Daniela Bresolin

DEVELOPMENT OF POLYMER SUPPORT FROM RENEWABLE SOURCES INTENDED FOR ENZYMES IMMOBILIZATION

Tese submetida ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutora em Engenharia Química.

Orientador: Prof.ª Débora de Oliveira

Coorientadores: Dr.ª Alexsandra Valério e Prof. Pedro Henrique Hermes de Araújo

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Florianópolis, 07 de junho de 2018.

Dedicated to my family.

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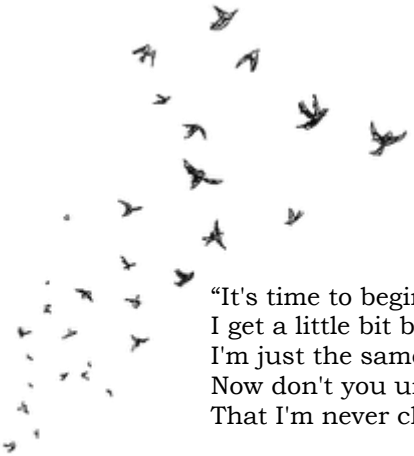

“It's time to begin, isn't it?
I get a little bit bigger but then I'll admit
I'm just the same as I was
Now don't you understand
That I'm never changing who I am.”

Imagine Dragons

ABSTRACT

The use of vegetable oils has been reported in numerous investigations for polymerization as they are considered a clean and environmentally friendly alternative. The oils are an interesting alternative for this purpose, because they allow modifications in the structure through reactions, as hydrolysis and transesterification, enabling the changes in the chemical structure with the arrangement to the desired polymer. Thus, it is possible to obtain a wild range of materials, which can be used as support for the immobilization of enzymes, making the process more environmentally favorable. At the first step of this work, a biopolyol was obtained by enzymatic glycerolysis reaction of castor oil using immobilized commercial Novozym 435 as biocatalyst. The obtained biopolyol, with a conversion of 64% in mono- and diacylglycerol, was applied in the synthesis of polyurethane foam. Then, the foam was used as support for lipase NS-40116 immobilization via entrapment technique in one single step, with 94±4% of immobilization efficiency. Free and immobilized NS-40116 in polyurethane foam lipases were characterized in terms of relative enzymatic activity using ρ-nitrophenyl-palmitate as substrate. The enzymatic derivative was applied in the fatty acid methyl esters synthesis using abdominal chicken fat as feedstock and it was possible to perform the reuse for 4 cycles with conversions around 60%. In a second step, poly(urea-urethane) nanoparticles were synthesized using the biopolyol produced via enzymatic glycerolysis catalyzed by Novozym 435. Nanoemulsions with a different solids content (20, 30 and 40%) presented a remarkable lower averages sizes, between 167-280 nm, and high stability were obtained using miniemulsion polymerization without the use of any additional surfactant. In the third step, the free lipase NS-40116 was used as biocatalyst to obtain biopolyols by glycerolysis in a solvent-free system. The glycerolysis product was used in the synthesis of PUU nanoparticles and applied as support for lipase NS-40116 immobilization in the surface of the nanoparticle, presenting an average size of 231 nm. In the last step, biopolyols produced in this work were used to obtain polyurethane foams, using the polymeric diisocyanate papi-27®. The foams were characterized, and it was possible to conclude that the biopolyols can be applied in the polyurethane foams synthesis if used with a high reactivity diisocyanate, promoting to the foam high resistance to compression strength.

Keywords: Biopolyol, lipases, immobilization, polyurethane, NS-40116, biocatalysts.

RESUMO

O uso de óleos vegetais tem se tornando alvo de inúmeras investigações na área de polimerização, pois são considerados uma alternativa limpa e ambientalmente correta, indo de acordo com as expectativas de uma ciência mais verde e menos poluente. Os óleos são ideais para esse propósito, pois além de serem considerados renováveis, permitem modificações em sua estrutura através de reações, como hidrólise e transesterificação, que fazem com que a conformação de sua estrutura se adeque ao polímero desejado. Com isso, é possível obter uma alta gama de materiais, os quais podem ser utilizados como suporte para a imobilização de enzimas, fazendo com que o processo possa ser considerado ambientalmente favorável. Na primeira etapa deste trabalho, um biopoliol foi obtido através de reação de glicerólise enzimática do óleo de mamona utilizando a lipase Novozym 435 como catalisador. O biopoliol apresentou 64%, em termos de composição, de mono- e diacilglicerol, e foi aplicado na síntese de espuma de poliuretana (PU). Após, a espuma foi utilizada como suporte para a imobilização da lipase NS-40116, utilizando técnica de aprisionamento em um único passo e apresentou 94±4% de eficiência de imobilização. A lipase livre e imobilizada foram caracterizadas em termos de atividade enzimática relativa utilizando p-nitrofenil-palmitato como substrato. O derivado enzimático foi aplicado na produção de ésteres metílicos utilizando gordura abdominal de frango como matéria-prima e foi possível realizar o reuso por quadro ciclos com conversões de aproximadamente 60%. Em um segundo momento, nanopartículas de poli(ureia-uretano) (PUU) foram sintetizadas utilizado o biopoliol produzido anteriormente. Nanoemulsões com diferentes porcentagens de sólidos (20, 30 e 40%) e tamanhos de partículas entre 167-280 nm apresentaram alta estabilidade quando obtidos por técnica de polimerização em miniemulsão sem o uso de surfactante extra. No terceiro passo deste trabalho, a lipase NS-40116, em sua forma livre, foi utilizada como biocatalisador para a obtenção de biopolióis por glicerólise em sistema livre de solvente. O produto dessa reação foi utilizado na síntese de nanopartículas de PUU e, então, a lipase NS-40116 foi imobilizada na superfície da nanopartícula, apresentando tamanho médio de 231 nm. Por último, os dois biopolióis, sintetizados anteriormente neste trabalho, foram aplicados para a obtenção de espuma de PU utilizando o diisocianato polimérico papi-27®. As espumas foram caracterizadas e foi possível concluir que espumas com alta resistência a compressão foram obtidas quando os biopolióis foram empregados na sua produção, em conjunto de um diisocianato de alta reatividade.

