydrolysis of fatty acids from the glycerol backbone of the oil. This is important because peracids are expected to be formed from these free fatty acids and regenerated as free fatty acids as the oxygen transfers to the unsaturated carbons to form the epoxides.

In acid catalyzed processes, it is common that the consumption of carbon-carbon double bonds is completed at relative low conversion of oxirane. However, reactions are aimed to be correlated by the decrease of iodine value by an equal amount oxirane oxygen. This means that there is a side product formation from ring opening reactions thus losing the desired reaction product. These ring opening reactions can be catalyzed by a variety of nucleophiles, and the rates

are dependent of the pH. When the pH is low, the protonation of the epoxide group is favored to carry out the ring-opening reactions[71].

Due to the increasing demand for epoxidized soybean oil and other epoxidized vegetable oils, production is estimated at more than 200,000 tons annually[59], and therefore optimization of the process is important. Several approaches have been explored to optimize the epoxidation reaction. These can range from the addition of neutralizing steps to reduce side reactions of the epoxide product[72], to the modification of the separation and purification processes to reduce cost of complicated post-processing operations[73].

For example, Kotlewska *et al.*[74] determined that including ionic liquids in the reaction mixture can increase the polarity and miscibility of the phases, leading to higher yields. This is achieved by hydrogen-bond-donating (HBD) liquids which exhibit a positive solvent effect for oxygen transfer by the peracids[74]. Nevertheless, addition of more reagents can increase the overall cost of the production. Others have focused on the reaction configuration, modifying the fed-batch reactor to continuous operation using a packed bed reactor. Santacesaria *et al.* carried out epoxidations in a reactor bed packed with stainless steel to have static mixing of the immiscible reactants. They validated a biphasic kinetic model and found that continuous operation is feasible at faster flow rates at high temperatures[66].

Other important topic for improvement of epoxidation at scale is related to the catalyst employed for peracid synthesis. Strong inorganic acids have been evaluated for their effectiveness to form peracids, H_2SO_4 be the most common catalyst used in commercial synthesis. Replacement of this strong mineral acid is important in order to reduce waste generation and to lessen corrosion of equipment[65,75].

Sienkiewicz *et al.*[54] refer to three conditions for an effective catalyst in oxidation of soybean oil, first selectivity of oxidation to epoxidized product, second the epoxidation reaction efficiency, and finally the simplicity to its removing from the post reaction mixture of final product[54].

The catalyst used and its efficiency is a critical parameter in every industrial process. Different types of catalysts have been suggested to optimize the Prileschajew reactions and increase the yields and efficiency of the epoxidation. These can be homogenous to the reaction such as the inorganic acids already employed. The drawbacks to their use is that they can lead to

ring opening reactions, thus suppressing the selectivity of the reaction[71,76], they are corrosive, and not recyclable.

Heterogeneous catalysts such as acidic ion-exchange resins and Ti-based catalysts have been tried as well with positive results in conversion rates[33,77]. Acidic ion-exchange resins are functional polymers that facilitate acid-catalyzed reactions by providing acidic sites in a solid heterogeneous medium[65].

The use of heterogeneous catalyst presents a more environmentally friendly alternative since the catalyst can be retained and recycled, therefore cutting post processing neutralization operations[65,76]. However, the use of Ti-based catalysts often do not have the pore sizes large enough to allow the reaction of large substrates such as triglycerides[65]. Acidic ion-exchange catalysts require large amounts of loading (15-20 wt%)[65] which makes their industrial application is limited, and have been found to undergo chemical and physical degradation and must be changed after 6-8 runs[78].

Other optimization parameters for epoxidation have focused on the peracid substrate. Among these is the common commercial peracid, m-choroperoxybenzoic acid (MCPBA) which has been reported as an effective catalyst for epoxidation[79,80]. This is a common peroxyacid that can be dissolved in the reactants for epoxidation[81]. However, it is usually not available at larger scale and therefore has not implemented by industry.

Overall, the main objective of improving the catalyst used is to increase the selectivity of the reaction without higher energy demands. A feasible alternative to achieve this is the use of biological based catalysts like cells or enzymes. The latter has been extensively studied for over 150 biocatalytic processes which are practiced industrially[73,75].

2.3 Enzymes as versatile epoxidation biocatalyst

2.3.1 Enzymes as biocatalysts

Enzymes are large macromolecules composed of polymers of amino acids that work as the catalyst of living organisms. Their catalytic activity happens within the active site, a structure often found deep within the proteins structure[82]. Enzymes can perform a wide range of reactions efficiently by reducing the activation energy to perform a reaction. It has been reported that enzymes can perform reactions as fast as substrates diffuse with reaction factors of 10^{13}([83,84]). Due

to this, the use of enzymes industrially has been increasing due to reduced reaction times, low energy inputs and cost effectiveness as well as to achieve ecofriendly characteristics[82].

Enzymes are classified in families depending on the reaction performed. Such activity is given by the crystal configuration of the active sites[85]. Although enzymes are known to be selective and substrate specific, some enzymes are known to catalyze a wide range of substrates[86]. One family of enzymes of great industrial importance is the α/β-hydrolase fold enzymes with a Ser-His-Asp active site.

Rauwerdink *et al.* elaborated on the different mechanisms employed by this family of enzymes and elucidating the differences between the 17 different reactions catalyzed, spanning from hydrolases mechanisms such as esterase, thioesters and lipases to lyases-type mechanisms using only general acid-based catalysis[85]. Additionally, esterase and lipases, which catalyze hydrolysis of esters, have overlapping catalytic activities with non-heme haloperoxidases, which catalyze oxidations by hydrogen peroxide via a peroxycarboxylic acid[86].

Lipases (triacyl glycerol acyl hydrolases EC 3.1.1.3) are one of the most frequently used enzymes in organic synthesis and are used in the synthesis of optically active alcohols, acids, esters, and lactones[87]. Lipases are very well known for their substrate specificity, stereoselectivity and enantioselectivity [88]. As industrial catalysts lipases and esterase have been used widely for polymeric synthesis, they allow high selectivity, and mild reaction conditions. Processes using different forms of lipases can be found from chemical industry to the medical high specialty production of therapeuticals[88]

Lipase-catalyzed reactions take place at the interface between the insoluble substrate and the aqueous phase, where the hydrophobic/hydrophilic interactions of the protein allow access of the substrate to the active site[87]. They naturally hydrolyze triglycerides releasing, fatty acids and glycerol but have been used *in vitro* for a number of other non-natural reactions including transesterification, esterification and epoxidation in non-aqueous and aqueous media, making them incredibly versatile and of potential industrial applications[89].

The versatility presented by this lipase is often posited to be due to the similarity of the catalytic triad to the active sites in serine proteases, which suggest that the mechanism is similar. The catalysis of a reaction begins with a nucleophilic attack on the carbon from ester bond of the susceptible substrate by the hydroxyl group in the serine residue, forming an acyl intermediate that

releases the water or alcohol from the lipid. Then the acyl intermediates is hydrolyzed, regenerating lipase[90].

One of the most used lipases is lipase B from the yeast *Candida antarctica* (CALB), it is well known for its catalytic promiscuity. As other lipases, CALB primary reaction is the hydrolysis of ester bonds but it has been suggested that it is an effective catalyst for a carboxylic acid like esterase, thioesterase, peptidase, dehalogenase, epoxide hydrolase, or halo peroxidase[91].

Further developments on bio-catalysis using CALB have been in terms protein engineering and immobilization support to increase stability of the enzyme. In general, the stability and reusability of the enzyme can be improved with immobilization techniques, where the protein is adsorbed by different means like covalent attachment, and entrapment in polymer or inorganic matrices[92]. The choice of using free soluble enzyme or immobilize it is a matter of cost and application.

The use of enzymes in immobilized form facilitates handling and increase the stability by their chemical conformation, allowing a robust applications in wide ranges of temperature and pH[93]. Most industrial applications using CALB employed an immobilized form of the enzyme. Commercially available enzymes incorporate resins with different pore size to reduce mass transfer limitation. One of the most reported commercially available immobilized lipases is N435 which is CALB in immobilized in microporous resins of (Lewatit VP OC 1600)[92]. N435 uses an ionic resin support often referred as Lewatitt which by itself has been used as catalyst in other processes. Other immobilization supports take advantage of the hydrophobic interactions of the support to increase the interfacial area in heterogeneous reactions as well as the catalytic effectiveness of the enzyme[83]. Other effective supports reported include Eupergit C®, Sepabeads®, and Immobead 150®. The latter is formed by a mesoporous methacrylate polymer with epoxy functions the particle size of the support ranges from 150-300 μm[94], and a pore size of 2-50 nm[95].

The difference in immobilization support can impact both the enzyme activity and the physical stability of the support during the process. Larger materials could potentially be more exposed and damaged by shear stresses in batch operation conditions. On the other hand, smaller materials and pore sizes increase diffusion limitations in and out of the support, thus decreasing the reaction rates of the enzyme.

One mayor drawback of the use of N435 has been related to desorption of CALB into the reaction medium during reaction, making multiple reuses unattainable[92]. Whereas Immobead 150®

is covalently bound and is less prone to leaching, but this can also be a drawback as the enzyme can be incorrectly bound to the support, rendering the enzyme inactive.

The use of lipases over chemical catalyst offer several advantages such as lower reaction temperatures, higher purity of the product, and reduced downstream processing. Their use is limited by high cost of immobilized enzymes compared to chemical catalyst[93]. Most industrial applications of lipases are related to modification of vegetable oils due to health concerns. In the chemical industry, use of lipases has been common for enantiopurity materials such as the purification of chiral amines, a process patented by BASF in 1995[96].

Considering the application of the enzyme in epoxidation and the intention to obtain pure epoxidized soybean oil the clear application of enzymes corresponds to immobilized form that can be retain for recycle and not be present in the final product. The use of lipases as catalysts in epoxidation has been proposed as a feasible alternative for epoxidation for more than 20 years. Enzymes such as lipase are very well known for their catalytic promiscuity[91,97], and known to perform non-natural reactions efficiently and under mild conditions.

2.3.2 Lipase-catalyzed epoxidation of soybean oil

The use of lipases as a biocatalyst for epoxidation allows for higher selectivity, and also decreases the hazardous reaction conditions and lowers energy requirements. Investigation by Sienkiewicz *et al.* points out that the use of enzymes allows more productivity in epoxidation[54].As mentioned before, the most reported enzyme for epoxidation is CALB. It is advantageous because it does not require additional cofactors and it works as part of interphase activation making the activity mechanism of high robustness[67,98].