Palavras-chave: Biopoliol, lipases, imobilização, poliuretana, NS-40116, biocatálise.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASTM – American Society for Testing and Materials
CalB – *Candida antarctica* fraction B
CG - Gas Chromatograph
DAG – Diacylglycerol
FAME – Fatty acid methyl ester
FTIR – Fourier transform infrared spectroscopy
ɤ – Yield of immobilization
IPDI - Isophorone diisocyanate
MAG – Monoacylglycerol
MDI – Methylene diphenyl diisocyanate
NPs – Nanoparticles
PBS – Phosphate buffer solution
pMDI – Polymeric methylene diphenyl diisocyanate
PU – Polyurethane
PUF – Polyurethane foam
PUU – Poly(urea-urethane)
RCF – Relative centrifugal force
SEM –Scanning electron microscopy
TAG – Triacylglycerol
TEM – Transition electron microscopy
TLL – *Thermomyces lanuginosus*
ρNPP – ρ-nitrophenyl palmitate

LIST OF SYMBOLS

% – Percentage
°C – Degree Celsius
µL – Microliter
µm – Micrometer
Dp – Particle diameter [nm]
g – Gram
h – Hour
K_m – Michaelis-Menten constant
$mg \cdot mL^{-1}$ – Milligrams per millimeters
min – Minute
mL – Millimeters
mM – Millimolar
mmol – millimole
nm – Nanometer
pH – Potential of hydrogen
rpm – Revolutions per minute
U/g – Unit per gram
V_{max} – maximum specific activities
wt% – Weight percentage

CONCEPTUAL DIAGRAM

WHY?

- The use of vegetable oils for the synthesis of polymers has been studied as a clean and environment friendly alternative;
- The modifications that vegetable oils can undergo make them attractive since the conformation of the polymer can be determined by these modifications;
- The potentiality application of these polymers is large and the capacity to be used as support for the immobilization of enzymes is one of them.

WHO DID

- There are studies on the immobilization of enzymes in polyurethane substrates, however, all of them using supports of petroleum source;
- To the best of our knowledge, there is no research that encompasses the use of MAG and DAG to obtain stable PUU nanoparticles without the use of extra surfactant;
- Studies on the use of lipase NS-40116 as catalysts for glycerolysis reactions have also never been reported;
- There are few works in the literature using enzymatic glycerolysis product as biopolyols.

HYPOTHESES

- Does the immobilization of lipase NS-40116 in rigid PU foam from biopolyol affect the activity and stability of the enzyme?
- Is it possible to obtain stable PUU nanoparticles without the use of high HLB surfactants?
- Is lipase NS-40116 able to be used to catalyze glycerolysis reactions?

METHODS

- MAG and DAG synthesis by enzymatic glycerolysis in a solvent-free system;
- Immobilization of lipase NS-40116 using entrapment and covalent binding techniques;
- Synthesis of NPs using high shear force via miniemulsion;
- Determination of catalytic activity of lipase NS-40116 using different techniques.

RESPONSES

- Determine the behavior of lipase NS-40116 in different reaction media;
- Evaluate the behavior of PUU NPs without the presence of extra surfactant;
- Optimize the experimental conditions that lead to greater conversion of triglycerides in MAG and DAG;
- Promote the effective immobilization of lipase NS-40116.

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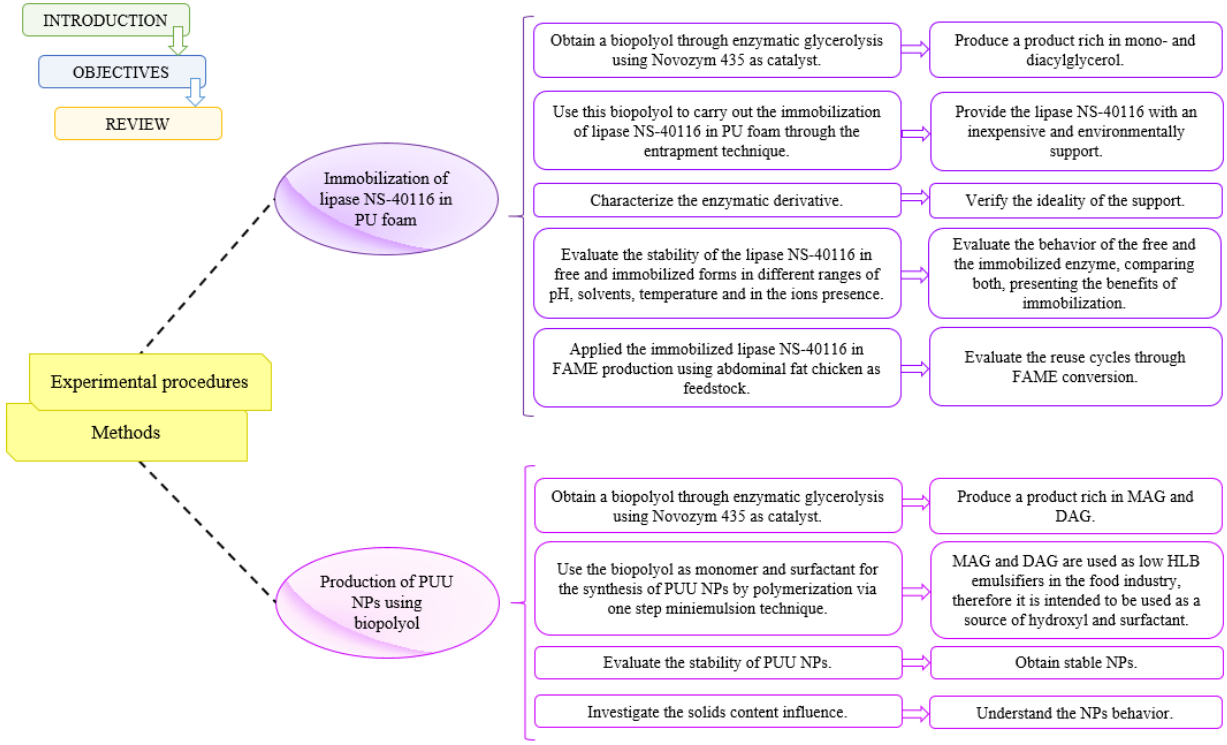