Lipase-catalyzed epoxidation of vegetal oil can be chemically described in three steps, first, breaking apparat an initial concentration of triglycerides into their respective fatty acids through the hydrolytic activity of lipases. This process involves the hydrolysis of vegetable oil, breaking the ester bonds in the glycerol backbone and consuming a water molecule to release one free fatty acid and a glyceride. Second, the released free fatty acid is converted into a fatty peracid by the lipase-catalyzed peroxidation in which hydrogen peroxide is consumed and the active oxygen is attached to the carbonyl carbon end of acid chain. Finally, the highly reactive fatty peracid oxidizes the unsaturated carbon double bonds (C=C) of the triglycerides thus forming the oxirane oxygen

ring. This process is summarized in figure 2, which shows the glycerol backbone in orange boxes, the active oxygen in blue and the oxirane oxygen of the epoxide in green.

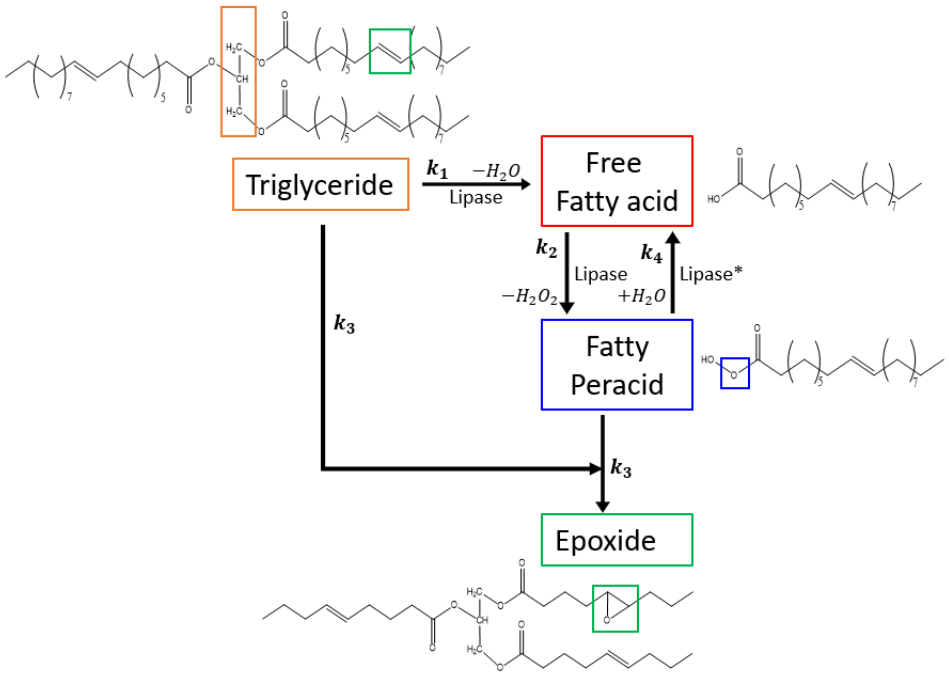

Figure 2 Reaction scheme in 3 steps for lipase-catalyzed epoxidation.

Effectiveness of the reaction, similar to acid-catalyzed epoxidation, is given first by the ratio of oxidant hydrogen peroxide to double bonds, which set the oxygen transfer balance of the reaction. Second, due to the high cost of the enzyme catalyst, the least amount of added catalyst is optimum. Optimization of these parameters have been investigated for the epoxidation of multiple substrates in different conditions[98–103].

For example, in the first subject, Orellana-Coca *et al.* determined that hydrogen peroxide is a critical parameter in chemo-enzymatic epoxidation since a lower concentrations will not provide the necessary oxidant for epoxide formation but an excess can easily inactivate the enzyme catalyst[102]. The loss of enzyme activity occurs when the concentration of hydrogen peroxide is too high, a characteristic that is necessary for high yields of epoxide. In general, Steinhagen *et al.*[100] have described the inactivation of the enzyme due to oxidation of disulfide bridges and have reported further protein engineering efforts to replace these disulfide bridges with amino acid less prone to oxidation. While a promising approach, no commercially available modified enzyme is now available.

Examining again the balance of double bonds to oxidants, it has also been a common practice to add an initial concentration of free fatty acids to accelerate the epoxidation reaction rates and reduce the generation of diglycerides and monoglycerides. The addition of free fatty is intended to accumulate peracids in the reactor rapidly. Lipase catalyzed peroxidation has been reported for more than 25 years. Björkling *et al.*[104] first evaluated the synthesis of peroxides from free fatty acids in different configurations as part of Baeyer-Villeger oxidation of ketones and alkenes as a mild and safer alternative[104]. The mechanism of the lipase is likely similar because of the structure of the active site in CALB [85].

Per-hydrolysis of the free fatty acids incorporates the use of serine, histidine, and aspartate to carry out peroxidation of the fatty acids. This mechanism has been described in two steps. First, the carbonyl carbon of the free fatty acid gets activated by catalytic serine forming an acyl intermediate. Then catalytic histidine removes the proton of hydrogen peroxide allowing the nucleophilic attack on the carboxylic acid to form the peracid, while the oxyanion hole of the active site stabilizes the reaction[105]. As mentioned above, the peracids that are generated are highly reactive, and their consumption can lead to productive epoxide formation or nonproductive free fatty acids conversion as shown in figure 2.

The second parameter of optimization, the minimum amount of enzyme to achieve high yields, was investigated by Vlček *et al.*[106] using soybean oil as a substrate. They found that enzyme concentrations lower than 4 wt% strongly affect the rate of formation of epoxide, and larger than this increase the rate of hydrolysis of soybean oil. This information is valuable to reduce unnecessary addition of enzyme.

2.3.3 Potential industrial implementation of lipase-catalyzed epoxidation

The benefits from the use of enzymes in epoxidation of soybean oil and more so in the use of high oleic soybean oil are clear. First, the process is performed in mild conditions. Second, the reaction is highly selectively and third, the epoxide is produced at stable conditions unlikely to result in degradation. Nevertheless, there is also important challenges to effectively use lipases, such as the loss of catalytic activity and the high cost of the enzyme.

As mentioned by Basso *et al.*, in an industrial process the cost contribution from an immobilized enzyme is dependent on the reaction kinetics and the number of cycles the enzyme is reused as an indirect measure of total productivity[93]. A technoeconomic analysis of the process

using lipases previously performed at Purdue University determined that the optimized process as was not economically competitive to acid-catalyzed epoxidation unless the loss of enzyme could be minimized to enable continuous epoxide formation for 100 days or more with one batch of immobilized enzyme.

Danov *et al.*[65] listed the most important drawbacks of using enzymes as catalysts for an industrial process grouped into four categories: (1) the use of large solvents concentrations, (2) the low catalytic activity towards triglycerides due to a steric hindrance with the interaction of the catalytic center and the large molecules substrate, (3) the rapid deactivation of lipases under certain conditions (high temperature, high concentration of the solvent and H2O2) and (4) the high cost of enzymes.

Regarding the first category, the large use of solvents has been refuted in research by Zhang *et al.*[52], by successfully carrying out the epoxidation of high oleic soybean oil at mild conditions in a solvent-free system using an enzyme catalyst obtaining yields higher than 90% in a solvent-free system. This shows that although the process is performed by the enzyme in a lipophilic-hydrophilic interface[57], mass transfer limitations by the two non-miscible liquids have little effect on the efficacy of the process[103].They additionally defined the optimum conditions for high yield epoxidation with as 4% wt. enzyme loading, 2:1 ratio of hydrogen peroxide to double bonds and added over 20 minutes and no initial concentration of free fatty acids.

The second factor, low catalytic activity towards triglycerides due to a steric hindrance with the interaction of the catalytic center and the large molecule of the substrate. This has been described by multiple authors[52,102], and can be addressed with the use of high oleic soybean oil, where high steric hindrance reactants such as linolenic acid are found in small concentrations thus reducing the impacting the reaction rates. Although the kinetics of the process using high oleic soybean oil have not been reported to our knowledge, the conversion rates of the three common fatty acids have been described by Zhang *et al.*[52], elucidating the change in rates given by the number of double bonds.

Finally, the last 2 drawbacks mentioned by Danov *et al.*[65], the rapid deactivation of lipase and the high cost of the enzyme are equally important and have not been fully elucidated to our knowledge. The scope of this project is to understand the decay of hydrolytic activity and decrease in epoxide production in batch epoxidation of high oleic soybean oil using CALB immobilized in Immobead 150®support. Additionally, understand the effect of parameters such as temperature,

hydrogen peroxide addition rates and concentration of hydrogen peroxide on the decay of the hydrolytic activity and epoxide product formation.

MATERIALS AND METHODS

3.1 Materials

High Oleic Soybean oil was kindly donated by Catania – Spagna Corporation, 3 Nemco Way, Ayer, MA USA01432-0227. Lipase B from *Candida antarctica*, recombinant from *Apergillus oryzae* and immobilized on Immobead 150 (Particle size around 300µm, enzyme activity ≥5000 U/g) was purchased from Millipore Sigma. Stainless steel meshes were purchase from McMaster-Carr. Wijs solution, acetic acid glacial ACS reagent, isooctane ACS reagent, hydrogen bromide solution 33 wt.% in acetic acid, potassium iodide, p-nitrophenyl acetate, and hydrogen peroxide solution 30% w/w, was purchased from Sigma Aldrich. T-Sac Tea Filters chlorine-free were purchased from local supermarket.

3.2 Methods

3.2.1 Chemo-enzymatic epoxidation of high oleic soybean oil

Epoxidation was carried according to Zhang *et al.*, a 250 mL three-necked, round bottom flask was placed in a water bath set to 35°C and on top of a magnetic heating stirring plate. High oleic soybean oil (50.0g) was combined with 2.0 g (4.0 wt. % of oil) of Lipase B immobilized on Immobead 150® support and agitated using a polytetrafluoroethylene-coated magnetic stir bar. Epoxidation reaction was carried out for 20 Hrs. Samples of 10 mL were taken at a predetermined time interval. After sampling reaction tubes were centrifuged at 3000 RPM for 5 minutes. After centrifugation, the three layers formed were separated, the supernatant oil on the top was placed in a different centrifuge tube and 10% w/w potassium sulfate was added and centrifuged again at 3000 RPMs for 5 minutes. The separated oil was stored at -20° C for further analysis. The enzyme retention was placed in a T-sac and set to dry for further analysis.