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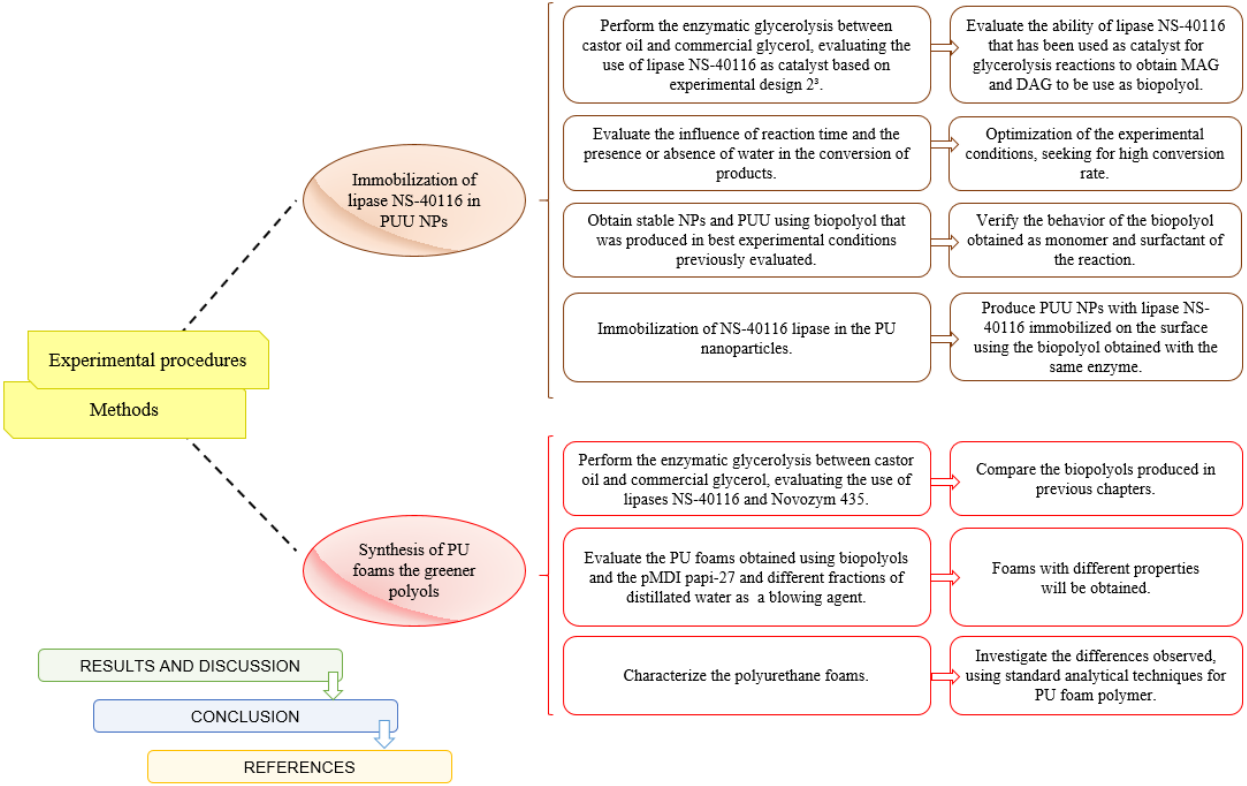

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1 INTRODUCTION

The use of vegetable oils to obtain polymers has become the guideline of many investigations in polymers field. This occurs due to environmental concerns, the high price of oil and the high use of polymers in the day by day of contemporary society (ZHANG et al., 2017a). As nature offers a considerable number of vegetable oils with distinct characteristics, the choice of the oil becomes of great importance when considering the polymer to be obtained and the physical characteristics that this polymer must have: presence or absence of saturations, acidity, carbon chain size, molar mass and the presence of hydroxyl groups, (MIAO et al., 2014; RAFIQ et al., 2015).

An important point that should be emphasized when vegetable oils are studied is the possibility of the structural modifications through reactions as for example, epoxidation, transesterification, hydrogenation, among others (Zhang et al., 2017b), which can be performed by using chemical and/or enzymatic catalysts. This type of modification is widely used to obtain monomers in improving the final characteristics of the polymers (SHARMA & KUNDU, 2006).

When it comes to obtain polymers using vegetable oils, the polyurethane (PU) is one of the most studied polymers. In order to obtain the polymer, a step polymerization reaction is carried out between the NCO (diisocyanate) and OH (polyol) groups (JANIK & MARZEC, 2015a). The polyol can be obtained from vegetable sources, and the oil can naturally present a hydroxyl group, as in the castor oil, with ricinoleic acid as major component (MUTLU & MEIER, 2010). Polyurethane foam from vegetable oils is commonly prepared by bulk polymerization, where the polyol, diisocyanate, blowing agent and vigorous stirring is generally used. As result of carbon dioxide (CO_2) formation by the reaction with the blowing agent and diisocyanate, there is the formation of cells, which the size may change according to the polyol type, blowing agent, stirring speed and diisocyanate (SHOAIB, 2014).

A method of obtaining polyurethanes nanoparticles (NPs) is by miniemulsion polymerization technique. In this type of polymerization, the system presents an aqueous phase, formed of water and a surfactant, responsible for the nanoparticles stability, and a dispersed phase, formed by the polyol and diisocyanate (ZANETTI-RAMOS et al., 2006; VALÉRIO et al., 2013). NPs have a size between 50-500 nm and this

submicron size is obtained through a high mechanical shear force (LANDFESTER & WEISS, 2010). In this case, due the water presence, urea bonds together the urethane bonds are formed, so the polymer obtained with this technique is named poly(urea-urethane).