3.2.2 Hydrolysis of high oleic soybean oil

Determination of non-selective hydrolysis of soybean oil was evaluated at the epoxidation conditions mentioned before. Separation of the individual hydrolytic elements was: selective enzyme activity hydrolysis evaluated from the commercially available lipase B on Immobead 150,

non-selective hydrolysis from the immobilization support was evaluated by heat deactivation of the enzyme in water at 65°C for 24 Hrs. followed by additional 24 Hrs. of drying time. Nonselective oil hydrolysis by degradation in aqueous media was evaluated by running the reaction without enzyme catalyst at the same epoxidation conditions. Lipase catalyzed hydrolysis was evaluated by acid number of the total acid values to take into account the non-selective hydrolysis of soybean oil.

3.2.3 Epoxidized soybean oil characterization

Characterization of epoxide product was performed following standard methods. Iodine number was performed following the standardized method ASTM D5554-15. Oxirane oxygen titrations were performed according to the American Oil Chemist Society (AOCS) following methods Cd 9-57. Acid number was determined through acid number titration following the process described by Hagström *et al.* using 0.1g of oil sample added to a 50mL flask and dissolved in 10mL of ethyl acetate. 4ml of phenolphthalein 1% v/v in ethanol was added, and the solution was titrated with 0.03M potassium hydroxide in ethanol until a change in color was observed. Calculation of acid values was calculated using equation XX.

$$Acid\ Number = \frac{c_{titrant} * v_{titrant} * MW_{KOH}}{m_{sample}}$$

Where $c_{titrant}$ is the concentration of KOH (M), $V_{titrant}$ is the volume of KOH, MW_{KOH} is the molecular weight of KOH (g/ml), and m_{sample} is the mass of the sample (g). All samples were analyzed in triplicate.

3.2.4 Enzyme recycling for evaluation of decay

Evaluations of decay were performed on the analyzed product for acid number, iodine value, and oxirane oxygen after consecutive reactions in batch conditions and following the epoxidation settings mentioned above. The reaction time for epoxidation was 12 hrs. After this time, the liquid was filtered through a 0.0046" steel mesh and placed in a 15ml centrifuge tube. The retained enzymes were washed with fresh high oleic soybean oil (50.0 g), and the reactions were re-initiated. This process was repeated for five consecutive cycles.

3.2.5 Residual enzymatic analysis

Analysis of residual enzymatic activity was performed through the hydrolysis of P-nitrophenyl acetate following the method by Kademi *et al.*[107], with slight modifications. After liquid filtration, samples were collected and let dry for 8 hours; 3.0 mg of dried enzymes were scaled and placed in a 2ml centrifuge tube. Reaction tubes were prepared with 980.0 µl of phosphates buffer (7.4 pH) and 10µl of 50mM solution of p-nitrophenyl acetate in acetonitrile. The tubes were let to react in a dark area for 10 minutes. After the reaction time, the liquid content was transferred to a cuvette and measured at 405 nm in a spectrophotometer.

3.2.6 Physical parameters and their effect on lipases stability and production decay.

The effect of several physical parameters was evaluated by three factors, first the concentration of hydrogen peroxide was evaluated by replacing the solution of hydrogen peroxide by di-ionized water and running the cycle under the same epoxidation settings as the method for enzymatic recycling and for 4 consecutive periods..

The second parameter to evaluate is the effect of hydrogen peroxide addition to the reaction, three different addition rates were evaluated for their effect on hydrolysis and epoxidation decay. Hydrogen peroxide addition flows were 1 hour (0.441ml/min), 3 hours (0.147 ml/min) and 6 hours (0.0715 ml/min). All addition of hydrogen peroxide was performed using a Harvard apparatus PHD 2000 pump and a Cole-Parmer clear disposable 25 mL clear syringe pump.

The third parameter to evaluate was temperature, analysis was evaluated at the normal 35°C of epoxidation and the second without temperature control which was monitored to be at 22°C ± 1° C.

The results were evaluated as the theoretical activity (%) on the comparison of decay rates and analyzed as the results after each reaction period therefore decay rates are given by the change from initial cycles for acid number, iodine value and oxirane oxygen.

RESULTS AND DISCUSSION

4.1 Lipase-catalyzed hydrolytic activity rates

In order to assess the enzyme-catalyzed hydrolysis of the high oleic acid soybean oil, we first needed to determine the initial amount of free fatty acids in the oil, the rate of natural breakage of triglycerides in aqueous media, and if there is any non-specific hydrolysis catalyzed by the immobilization support. Separation of these parameters will help validate lipase activity and study it individually for its stability in high oleic soybean oil hydrolysis as part of an epoxidation.

Element discrimination analysis shows that background hydrolysis does not exist over the reaction period analyzed, but there is an initial FFA concentration, which remains constant when no enzyme catalyst is present through the reaction period (12 Hrs.). Figure 1 shows the values for free fatty acids (% of total fatty acid) obtained from an acid number analysis. Reaction conditions were kept the same, following the previously optimized epoxidation conditions, 35°C, constant stirring at 400 RPMs, batch reactor configuration using high oleic soybean oil, and 30% (w/w) hydrogen peroxide solution.

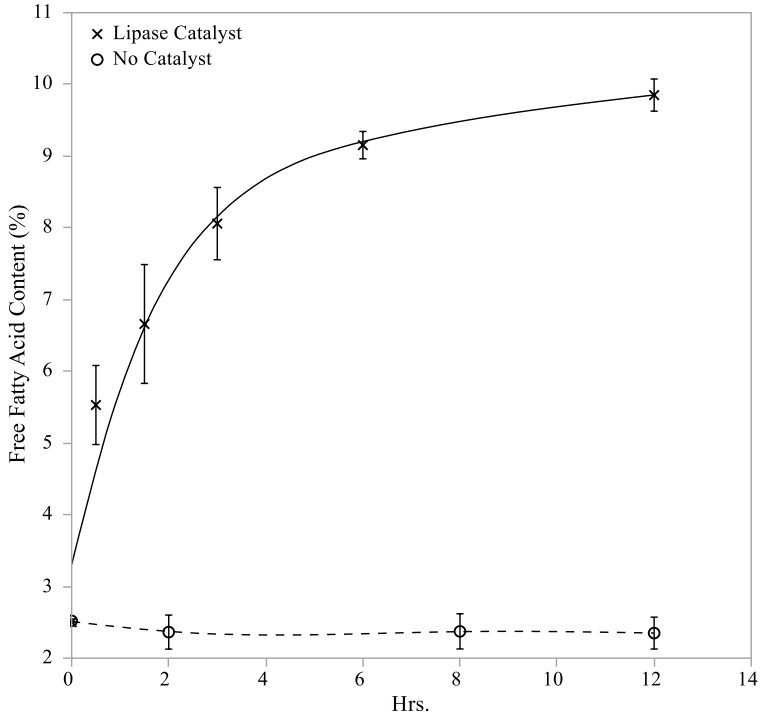

Figure 3 Hydrolysis of high oleic soybean oil using non-catalyzed methods (non-selective immobilization support and oil hydrolysis when in agitation with water) and comparison to catalytic lipase activity. Batch reactor 35 °C, 400RPM.

The accumulation of free fatty acids in oil is given from hydrolysis of triglycerides. To represent the different sources of hydrolysis in terms of kinetics, we use a complex constant *f* that represents the combined action of the hydrolytic elements mentioned above, including lipase-catalyzed hydrolysis, and non-catalyzed natural background hydrolysis along with non-selective ionic resin aided hydrolysis. It can be observed from figure 1, that the concentration of free fatty acids at time 0 (FFA_0) is 2.54%, this concentration is retained constant over the reaction periods of 12 hrs.

The constant value starting from FFA_0 shows the stability of high oleic soybean oil at the given conditions is not surprising, high oleic soybean oil in contrast with regular soybean oil has a reduced number of polyunsaturated soybean oil which has been developed for high heat and oxidative stability[108], therefore the short time of reaction and low temperatures did not significantly affect the amount of free fatty acids.

The kinetics of the lipase-catalyzed hydrolysis have been investigated extensively[109–111] and it has been described to occur in a stepwise manner starting with the hydrolysis of triacylglycerol (TG) to diacylglycerol (DG), following to monoacylglycerol (MG) and ending in glycerol. This suggests that there are different rates per each glyceride released. Nevertheless, the scope in this research focuses only on the released free fatty acids not considering the glycerol backbone configuration. Therefore, just as Cheirsilp *et al.*[112], we assumed that there is no significant change between the hydrolysis rates of TG, DG or MG as a single element is quantified in the analysis. This approach is often used in the chemo-enzymatic synthesis of biodiesel where all free fatty acids released are treated under a single constituent regardless of the source[112].

As observed the lipase-catalyzed hydrolysis increases rapidly over 6 hours and then reaches a plateau after 9% free oleic acid in the system. The plateau reached in lipase-catalyzed hydrolysis after 6 hours shows that as reaction progresses the produced FFA leads to product inhibition of the hydrolysis [113,114]. This inhibition could also facilitate the per-hydrolytic activity of the enzyme to produce peracid intermediates from the FFA available in solution. Additionally, if we assume that there is a high turnover rate of peracid due to oxygen transfer from H_2O_2 to fatty peracid to form epoxides, product competition could potentially shift the balance of the reaction towards recycling of FFA to peracids.

Finally, in terms of analysis, the total released free fatty acids can be adjusted by subtracting the non-catalyzed FFA, this allows the adequate quantification and analysis of the

enzyme hydrolytic activity and its stability over longer periods of time in a flow-through reactor or over subsequent uses of the enzyme across multiple batch reactions.

4.2 Loss of hydrolytic activity in batch epoxidation

Epoxidation is an heterogeneous process that occurs in three phases: organic, aqueous and the immobilized enzyme, these phases are continuously in contact to produce the epoxide product but this also means that the main issue is related to poor stability of the enzymatic activity to perform the reactions for long periods of time. Nevertheless, the analysis focused on optimum yields does not take into consideration this important factor. Therefore, evaluations of stability or present state of enzymatic activity lifespan are important for scale-up of the process.

As mentioned before, analysis of the stable life span of hydrolytic activity must be adjusted by removing the initial concentrations of free fatty acids after endpoint quantification of consecutive batches. This means that the function of decay is reflected only by the change of released free fatty acids after consecutive batches and in terms of residual enzymatic activity as determined by the hydrolysis of p-nitrophenyl acetate (PNPA).

The function of decay of lipase hydrolytic activity is presented in Figure 6, the reaction conditions are kept as the previously determined optimum yield conditions, and reaction periods are set to 12 hours, after which, liquid materials are filtered and fresh substrate is added to the retained immobilized enzyme. No rinse or dry period are used on the enzyme catalysts prevent potential loss of activity of the enzyme from this additional step.

Figure 6 shows the decay of theoretical hydrolytic activity evaluated by the change of released free fatty acids after each epoxidation batch. It can be observed that the hydrolytic activity (%) follows a strong decay in the first 2 reaction cycles and within the first 20 hours of epoxidation conditions.