In the last years, several investigations involving the immobilization of enzymes using the polyurethane foam (SILVA et al., 2013; SANTIN et al., 2014; BUSTAMANTE-VARGAS et al., 2015; NICOLETTI et al., 2015; NYARI et al., 2016) and poly(urea-urethane) nanoparticles (CIPOLATTI et al., 2014, 2016, CHIARADIA et al., 2016 a,b) have been performed. However, although these studies are in focus, the sources of monomers for these polymers are, in most cases, from the petrochemical industry, showing that investigations involving greener routes are still required. All this interest is due to the main characteristics as low cost of the polymer matrix and the high cost of enzymatic catalysts. Enzymes are important biocatalysts in the industry, mainly by the characteristics of high catalytic activity, selectivity, specificity and high activity under very mild environmental conditions. However, when enzymes are presented in a free form the reuse of this catalyst is impracticable, which makes the process expensive and, in some cases, not feasible. Thus, the immobilization process becomes highly attractive, due to the reuse, easy separation of the immobilized enzyme from the final product, avoiding intermediate purification processes, and also the creation of a protective barrier against enzyme denaturation (BARBOSA et al., 2015; CARVALHO et al., 2016).

In relation to the enzymes, lipases are used in numerous catalytic processes, generating a great deal of interest in their immobilization, mainly due to their affinity to catalyze reactions involving vegetable oils. When the lipase is immobilized, the surface contact of the catalytic and reactants can increase and the conversion of the products can be higher (MEHTA et al., 2016).

Thus, the purpose of this study was investigated the production of green polyols via glycerolysis reaction using enzymes as catalysts and the synthesis of polyurethane foams and poly(urea-urethane) NPs, using these biopolyols. In addition, the enzyme NS-40116 was immobilized on the foam and on the surface of the NPs.

1.1 OBJECTIVES

The goal of this work was the production of a biopolyol by enzymatic glycerolysis and use the obtained biopolyol in the synthesis of polyurethane foam and poly(urea-urethane) nanoparticles aiming at their use as a support for lipase NS 40116 immobilization. Therefore, specific objectives of this work are:

✓ To prepare biopolyols using enzymatic glycerolysis using the lipases Novozym 435 and NS-40116 as catalytic;

✓ Use of biopolyols to produce polyurethane foam and poly (urea-urethane) nanoparticles;

✓ Investigate the immobilization of lipase NS-40116 using the polyurethane foam as support and stability evaluation of the free enzyme and the immobilized enzyme;

✓Obtaining of monodisperse nanoparticles without the use of extra surfactant;

✓Immobilization of NS-40116 lipase on the surface of nanoparticles.

To facilitate comprehension, this work was divided into chapters. An overview was in Chapter II, where was approached the production of polymers using renewable sources and enzyme immobilization techniques. Chapter III describes the preparation of the biopolyol using the Novozym 435 lipase as catalyst, the preparation of the enzymatic derivative of lipase NS-40116 (immobilized on the foam), the characterization of the enzyme in relation to the stability in different media and application of the same to the production of FAME. The preparation of poly(urea-urethane) nanoparticles using miniemulsion technique without the presence of extra stabilizer was discussed in Chapter IV. In order to reduce the costs of biopolyol production, the lipase NS-40116 was applied as a catalyst in the enzymatic glycerolysis reaction of Chapter V, so the biopolyol was used to obtain an enzymatic derivative of the same lipase, where immobilization occurred on the surface of the NPs. In Chapter VI, both biopolyols obtained in this work were applied

to obtain PU foams and the polymer was characterized in terms of morphology, compressive strength and mass loss when subjected to high temperatures. Finally, Chapter VII presents the final considerations and future perspectives.

CHAPTER II

2 REVIEW

In this chapter, it will be presented a brief review of the relevant issues about this thesis. Firstly, we will present concepts about enzymes, mainly lipases and most used enzyme immobilization techniques. Secondly, polyurethane will be discussed, which is one of the immobilization supports that has been used in this work. Finally, it will be considered the synthesis of polyols from vegetable source by enzymatic glycerolysis reaction, aiming the application in the polymerization of polyurethane foams and nanoparticles.

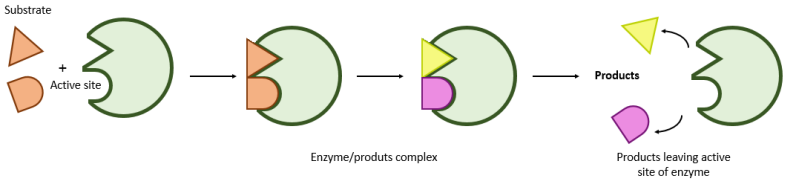
2.1 ENZYMES

Enzymes are important biocatalysts in the industry, mainly due to the characteristics as high catalytic activity, selectivity, specificity and high activity under very mild environmental conditions (BARBOSA et al., 2015; CARVALHO et al., 2016; PELLIS et al., 2018). The range of enzymes use is extensive; agricultural, pharmaceutical, fuel and food industries, e. g. (ILLANES et al., 2012; PRICE et al., 2016; SASSON; MALPICA, 2018). In the last years, the search for clean technologies in the industry has encouraged the study of these proteins in different applications than the usual.

The enzyme functionalization occurs when the active site is bound to the substrate forming the products. When the product is released from the enzyme surface, it is regenerated, and the reuse can be applied for several times. For this reason, Emil Fischer (1894) proposed a key-lock analogy to the functioning of the enzyme. According to the theory, only with the appropriate substrate, the enzyme will activate its site. In 1954, Koshland proposed to adapt the first theory, named Induced Fit Theory (Figure 2.1), when was the assumed that the enzyme is partly flexible, being able to be induced to the proper alignment of the active site, although only the proper substrate is capable of inducing the proper alignment of the active site (KOSHLAND, 1995; LICHTENTHALER, 1995). Initially, the enzyme binds the active site to the substrate by non-covalent interaction, including hydrogen bonds, ionic bonds, and

hydrophobic interactions. When the substrate is bound to the active site of the enzyme, multiple mechanisms can catalyze the conversion product (COOPER, 2000).

Figure 2.1 – Enzymatic catalysis of a reaction between two substrates based on Induced Fit Theory, adapted from key lock analogy.



Source: The Author.

Like other proteins, the enzymes may be submitted to mutational, induce evolution and therefore, new functions. Three principal properties can facilitate this event: (a) Trade-off between stability, which affords mutational robustness and flexibility; (b) Substrate ambiguity, that allows an enzyme can recognize alternative compounds that are related to the native substrate and catalytic promiscuity enabling a single enzyme to catalyze multiple chemically distinct transformation; and (c) Epistasis, preventing the enzyme establishment a new interconversion without resuming the last route (KALTENBACH; TOKURIKI, 2014; SCHULENBURG; MILLER, 2014).

The most common classification of enzymes is based on the key type of chemical reaction are able to catalyze, being them: (1) Oxidoreductases, characterized to performed oxidation-reduction, in which oxygen and hydrogen are gained or lost (ex: lactate dehydrogenase); (2) Transferases, that carry out functional groups, as acetyl, amino or phosphate group (ex. acetate kinase); (3) Hydrolases, which catalyzes the hydrolysis reaction (ex: lipase and sucrose); (4) Lyases, which is featured for removal groups of atoms without hydrolysis (ex: oxalate decarboxylase); (5) Isomerases, which is based on a rearrangement of atoms within a molecule (ex: alanine racemase); and (6) Ligases, which, using generally the energy derived from the breakdown of ATP, join two molecules (ex: DNA ligase). Therefore, each enzyme

has a specification, most times working at moderate pH and temperature (AEHLE, 2004; STRAATHOF; ADLERCREUTZ, 2005).

2.1.1 Lipases

Lipases are triacylglycerol ester hydrolases and have gained greater prominence over the years, mainly for be able to catalyze synthesis reactions of industrial importance. The wide range of reactions performed for these proteins includes epoxidation, esterification, transesterification, and alcoholysis, and consequently, can be applied in the production of biofuels, perfumes, cosmetics, foods and flavors developments (JAVED et al., 2018). Because of the large applicability and commercial availability, lipases have been the subject of numerous investigations in the academic area (MIRANDA; MIRANDA; SOUZA, 2015; SALIHU; ALAM, 2015). Although they are found in unicellular and multicellular organisms, lipases originated from yeasts and fungi are the most important catalyst for industrial applications (GUPTA et al., 2015b; PELLIS et al., 2018).

The two main important reactions catalyzed by lipases are (REIS et al., 2009; SHARMA; KANWAR, 2014):

*i.*Hydrolysis:
$RCOOR' + H_2O \rightarrow RCOOH + R'OH$

*ii.*Synthesis:
(a)Esterification
$RCOOH + R'OH \rightarrow RCOOR' + H_2O$
(b)Interesterification
$RCOOR' + R''COOR^* \rightarrow R''COOR^* + R''COOR'$
(c)Alcoholysis
$RCOOR' + R''OH \rightarrow RCOOR'' + R'OH$
(d)Acidolysis
$RCOOR' + R''COOH \rightarrow R''COOR' + RCOOH$

In spite of performing a great number of reactions, lipases have some specificity, so it is necessary to consider the substrate specificity, enantioselective and regioselectivity of the enzyme. The substrate specificity has great importance to improve the efficiency of the catalyst

processes and depends on the type of the lipase, generally from the natural source, as glycerol, ester or triacylglycerol. For example, lipase A from *Candida antarctica*, has more affinity with eladic acid over oleic acid, while lipases from *Candida rugosa* prefers oleic acid over eladic acid. Also, lipases can discriminate enantiomers in a racemic mixture due to the enantioselectivity, enabling, for example, the synthesis of R-isomer of aspartame, which tastes sweet, whereas the S-isomer tastes bitter. It is important to highlight, that this feature depends upon the substrate.

The regioselectivity of lipases can be subdivided into three segments: (a) non-specific lipases: monoacylglycerols and diacylglycerols, that are the intermediate products on the hydrolysis of triacylglycerol into fatty acids and glycerol in a random way; (b) specific 1.3 lipases: only hydrolyze triacylglycerol at C1 and C3 glycerol bonds producing 1.3-diacylglycerol and 1- or 3-monoacylglycerols and; (c) specific or selective fatty acid types: the lipases show the capacity to hydrolyze fatty acid esters located at any triacylglycerol position (BARROS; FLEURI; MACEDO, 2010; KAPOOR; GUPTA, 2012; SHARMA; KANWAR, 2014).

The lipases, as the other enzymes, can be in free and immobilized form. In the free form, the enzyme consists of a liquid formulation, generally stabilized by sorbitol, to prevent the denaturation. This form presents the advantage of easy and low-cost preparation (AGUIEIRAS; CAVALCANTI-OLIVEIRA; FREIRE, 2015). However, the use of the enzyme in free form presents some disadvantages, as instability in reaction medium and difficulty of reuse. Because of that, the immobilization of these proteins becomes attractive from the commercial point of view.

2.1.1.1 Lipase NS-40116

The lipase NS-40116 is a liquid formulation from modified *Thermomyces lanuginosus*, presenting some literature reports, mostly regarding biodiesel obtainment. Price et al. (2016) performed experiments on a pilot plant (80 L and 4 m^3) and a plant data (40 m^3) using cooking oil to produce this biofuel by this free commercial lipase. Their study aimed to evaluate the difference between using fed-batch operation system and continuous stirred tank reactor. They concluded the fed-batch showed the best results. Silva et al. (2016) evaluated different

concentrations of enzyme NS-40116 for the conversion of abdominal chicken fat into methyl esters. The authors concluded that reaction time of 20 hours with 0.7% (w/w) showed the most favorable results. Santos (2016) studied the enzymatic production of biodiesel from *macauba* pulp oil using the free lipase as a catalyst. The author concluded that it was possible the production of methyl esters using the lipase NS-40116. Fagundes (2016) evaluated the enzymatic catalysts using alternative raw material (residue from the extraction of soybean oil). The results demonstrated that the best response for esterification of fatty acids was 37 °C, 2 equivalents of methanol (divede in 6 additions), 3% of water (m/m), using as catalyst the liquid formulation of lipase NS-40116 as catalyst.