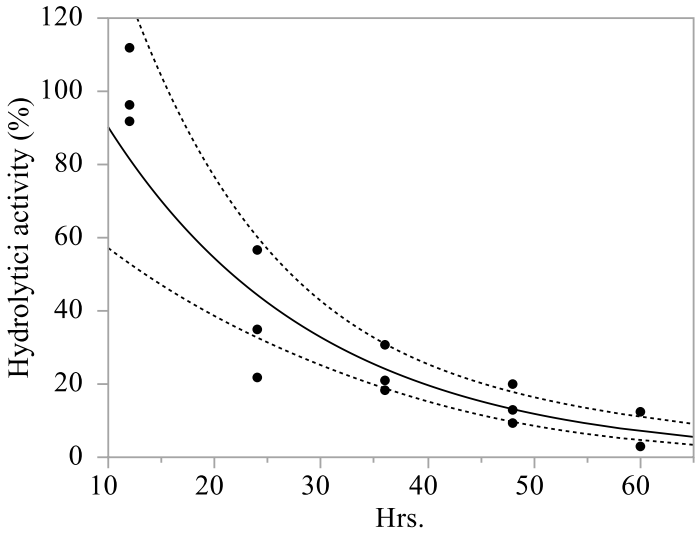

Figure 4, Decay of theoretical activity calculated as X = [FFA_X / FFA_1] × 100. FFA_1 represents the released free fatty acids after batch 1. Initial concentration of FFA (FFA_0) calculated at 2.54. This value was adjusted for analysis of lipase activity. Reaction cycles set to 12 hours, 35°C (±1°C) and 400RPM constant agitation. Solid curve represents the fit as Log values of hydrolytic results. Dotted line represents the confidence intervals at 95% from the semi log fit.

The decrease in hydrolysis is measured through the acid values in the oil and quantified as the percentage of free fatty acids in the system. Figure 3 shows the rate of decay for hydrolytic activity with continuous recycling of the lipase catalyst.

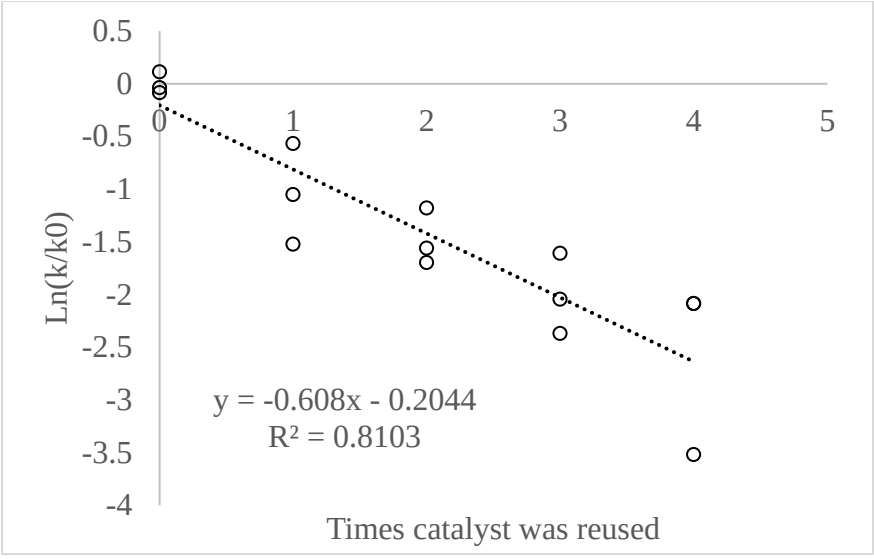

Figure 5, Linearization of the exponential decay of enzymatic hydrolytic activity (%). Half-life ($T_{\frac{1}{2}FFA}$) calculation at 13 Hrs. from the initial results.

From the continuous recycling of the catalyst we can estimate the half-life of the enzyme to be 25 hrs. This is calculated from the decay rate per cycle times the reaction conversion time (12 hrs.). The loss of enzymatic activity in the hydrolysis of oil follows a first order reaction decay and can the result of multiple causes such as temperature deactivation, lipase oxidation by high concentrations of hydrogen peroxide, or sheer stress of the solid immobilization support after continuous agitation the combinations of these factors.

For example, Aouf *et al.* found that lipases are rather stable in 6-12M of hydrogen peroxide at 20°C whereas at 60°C the activity is lost rapidly[98]. Increase in temperature has been tested as a detrimental condition on enzymatic activity in several lipase-catalyzed processes[67,115,116], but this parameter is also found in many research as a yield optimization parameter[52] because high concentrations of hydrogen peroxide are required for high epoxide yields.

Another important parameter of enzyme deactivation is hydrogen peroxide concentrations. As a strong oxidant, high concentrations of H_2O_2 have been described as main reason of loss of activity due to protein oxidation of the aminoacidic residues within the lipase active site[117].

Oxidation of the lipase active site and reduction of hydrolysis impacts the overall reaction rates and yields of epoxidation, because the hydrolysis is a prerequisite for the formation of peracid of free fatty acids (PFFA) which later oxidize double. Therefore, fewer FFA available represents less source of PFFA. Additionally, even though hydrolysis does not need H_2O_2 as a substrate, the high concentrations in the system are detrimental to the protein structure which is also an important characteristic of the process.

Other than reducing the total concentrations, some research has pointed out that slowing the addition rates of H_2O_2 and therefore reducing the initial concentrations in the system to the required conversion levels while still maintaining the mass balance for optimum yields could slow down the deactivation of the biocatalyst[67,103,118], but this does not show consistent results in terms of enzyme stability. The slowed addition of hydrogen peroxide is yet practiced at a conservative level of 20 minutes due to the highly exothermic reaction that takes place in chemical epoxidation, in which strong acid conditions aid the transfer of oxygen from hydrogen peroxide to the acid catalyst to form the peracid. Perhaps further engineering approaches could be addressed towards the systems design to decrease the exposure of oxidant species to the enzyme catalyst.

It is important to mention that although the chemo-enzymatic epoxidation is still an exothermic reaction, the limitation of initial H_2O_2 concentrations is focused on reducing the

exposure of the lipase to high oxidant concentrations over the reaction period. This highlights the difficult mass balance in the process and the extent of use for the enzyme catalyst in epoxidation.

To elaborate on this, the mass balance would ideally be performed around the oxygen transfer reactions of the process, from hydrogen peroxide as a substrate to oxirane ring. In our case the system is designed to operate in limited regime to calculate conversions from double bonds. The modification of the scope of the research to oxygen use efficiency is underway.

4.3 Epoxide conversion and its correlation to degree of unsaturation

The physicochemical properties and free fatty acid composition of high oleic soybean oil result in the following proportions of the main unsaturated free fatty acids, linolenic acid (C18:3) (2.0%), linoleic acid (C18:2) (7.0%) and oleic acid (C18:1) (76.0%) and iodine value was experimentally measured at 89.53.This fatty acid composition is different than soybean oil (SBO) as it can be observed in table 1 from the comparison to regular SBO.

Oil	Oleic Acid (18:3)	Linoleic Acid (18:2)	Linolenic Acid (18:3)	Double Bonds	Mean Iodine value
SBO[49,51]	25.5	62.4	-	4.5	130.0
HOSBO	76.0	7.0	2.0	3.0	89.53

Based on this, the estimated theoretical oxirane oxygen content was 5.34%. This concentration of oxirane ring validates the epoxidation of high oleic soybean oil and its application in chain polymer synthesis or low cross-linkage materials and as plasticizers or paint additives[101]. Chemo-enzymatic epoxidation at batch conditions is analyzed as the conversion between double bonds to oxirane oxygen yields of the epoxide product. A correlation between the decreasing double bonds and the increasing yields of epoxide shows the selectivity of the enzymatic process and stability of the product. Results for the conversion of high oleic soybean oil can be observed in figure 4.

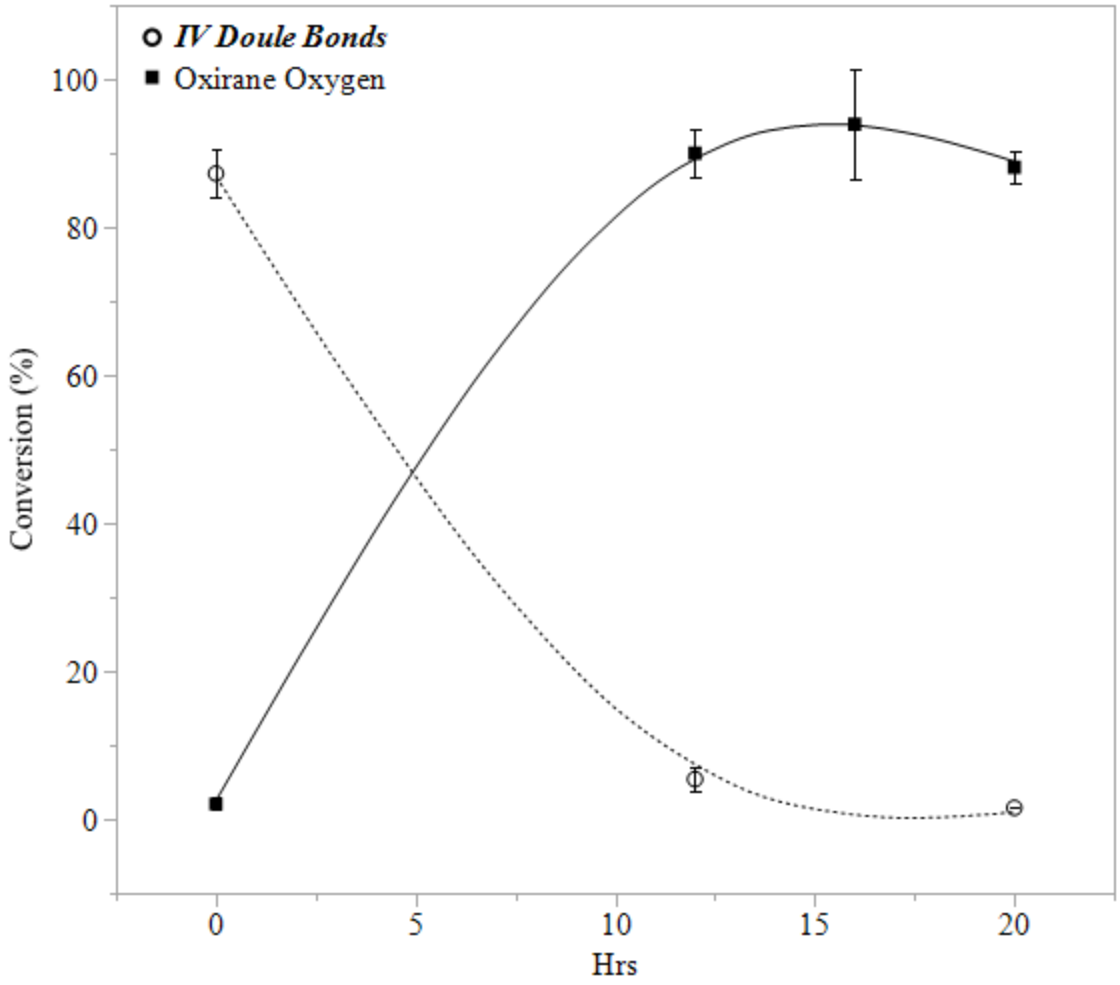

Figure 6 Enzyme-catalyzed epoxidation of high oleic soybean oil selectivity values on batch reactor over time 35°C. Conditions 2:1 H2O2 to C=C bond molar ration, steer speed of 400RPM. Error bars constructed at 1 Std Deviation from mean.

Figure 4 shows how both reactions advance steadily for the first 12 Hrs. After this, both reactions begin to plateau around 90% conversion for epoxide product and 7.0% for available double bonds. The continuous progress of the conversion of both reactions could suggest that transport limitation of the immiscible phases is not critical for the process conversion. This was previously reported by our group in which high yields were achieve in a solvent-free system[52].

The related conversion amounts, 90% for oxirane conversion and 7% double bonds, validate the selectivity of chemo-enzymatic epoxidation. This benefit of the reaction is well documented[57,102,104,119,120]. Enzymes are known to be substrate specific and have defined

configuration of aminoacidic residues in the active site to follow the several steps of the mechanisms.

As mentioned previously, the lipase mechanism in epoxidation involves two different reactions happening in the same active site. CALB is part of the α/β hydrolase fold family and the characteristic active site has a Nucleophile-His-Acid catalytic triad[91]. This allows the hydrolysis of ester bonds and the peroxidation activity.

Zhou *et al.*[121] modelled the molecular basis of the per-hydrolytic activity of CALB using octanoic acid (C8:0), the process initiates with the stabilization of the carbonyl carbon within the oxyanion hole of the CALB active site where the nucleophilic attack by Ser residues forming an acetyl-enzyme intermediate. Simultaneously, H2O2 is stabilized in the active side by Gln, Asp and Hist residues to attack the acetyl-enzyme intermediate and achieve the formation of PFFA.

Additionally, is worth mention that it is not clear if the enzyme has a direct participation in the epoxidation reaction, some research points out that CALB can perform direct epoxidation when no carboxylic acids or esters are present[85], in our case the presence of said species along with hydrogen peroxide leads to peracid formation which can react with double bonds by matters of thermodynamics.

Another essential part of this effectiveness is the mild reaction conditions employed in chemo-enzymatic epoxidation. Kahlerras *et al.*[122] describe that further characterization of epoxidized vegetable oils happens with ring opening of epoxy groups, which can be achieved with alcohols or water in the presence of acid catalysts, with organic acids, inorganic acids and by hydrogenation[122].

Ring opening reactions are dependent on nucleophilic catalysts and facilitated at low pH for easier protonation, Cai *et al.*[71] describe the kinetics of ring-opening reaction and defined that the acid-catalyzed conditions allow an easy attack on the epoxide groups. The scheme of possible derivatizations conditions and products for epoxide groups can be observed in Figure 5, where acid conditions facilitate side product formation.

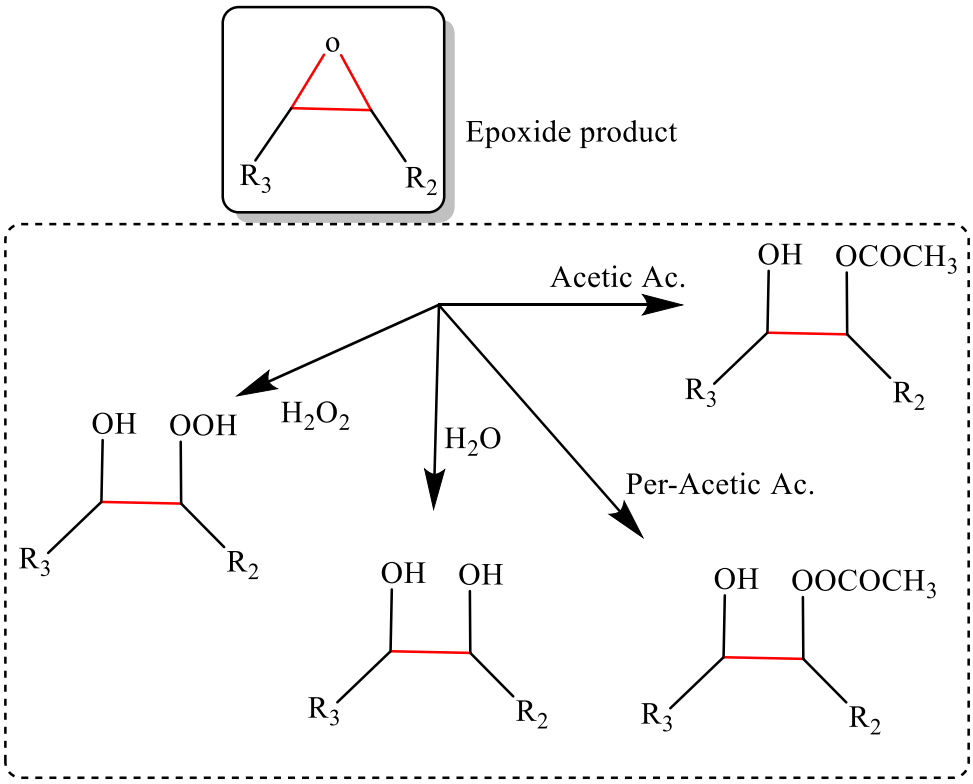

Figure 7: Schematic of possible ring-opening reactions in acid catalyzed epoxidation of oil. Adapted from Cai *et al.*[71].

Overall most vegetable oil polyols are produced by ring opening reactions with a proton donor[49] and therefore presence of medium to long chain fatty acids and peracids is less likely to achieve the required protonation. This is consistent with the results presented by Orellana-Coca *et al.* where in one-pot esterification and epoxidation of soybean no ring opening reaction was observed[123].

Finally, an important factor of the efficacy on conversion is in fact the use of high oleic soybean oil. Most reactions for oleochemicals use double bonds as building blocks for polymer network construction[124], therefore, reducing the proportion and location of unsaturated double bonds modifies the applicability of the oleochemical but also enhances the conversion rates of chemo-enzymatic epoxidation.

The use of high oleic soybean oil reduces reaction times because there is a lower concentration of double bonds from polyunsaturated fatty acids such as linoleic (C18:2) and linolenic (C18:3) and a higher concentration of high conversion monounsaturated oleic acid (C18:1). Results by Yan *et al.*[46] showed a rate reaction similar for oleic and linoleic and an increase

for full epoxidation of linolenic acid when using chemical epoxidation, this results were later validated by Zhang et al by GC-MS analysis of the epoxy fatty acids synthesized[52]. This additionally allows for a more complete conversion.

4.4 Loss of epoxide production from chemo-enzymatic activity

Although the efficiency of the process is adequate for single batch production, it is critical to retain stable enzymatic activity for longer periods of time and several batches. The conditions implemented in the process have been optimized in terms of yields, therefore the result of continuous reactions on the same enzyme load can lead to deactivation.

Results shown in Figure 4, show the decay of epoxide product formation its correlated to the loss of double bond conversion, this validates the effectiveness of the chemo-enzymatic epoxidation by the selective substitution of oxirane rings in the available double bonds. Both values are represented as the theoretical activity, a value calculated as the decay of both results (OO and IV) from the initial batch (t<12Hrs) and considering as 100% activity from the lipase, to measure change in the consecutive batches.

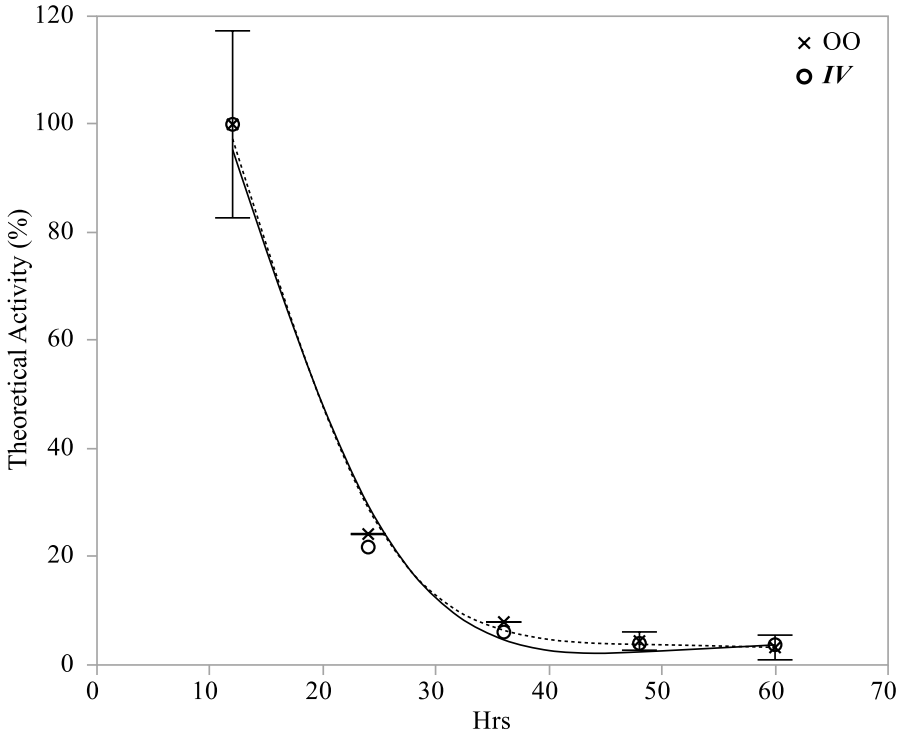

Figure 8 Decay of theoretical activity of epoxide production (OO) and double bonds (IV), calculated from the change in results per consecutive runs. Values for double bonds were calculated as $IV=[IV_0-IV_X/IV_1] \times 100$, where IV_0 is the iodine values obtained from raw high oleic soybean oil, IV_X is the Iodine value obtained at the cycle in question and IV_1 the iodine value conversion at the first cycle. Epoxide product decay was calculated as $OO = [OO_{ex} / OO_{th}] \times 100$ where OO_{th} is the theoretical oxirane ring obtained from the IV_0 and OO_{ex} the experimental determination. Reaction conditions: 5 cycles of 12 hours, 35°C (±1°C) and 400RPM using high oleic soybean oil.