Honaiser (2017) investigated the lipase immobilization on a hydrophobic matrix obtained by sol-gel technique. The basic immobilized with PEG was considered the most efficient, presenting higher enzymatic activity, higher yield, higher number of recycles and thermal stability. Dantas (2017) evaluated the best method to interact polystyrene particles and lipase NS-40116 and different methods of immobilization were studied: support adsorption, entrapment, and covalent bond by treating the particles with glutaraldehyde. The immobilized derivative was effectively used in the production of free fatty acids from soybean oil, and also showed application potential in transesterification and esterification reactions using abdominal chicken fat as substrate. In the hydrolysis of soybean oil, the derivative demonstrated operational stability after five cycles of use.

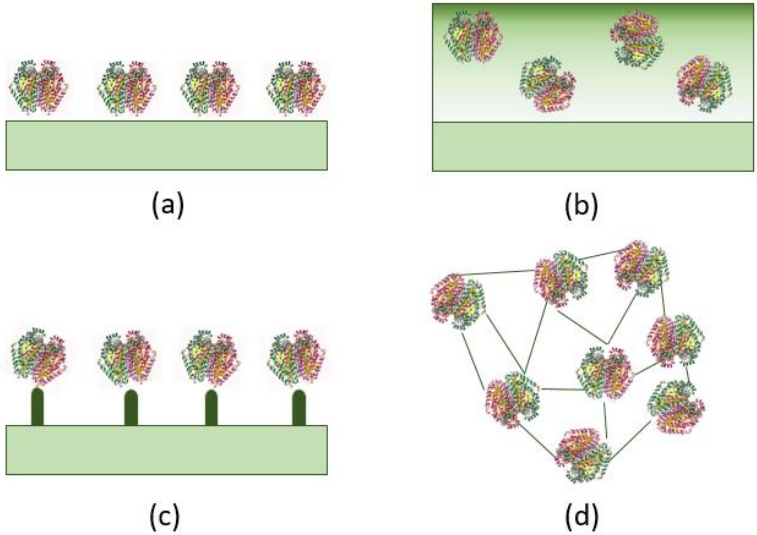
In a recent study, Facin et al. (2018) immobilized the lipase NS-40116 in PU support obtained through monomers from petrochemical sources. Free and immobilized enzymes were compared in terms of hydrolysis of soybean oil. Immobilized enzyme by entrapment was evaluated in successive cycles of reuse showing catalytic activity above 50% even after 5 successive cycles of reuse, confirming the efficiency of immobilization process.

2.1.2 Enzyme Immobilization

As the reaction medium conditions are crucial to allow the enzyme catalyzes and generate products, some aspects have been studied to obtain the full capacity of these proteins, and one of the opportunities is the

immobilization, improving stability over soluble enzyme forms, favorable alterations in optimal values of pH and temperature, and provides the possibility of reuse, lowering the operating costs. Furthermore, the immobilized enzyme may change the physical and chemical properties and make possible the co-immobilization with other enzymes. Although essential factors should be taken into consideration as whether to immobilize enzymes as the selection of immobilization supports, and conditions/methods of immobilization, some qualities required for support are biocompatibility, resistance to microbial attack, inertness towards enzymes and low cost (NISHA; KARTHICK; GOBI, 2012; DICOSIMO et al., 2013; MOHAMAD et al., 2015). Different techniques are used for immobilization and the most common are shown in Figure 2.2.

Figure 2.2 – Schematics of the four most common enzyme immobilization techniques: (a) physical adsorption, (b) entrapment, (c) covalent binding and (d) cross-linking.



Source: The Author.

The physical adsorption is the oldest and easiest technique for enzyme immobilization. It is characterized by weak non-specific forces

such as hydrogen bonding, Van der Walls forces, ionic and hydrophobic bonds. In addition, this technique makes possible the support regeneration when enzymatic activity decays. Nevertheless, as the forces are very weak, this method has the possible disadvantage of leaching of the enzyme (NISHA; KARTHICK; GOBI, 2012; MOHAMAD et al., 2015).

Entrapment is an inexpensive and fast immobilization method. Moreover, mild conditions are required for reaction process. The immobilization is performed during the formation of the entrapment matrix. Thus, the method is irreversible and presents a physical interaction (ADLERCREUTZ, 2013; NICOLETTI et al., 2015; MEHTA et al., 2016).

The immobilization by covalent binding occurs through direct binding of the enzyme with the solid support. The binding is strong, thereby there is no leaching of the enzyme, and the enzymatic activity will only be affected by the natural loss of activity due to the reuse. Although it presents various advantages of stability, the method is considered high cost and presents difficulty to preparation (ASGHER et al., 2014; AHMAD; SARDAR, 2015; SHUAI et al., 2017).

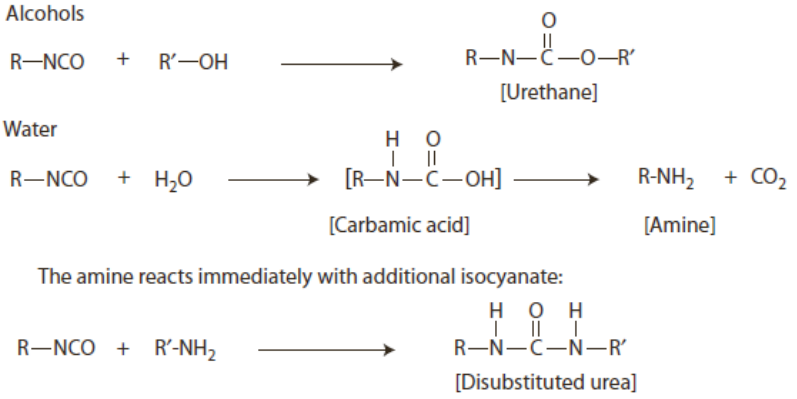
To perform the cross-linking immobilization, the agent glutaraldehyde is the most used in the presence of an inert protein, such as gelatin and albumin. The method can be divided into two types: cross-linked enzyme crystals and cross-linked enzyme aggregates. The first one is carried out with the crystallization of enzyme, where crystal sizes of 50 - 150 μm is preferred. This immobilization offers high stability due to protein-protein interaction within the crystal, but it is necessary enzymes with a high degree of purity to an effective crystallization. The second one is prepared by precipitation of the enzyme in a solution and have better storage stability, however, presents severe diffusion limitations and lacks mechanical strength. The two methods present limited application because the gelatinous medium (ADLERCREUTZ, 2013; BHATTACHARYA; PLETSCHKE, 2014; GUPTA et al., 2015a).