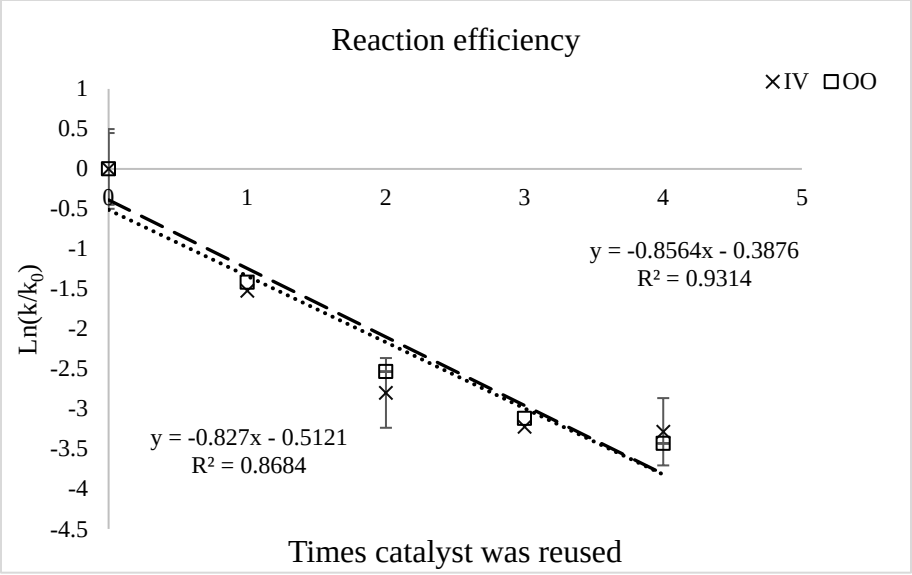

Figure 9 Analysis of decay for determination of half-lives. $T_{\frac{1}{2}IV} = 9.7$Hrs., and $T_{\frac{1}{2}OO} = 10\ Hrs.$ from initial cycle (12 Hrs).

From the linearization presented in figure 7, we can see the decrease in epoxide yields as a first order reaction as a function of the recycling of the catalyst. The rates found show that half-lives obtained for epoxide production yield is of 22 hrs. This is calculated from the decay obtained per cycle multiplied by the reaction time (12 hrs.)

As mentioned before, the parameters affecting the loss of activity are varied, and different approaches have been investigated to increase the stability of the enzyme. For example, the use of anhydrous hydrogen peroxide was proposed to minimize the exposure of the hydrophilic active site of the enzyme to peroxide, but the results did not validate the hypothesis[67].

It has been proposed that the presence of hydrogen peroxide is detrimental to the enzyme catalyst by causing oxidation of the aminoacidic residues[101,125,126]. The lipase catalytic site contains histidine (His), serine (Ser) and aspartate/glutamate (Asp/Glu) residues, these are essential to perform the epoxidation process and are sensible to oxidation

Addressing the different drawbacks from enzyme deactivation have been addressed also using additional enzyme loadings in the system. For example Orellana-Coca *et al.*[67] describe the use of a system with an excess loading of lipase to reduce the productivity problem. Nevertheless, this strategy undermines the final goal of reducing cost from enzyme catalyst use in the process.

4.5 Physical parameters affecting the decay of activities

The analysis of parameters to retain enzymatic activities for longer periods of time is critical for industrial implementation of the process. Nevertheless, addition of extra reagents will consequently increase operation costs in extra purification steps and cost of additional reagents. For this reason, we explore the effect of physical parameters such as hydrogen peroxide addition rates, as well as the validation of its oxidation ability and temperature control of the process.

First, to test the effect of change temperature, we performed a 2-level design at 35°C and a non-heating assay at 22°C (±1°C). Higher temperatures have been tested extensively for yields of epoxide and found detrimental for lipase activity and loss of hydrogen peroxide[52]. The second parameter to assess is the presence of hydrogen peroxide, evaluated for its effect on activity in a two-level experiment, one using 30% hydrogen peroxide and the other using de-ionized water. The amount of hydrogen peroxide has been reported as the greatest influence on the rate of reaction and loss of enzymatic activity due to it highly oxidative characteristics[102,127]. Finally, as a control of hydrogen peroxide concentrations throughout time we evaluated 3 different rates of controlled addition of hydrogen peroxide over 1, 3- and 6-hours or (0.441 ml/min), (0.147 ml/min (0.0715 ml/min) respectively.

Analysis of these results are shown in table 1, and values are reported as statistically significant when *p*-value is smaller than or equal to 0.05 ($p \leq 0.05$) the exact p-values were obtained using JMP Pro 14. As observed, the effect of hydrogen peroxide is evident with a $P<0.001$ for both activities. This is not surprising since hydrogen peroxide is critical for the formation of per-carboxylic acid and subsequent epoxidation.

In terms of its controlled addition rates, the analysis of this factor does not reflect a statistical significance on the decay of epoxide and hydrolytic activities, with P>0.5.

Finally, the analysis of temperature on decay had conflicting values with a significant effect on hydrolytic activity but not significant for epoxidation activity.

Table 2 P-Values (α=0.05) given by the analysis of variance on the decay of activity given by lipases

PARAMETER	HYDROLYSIS	EPOXIDATION
H_2O_2 Presence	P<.001	P<.001
H_2O_2 addition rate	P=.5758	P=.6832
Temperature	P=.0063	P=.1318

4.5.1 Hydrogen peroxide presence in enzymatic activity

Regarding the presence of hydrogen peroxide, both reactions show statistical significance. These results are not surprising but should be elaborated separately. First, when hydrogen peroxide is used as oxidant in bio-catalysis it leads to deactivation of the enzyme. Approaches to increase stability have been taken in measures of molecular biology as well as immobilization strategies of the biocatalyst enzyme in different supports. Zhao *et al.* used mutagenesis of the to increase the robustness of the enzyme against H_2O_2[128]. Nevertheless, in terms of engineering, the new variant enzyme would also have to be studied for immobilization supports, physical stability, and effectiveness in epoxidation therefore this solution poses a long-term set of tasks to finally lead to a practical application. On the other side, the election and use of a commercially available immobilized enzyme allow us to obtain a standardized product with defined characteristics in its application; therefore, optimization strategies can be implemented into the process configuration rather than the biocatalyst.

The second part of the analysis is in terms of the reaction conditions. Even though the H_2O_2 concentrations oxidizes the enzyme, and thus impairs the reaction cascades for epoxidation. The experimental analysis depends on the reaction taking place. Figure 8 shows the schematic of the reactions required for chemo-enzymatic epoxidation. It can be observed that H_2O_2 is a required substrate of the lipase per-hydrolytic activity for the synthesis of PFFA, leaving as reduced product water. This is not the case for the natural hydrolytic activity of lipases. As mentioned before, the mechanism of both activities has similarities, but the products obtained are different and their production modifies the mass balance of the system.

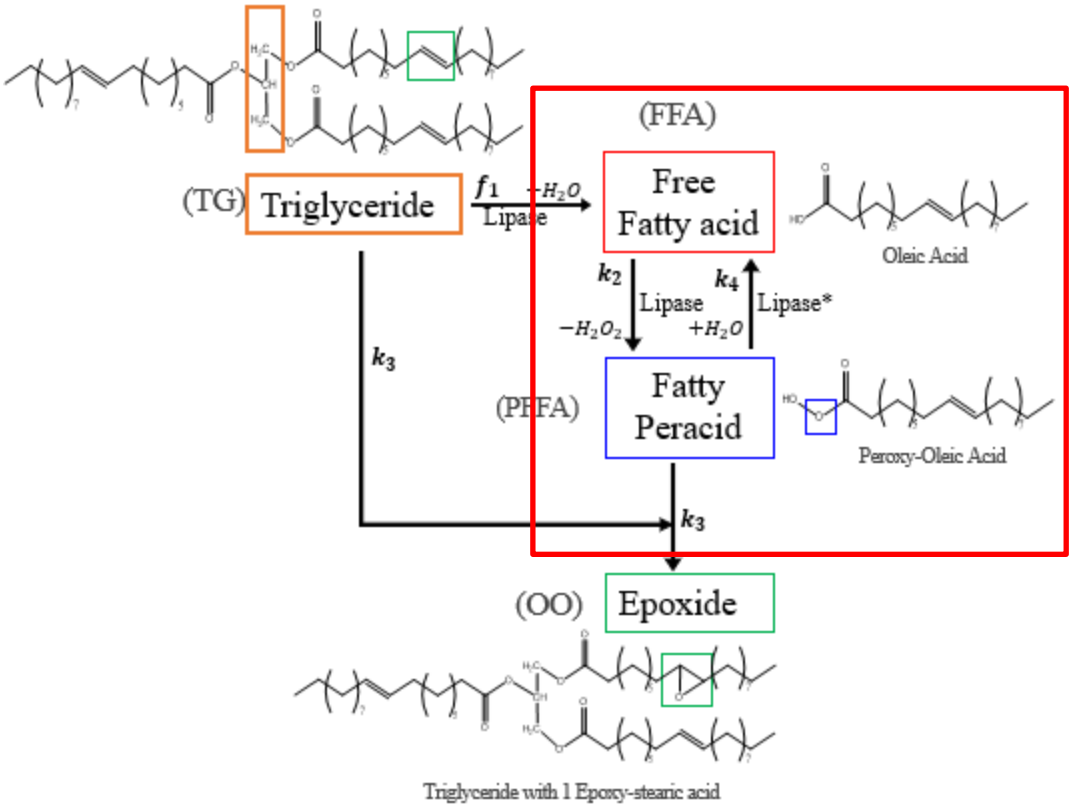

Figure 10 Schematic representation of chemo-enzymatic epoxidation.

The presence of hydrogen peroxide impacts the analysis of both activities differently, hydrolysis does not need hydrogen peroxide as a substrate to break the ester bonds. Therefore, not having H_2O_2 allows continuous hydrolysis over time and at stable rates. Whereas in epoxidation, hydrogen peroxide is the active oxygen substrate required for the synthesis of PFFA. Therefore, the lack of it will not produce any epoxide over time. The results showed in table 4 show the statistical significance of hydrogen peroxide on the decay of both activities were behavior of hydrolysis shows longer stability and epoxide production remains constant at zero values.