The most common methods of immobilization can be used in various media including inorganic (clays, zeolites, and mesoporous silicas), natural, and synthetic polymers. Nowadays, polymers have gained notoriety as support for enzymes immobilization. Among the most used in recent studies is the polyurethane, which has distinctive characteristics compared to other polymeric matrices for the low cost (NICOLETTI et al., 2015; CIPOLATTI et al., 2016; NYARI et al., 2016).

2.2 POLYURETHANE

Polyurethane is a versatile polymer, enabling applications from building insulation to scaffolds (JANIK; MARZEC, 2015; NOREEN et al., 2016). The polymer has formatted by monomer repeatedly unit of urethane linkages in its structure, which are non-regularly distributed, therefore differentiating it from other polymers (HOOD et al., 2010). This polymer is obtained by a reaction of a diol (OH) and diisocyanate (NCO) in a polyaddition polymerization (BILLIET; FOURNIER; DU PREZ, 2009). In the presence of water, it is possible to observe the formation of urea grouping. This occurs due to hydrolysis of the diisocyanate, resulting carbamic acid which then, because of the low stability, generates an amine, and this amine reacts with another diisocyanate to form an urea grouping, as presented in Figure 2.3 (SZYCHER, 2013).

Figure 2.3 – Reaction of polyurethane and polyurea formation.



Source: Adapted from Szycher, 2013.

It is possible to divide this polymer into several types: flexible polyurethane foam, rigid polyurethane foam, coatings, adhesives, sealants and elastomers, thermoplastic polyurethane, reaction injection molding, binders and waterborne polyurethane dispersions. Furthermore, PU presents a wide range of applications, like apparel, appliances, automotive, building and construction, composite wood, electronics, flooring, furnishings, marine, medical and packaging. On a global scale,

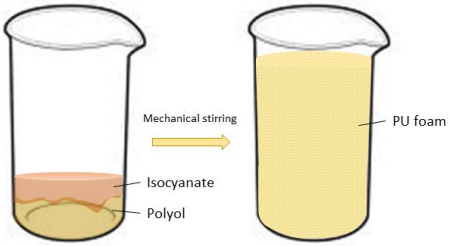
this polymer is used in large quantities, representing economic importance in this segment, employing over 800,000 people in the European Union and representing the sixth position in the market. Thus, most of the producers of this polymer has been searching for renewable alternatives, reducing the environmental impacts, investing more in programs and studies for new sources and routes to obtaining PU investigation (SZYCHER, 2013; ISOPA, 2016).

2.2.1 Polyurethane foam

The most common mold of polyurethane is foam. Foaming systems are classified into three types, based on the type of chemicals used in the synthesis process. The one-step system consists in the mixed of the polyol (containing surfactant, blowing agent and catalyst) with isocyanate, leading the formation of the foam. The second, quasi-prepolymer system, is characterized by a premix of polyol (no additives) with isocyanate and, after, is added more polyol containing the blowing agent, surfactant, and catalyst. The last one, full-prepolymer system, is not industrially used. It is performed by mixing the polyol with the isocyanate, and after mixing, the additives are added such as the blowing agent (SHOAIB et al., 2014). The first cited method is frequently used in industry.

The bulk polymerization technique is used (Figure 2.4) to obtain the polyurethane foams. In this type of technique is required vigorous agitation because there is an increase of viscosity system, making the conversion depending on the homogeneity of the reaction medium.

Figure 2.4 – Bulk polymerization to obtain polyurethane foam.



Source: The Author.

The PU characteristics depend on the chemical composition of polyol, polyol and isocyanate functionality, crosslink density, foam density, urea content and cell structure. The diisocyanates generally used to obtain PU foam are methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI) and, due to high reactive with the active hydrogen, results in rigid and semi-rigid foams (SZYCHER, 2013; KRAITAPE; THONGPIN, 2016). The polyol, on the other hand, can be produced or purchased. In recent years, studies involving the use of vegetable oils as a polyol has been increased (DWORAKOWSKA et al., 2014; LI et al., 2014; ZHANG et al., 2014). Since this is a polycondensation reaction, the functionality of the polyol will directly affect the structure of the polymer (ZHANG; JUNE; LONG, 2012).

2.2.2 Polyurethane nanoparticles

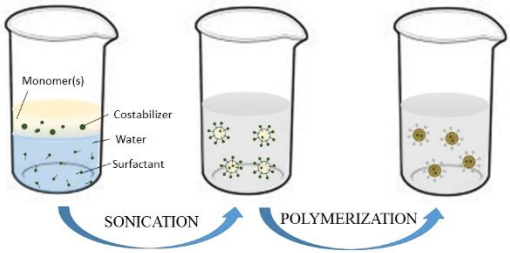
Studies involving polymeric nanoparticles (NPs) have emerged in recent years, especially due to a wide variety of potential applications in the areas of physical, chemical, biological, health sciences and other interdisciplinary fields of science and engineering (BOUR et al., 2015). Considering the versatility and wide application of the polyurethane, this polymer has become the target of several studies, when the miniemulsion technique can be used to NPs synthesis.

This polymerization technique is defined as dispersions with drops that have a stable particle size between 50-500 nm. To reach this submicrometer size, the reaction is subjected to a high mechanical shear force, such a sonication or high-pressure homogenization (LANDFESTER; WEISS, 2010). The polymer particles are formed via droplet nucleation and the miniemulsion system is obtained from a continuous and a disperse phase. A surfactant is required to prevent the droplets and particles from coalescence, due to the small particle size (CAO; ZIENER, 2013). Figure 2.5 illustrates the technique of direct miniemulsion polymerization when performed with hydrophobic monomers.

The poly(urea-urethane) nanoparticles started to be studied when Tiarks, Landfester & Antonietti (2001) published a research in which they conducted the synthesis of nanoparticles in aqueous dispersion, obtaining particle sizes ranging between 202 and 232 nm. Years later, using miniemulsion polymerization technique, Zanetti-Ramos et al. (2006)

investigated the formation of PUU-NPs using vegetable oil as polyol and sodium dodecyl sulfate (SDS) as surfactant, obtaining particle size near 280 nm. Valério, Araújo & Sayer (2013) studied the influence of reaction temperature, polyol type, and surfactant type and concentration in the stability of PUU nanoparticles containing *açaí* oil. The authors concluded that reaction temperature and polyol type are significant parameters to be evaluated in the synthesis of PUU-NPs.