Overall, this validates the negative effects of hydrogen peroxide on enzyme activity just by analyzing the change in hydrolysis decay rates. These results have been evaluated as well in terms of the structural changes of the enzyme configuration in contact with H_2O_2, Törnvall et al, described that hydrogen peroxide leads to oxidation of methionine, tryptophan and cysteine residues which breaks disulfide bridges and modifies the protein secondary structure[129].

Possible configurations are then related to slowing the addition rates of hydrogen peroxide and will presented next.

4.5.2 Controlled addition of hydrogen peroxide

Slowing the addition rates of hydrogen peroxide has been a process modification employed to reduce the initial concentrations of H2O2 without modifying the stoichiometric balance of the reaction, therefore, maintaining an excess of hydrogen peroxide per double bonds available. Theoretically, understanding the peracid generation rates will help define adequate hydrogen peroxide rates to reduce the possibility of a bottleneck from a lack of available hydrogen peroxide.

Evaluation of the three rates (1, 3, and 6Hrs) is helpful to evaluate the results in terms of epoxide production over several batches.

As results on initial decay of both hydrolytic and epoxide activity the first 24 hours are of important change in enzymatic activity. Analysis of variance for hydrogen peroxide addition rates weighted by times show that within the first 12 hours faster addition rates have a more pronounced decay of activity, but overall change of hydrolytic activity show that the use of different addition rates after the first 12 hours have no statistical difference to the resulting mean values. In terms of epoxide production, the rates of hydrogen peroxide had similar decay of productivity, and in contrast with hydrolysis the decay within the first 24 hours, had similar behavior for the 1- and 6-hour addition rates. Statistical significance, as well as previous cases, is strong for cross evaluation of hours of reaction and the addition rates. Interestingly, the faster addition of hydrogen peroxide had similar yields to the slowest addition rates.

4.5.3 Effect of temperature control on enzymatic activity decay

Temperature optimization is a critical parameter to explore in terms of conservation of enzyme. Traditionally temperature is set to influence the rate of a chemical reaction, reducing times of conversion by the Arrhenius equation[25]. Nevertheless, this focuses on the product and does not consider catalyst stability. Thus, reducing the temperature potentially decreases the yields and increases stability of the enzyme.

Although lipase B from *Candida antarctica* is known for great thermal stability, the aqueous phase of the system reaction can allow a more flexible conformation[92] allowing higher

contact with hydrogen peroxide and therefore make it prone to denaturation. Changes in immobilization support can modify the stability at different temperatures, but not so much when in contact with hydrogen peroxide[98].

In our analysis shown in Table 4, the effect of leads to statistical differences in hydrolytic activity decay but not in epoxide production decay. Effective control of temperature as a stability parameter is essential both in terms of the energy cost of the process as well as enzyme activity retention. Frequently short-timed use benefits more from high temperatures, but in the long run, it might be more suitable to have more extended use and lower yield in order to retain the enzyme given that represents the highest value material.

Temperature is very important parameter in any enzymatic process but to elaborate on these results we also need to look to each activity in separate. Considering hydrolysis of oil,, at higher temperatures reaction rates can be increased from and open configuration of the enzymatic structure but increasing the temperatures can also lead to inactivation[90]. It has been shown that temperature has an effect on the rate of hydrolysis of the enzyme[130], which results in a decrease of hydrolysis of oil. In our case by reducing the temperature to 22°C we found a decrease of FFA%, this lower production of FFA from oil can also be reflected in a slower decay rates over time. Statistical significance of temperature for enzymatic hydrolysis in was evaluated in a single factor ANOVA at the different temperature conditions.

The analysis of epoxide production decay becomes more complex due to the cascade of reactions and intermediates that take place. As it can be observed in figure 8, the formation of epoxide consists of two steps, first the formation of PFFA, and second, the formation of oxirane ring. Results in this experiment are expressed in terms of epoxide produced over consecutive batches, so it doesn't consider production and efficiency of intermediates.

Analysis of epoxide decay from both temperatures show that there is a higher variability at the high yield temperature (T_Y = 35°C) than conservation temperature (T_C = 22°C). This is given by higher yields obtained at T_Y which then decay as it was showed previously. For T_C the values are more uniform, but the decay is still present in the first 24 Hrs. of use of the lipase. Decay of activity at both T_Y and T_C is shows that temperature alone is not a statistically significant parameter from this experimental design. A deeper understanding of the kinetics within the reaction cascade of epoxide formation is critical to fully understand the effect of temperature on both exothermic reactions.

Other important parameter affected by changes in temperature is viscosity, epoxide formation is followed by an important increase in viscosity increase within the reactor. Additionally, viscosity is an important mass transfer limitation parameter and a sign of side product formation from phase separation reactions[25]. In terms of temperature, the analysis shows lipase deactivation in high oleic soybean oil epoxidation is not equally affected and optimization of the process can be benefitted from a different reactor configuration.

CONCLUSIONS

The chemo-enzymatic epoxidation process can effectively achieve conversion rates higher than 90% in high oleic soybean oil in solvent-free batch reactor conditions. This makes the process a feasible alternative for green epoxide formation.

Regarding the use of immobilized lipases, and specifically, the immobilization support Immobead 150® does not significantly participate in non-selective hydrolysis of high oleic soybean oil. This is also a benefit because diglycerides and monoglycerides can be challenging to separate from the final product and are considered undesired reactions of the oxidizedproduct[106,131].

The results given in acidity of the free fatty acid released, highlights the great diversity in the final product in terms of diglycerides and monoglycerides. This can be a negative characteristic for polymer applications and special monomeric applications. Acid-catalyzed epoxidation produces a low acid value (<0.6%), while lipase-catalyze epoxidation has been found with higher acid values[132].

In terms of epoxide yields, the use of *Candida antarctica* lipase B immobilized on Immobead 150® can achieve high yields of epoxide product after 12 hours of reaction in aqueous hydrogen peroxide. Additionally, there is no significant loss of epoxide product due to ring-opening reactions due to the mild conditions used.

In the second part of the study, the enzymatic activity evaluations show the decays in hydrolytic activity and epoxide yields, with half-lives of 25 and 22 hours, respectively. These values must be increased at least 3X to contemplate a scale-up of the reaction using lipases for industrial applications.

There is an essential set of intermediate reactions that incorporate PFFA, such as conversion rates and possible accumulation conditions that are not fully understood and are being evaluated for future work. Defining the optimum conditions of peracid production and use is essential to have a minimum amount of hydrogen peroxide present in the reaction and thus reduce the oxidation of aminoacidic residues in the active site.

The evaluation of the reactor parameters that influence enzymatic activities' decay validates that hydrogen peroxide is the most critical cause of enzyme deactivation represented by the effect of its presence on the decay of hydrolytic activity. These results align with those found by Hagström *et al.*[133], pointing out that hydrogen peroxide is the main bottleneck in chemo-

enzymatic epoxidation due to enzyme inactivation. Additionally, limiting hydrogen peroxide contact to the enzyme without reducing yields and increasing reaction times is unlikely under batch reactor conditions, as previous research by Zhang *et al.*[52] points out, we must transition to the continuous production of epoxides or a sectioned reaction in which hydrogen peroxide conversion is performed more efficiently.

Furthermore, the evaluation of addition rates of hydrogen peroxide shows inconclusive results for its significance on the decay of both hydrolytic and epoxidation decay. The reduction of hydrogen peroxide aims to reduce hydrogen peroxide initial concentrations, allowing a longer oxygen transfer process to epoxides. Our results show that there is no significant evidence to suggest that reducing the initial concentrations of hydrogen peroxide will reduce the decay of enzyme activity.

Finally, the effect of temperature on the decay of enzyme activities is complicated. Rates of hydrolysis are reduced at T_C; this decreased FFA production shows a decreased decay of hydrolytic activity. As per epoxide production, the conversion yields at T_C also decreased, showing a lower variability of results from conservative initial production to the enzyme's deactivation. However, in contrast with hydrolysis, the temperature did not show statistical significance to affect the overall epoxide production decay rates.

The effect of temperature also validates that optimization requires individual analysis and optimization of the enzyme activities. Understanding the kinetics of PFFA production is essential to increase the enzyme's stability through efficient use in oxygen transfer processes.

6. FUTURE WORK

The continuation of this research can go in multiple directions. The lifespan of the enzyme is a critical parameter to improve to have a feasible industrial application of the epoxidation process. Several approaches can be implemented to improve the stability of the enzyme, first the kinetics of the reactions should be looked closely, the evaluation of parameters shows a close relationship in epoxide production and temperature. As explained before, epoxide formation is a complex process that requires the formation of peracid intermediates as epoxidation catalyst.

Understanding the kinetics of per-hydrolysis could reveal the possible bottlenecks and potential configuration to reduce oxidation of the lipase. Additionally, the effect of the accumulation and conversion of peracids on the lipase activity is not well understood and could also participate as an active oxidant to the enzyme.

On that note, it is important to reduce the contact of the enzyme catalyst with high oxidant concentrations for prolonged times. From the schematics of chemo-enzymatic epoxidation it is possible to observe that PFFA are reactive enough to catalyze the production of epoxide through random collision reactions. Batch conditions impedes these processes from occurring without the enzyme, therefore different configuration of the reaction should be explored.

In epoxidation formation specifically, there are two reactions that take place and can be observed from the Figure 8, first the formation of PFFA through the reaction of H_2O_2 and FFA, and second, the formation of the oxirane ring through the reaction of PFFA with DB in the oil. Both of these reactions are exothermic, and evaluations performed on acid-catalyzed epoxidation by Monono *et al.*[25] have calculated that the energy released from the reaction of double bonds with PFFA to form epoxide is 15 times larger than PFFA formation from oil and hydrogen peroxide. This would suggest that the reaction kinetics could present a bottleneck in the formation of epoxide rather than the formation of PFFA.

This could be improved by the modification of agitation and temperature parameters in the process. Both conditions are detrimental to the enzyme catalyst, therefore a separate reactor configuration could improve this specific step while keeping the mild conditions for the formation of PFFA.

To validate the formation of PFFA we have performed a preliminary analysis of the per-hydrolytic reaction. This was performed using as model substrate octanoic acid, a saturated fatty

acid of 8 carbon chain length (C8:0). The model was selected because of the lack of double bonds in the chain therefore epoxidation is not possible and additionally the medium length of the chain in octanoic acid allows it to be liquid at room temperature conditions and stable in peracid form[134].

Preliminary results of octanoic acid can be observed in Figure 9, following the quantification methods by Sitko el al.[134] and represented as yields from peroxide moles produce from initial concentration of the acid moles used.

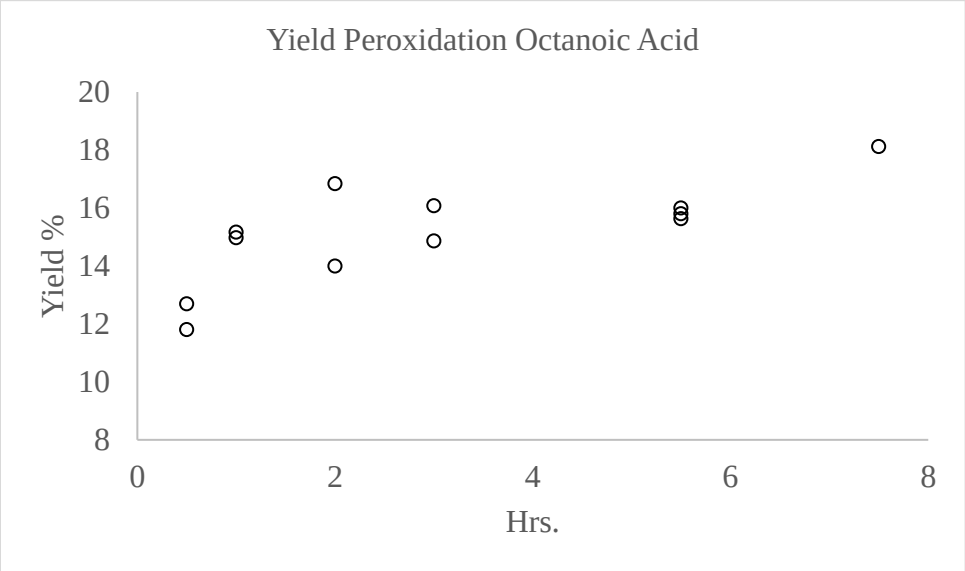

Figure 11 Per-hydrolysis of octanoic acid. Yields calculated as (mM Peracid/mM Acid) * 100 Reaction conditions 4% Enzyme loading, 01:1.4 molar balance of octanoic acid to hydrogen peroxide, 22°C and 300RPM.

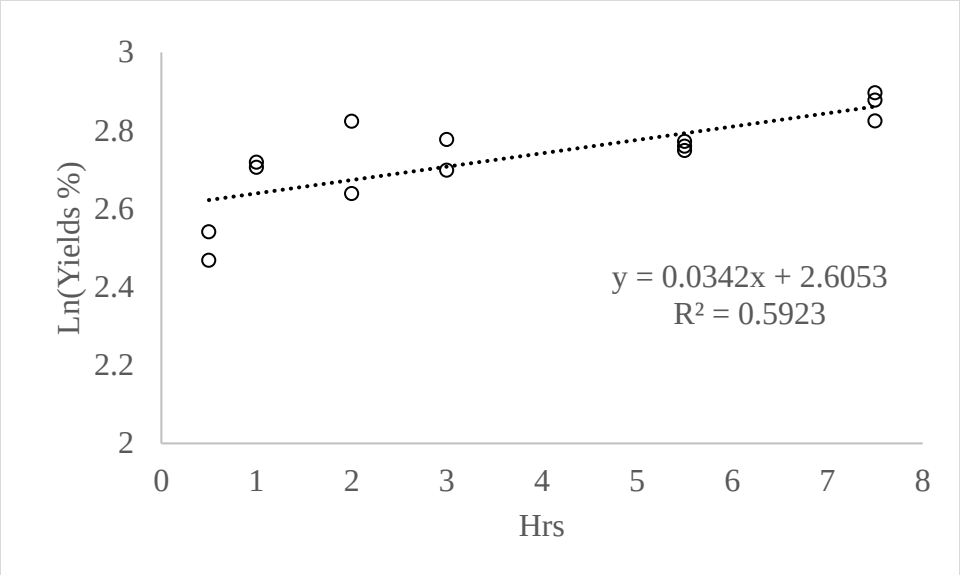

Figure 12 Peracid production rates using model substrate octanoic acid.

The reaction is shown to rapidly increase to 12% yields within 30 minutes and slowly increase to a final 17% after 6 hours. Additionally as observed in Figure 10, the results found (*k=0.0342*) in terms of rates probably do not represent the reality because of the lack of samplings prior to 30 minutes, and shorter times must be analyzed to accurately found the rate of the reaction. Additionally, the peracids produced must be evaluated in terms of mass balance against the available moles of active oxygen from hydrogen peroxide. In that sense, both PFFA production and hydrogen peroxide must be quantified over time to understand the efficiency in use of oxygen.

Additional preliminary results were analyzed in terms of epoxide production, from the produced peracids and tested for their effectiveness without additional reactants the results were compared to acid catalyzed peracid synthesis. High oleic soybean oil and the separated peracid products were placed in batch reactor at 50°C and 500RPMs. Preliminary results in terms of oxirane production are show in Figure 11.

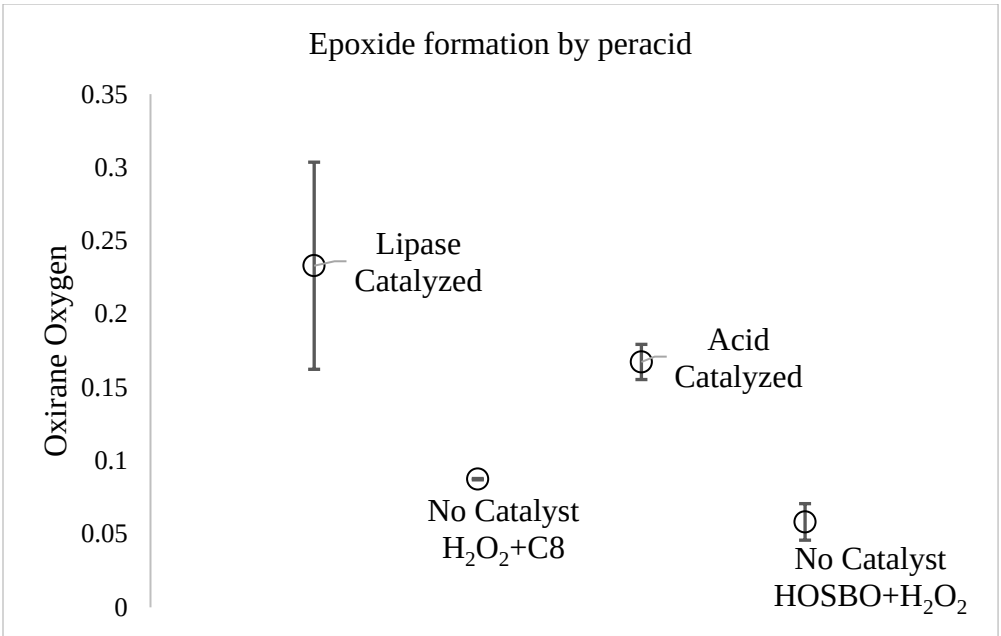

Figure 13. Changes in oxirane production from previously synthesized peracids.

This information suggest that we can possibly employ a process similar to acid-catalyzed epoxidation in which peracids are synthesis in situ and then used as catalysts for epoxidation.

Maximizing the use of enzyme catalyst to synthesize PFFA in a continuous flow reactor and allowing the hydrogen peroxide to flow in and out. Even though the enzyme catalyst retains the same lifespan, in terms of active oxygen transfer the process could become more efficient. This approach has already been explored by authors like Meyer *et al.*[99] using a continuous stirred tank reactor to retain per-hydrolytic activity for longer periods of time, resulting in 50hrs of continuous activity using CALB.

We propose the use of a packed bed reactor for easier modification of conversion given by modifications on volume and length of the column as well as flow velocity in addition to the oxidant ration to oil.

This allows us to suggest than a more feasible configuration could arise from 2 separate reactor conditions. First the chemo-enzymatic production of PFFA under mild reaction conditions in a packed bed reactor, and second a continuous stirred tank in which the thermodynamic conditions are optimized to oxidize double bonds using the produced PFFA in combination with the vegetable oil.

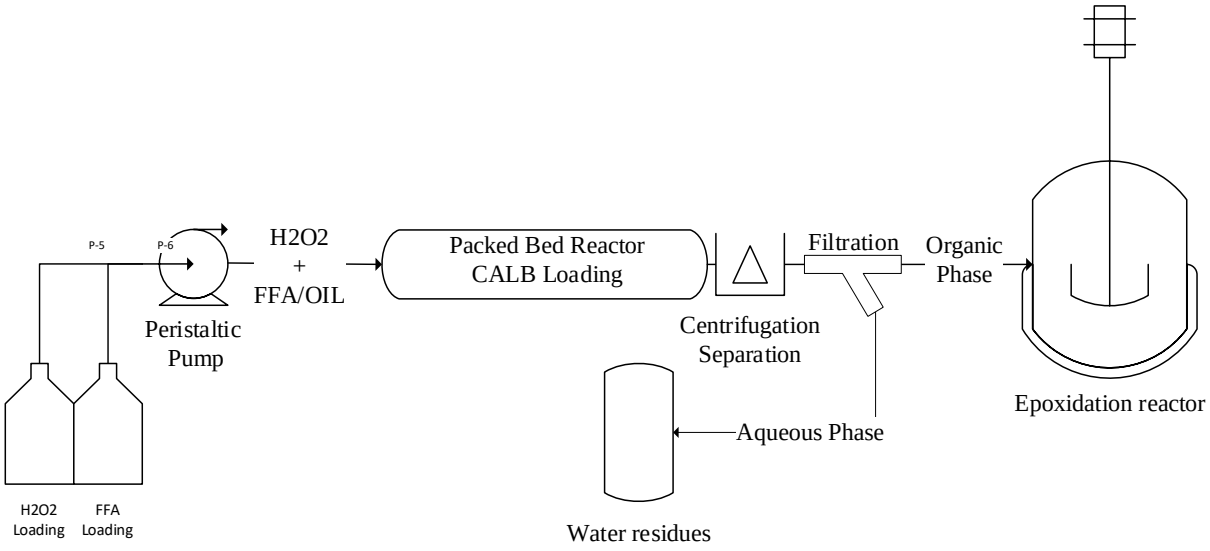

Figure 14 Proposed configuration of scale-up process in chemo-enzymatic epoxidation.

A system like the one presented in Figure 12, could allow a more efficient use of the lipase in the epoxidation process. Separating the system in two parts which allows both high yields and conservation of the enzyme catalyst producing PFFA and transferring it to a batch of soybean oil for epoxidation.

Additionally, this facilitates the estimation of the kinetic parameters, by individually producing the PFFA intermediates to quantified rate and depending on the bed reactor bed, void fractions, and flow velocity. From that value we can account for the effective oxygen transferred from aqueous hydrogen peroxide to the FFA, and finally from PFFA to epoxide production.

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