Figure 2.5 – Synthesis of polymeric nanoparticles via the direct miniemulsion technique.



Source: The Author.

Some authors investigated the bioavailability and biocompatibility of these NPs. Valério et al. (2014) studied the degradation of PUU NPs in 15 mL buffer solution (pH 7.0) incubated in an oven at 37 °C in a regular interval of 3, 7, 10, 15 and 30 days. It was possible to observe the decrease of molar mass in the polymers using PEG as polyol and the increase of molar mass when castor oil/PEG was used. Morral-Ruíz et al. (2014) studied the hemocompatibility and cytotoxicity of polyurethane and polyurea NPs based on surfactant polyoxyethylene derivative from castor oil, suitable for endovascular applications. The authors drew attention to the choice of surfactant used in the polymerization reaction to achieve the inactivation of nucleophilic groups which have been bounded by the appearance of acute hypersensitivity reactions in susceptible patients. The results indicated the potential of these nanosystems as promising drug carriers. Morral-Ruíz et al. (2015) investigated the possibility of the use of PUU-NPs as combined targeted therapy and detection strategy in human hepatocellular carcinoma.

2.2.3 Enzyme immobilization in polyurethane support

Polyurethane presents wide applicability for enzyme immobilization. The entrapment method is most commonly used when the PU is used as the polymer support. In addition to the inexpensive and easy immobilization technique, this polymer has several advantages, such as the possibility to be obtained from renewable sources, biodegradability and low cost (MIAO et al., 2014). Thus, studies using PU as the enzyme immobilization matrix has become increasingly common.

In the last years, PU foam was used for immobilization of many distinct enzymes. Silva et al. (2013) evaluated the stability of the activity of commercial inulinase from *Aspergillus niger* immobilized in polyurethane foam. The authors reused the immobilized enzyme for 24 cycles for the sucrose and inulin hydrolysis maintained 49.7% and 49.4% of enzymatic activity, respectively. Santin et al. (2014) used the polyurethane foam as support for *Candida antarctica* B (CalB) immobilization and performed esterification of fatty acids under ultrasound irradiation obtaining 81.6% of conversion, showing the technical viability for biodiesel production. Bustamante-Vargas et al. (2015) studied the immobilization of commercial pectinase from *Aspergillus niger* in rigid PU foam for application of the enzymatic derivative on the pectic oligosaccharides hydrolysis. The immobilized enzyme was reused consecutively for 6 catalytic cycles keeping 35% of the initial activity. Nicoletti et al. (2015) investigated different methods for immobilization of CalB in polyurethane foam using entrapment, adsorption, ionic adsorption, and ionic adsorption with a crosslinker agent. The immobilized enzyme was applied in the production of geranyl propionate and presented better results regarding conversion using the immobilized lipase by entrapment and adsorption techniques (83.5% and 95.9%, respectively). Nyari et al. (2016) evaluated the characterization of the CalB immobilized in PU foam and applied on enzymatic synthesis reactions of geranyl oleate, geranyl propionate, and ethyl oleate esters.

Enzyme immobilization investigations using poly(urea-urethane) nanoparticles as support has featured interest to researchers. Cipolatti et al. (2014) studied the immobilization of CalB on PEGylated PUU NPs by one-step miniemulsion polymerization. The authors evaluated the effect of sonication amplitude and time on enzymatic activity and concluded the time of sonication which led to the highest enzymatic activity was 2

minutes at 70% of amplitude. Cipolatti et al. (2015) immobilized *Thermomyces lanuginosus* (TLL) lipase in PUU-NPs under the same conditions amplitude and sonication time above mentioned. The enzymatic derivative immobilized in PUU-NPs were applied in ethanolysis of ethyl esters from fish oil, proving to be effective for this reaction. Cipolatti et al. (2016) investigated the kinetic properties, thermal stability and the effect of pH on the enzymatic activity of immobilized TLL in PUU-NPs using different diol: poly (ethylene glycol) with nominal molar mass of 400, 4000 and 6000 Da, polycaprolactone diol with molar mass 530 Da, and polyethyleneimine 25,000 Da.

As presented above, although several studies have been conducted using PU as support for enzymes immobilization, most of them are carried out by using polyol from the petrochemical industry, showing that investigations in this area are still required.

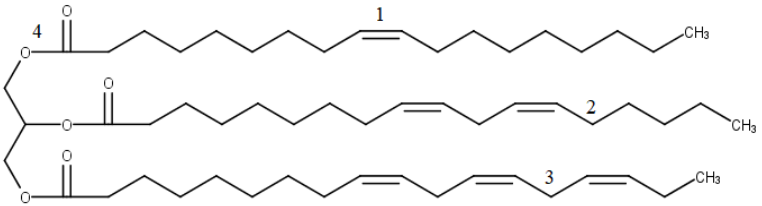
2.3 VEGETABLE OILS

Polymers have been the aim of countless studies, searching the replacement of the petrochemical monomers for renewable sources. In this perspective, the vegetable oils are the most used source, mainly for presenting a several attractive characteristics than the other bio-based polymers, as easily of conversion the vegetable oil in monomers with different characteristics, similar final polymer properties to petroleum-based and development of environmentally benign products (MIAO et al., 2014; SHARMIN et al., 2015). Furthermore, the polymers from vegetable sources are, according the 7th principle of the *12 Principles of Green Chemistry* (Use of Renewable Feedstocks) and the 12th principle of the *Principles of Green Engineering* (Renewable Rather than Depleting), promoting over the past decade great advances and reducing the environmental impact (ANASTAS; WARNER, 2000; HOTTLE; BILEC; LANDIS, 2013).

The vegetable oils are mostly composed of triglycerides and may have variations in their molecular structure, carbon chain, instauration and presence of functional groups (MUTLU; MEIER, 2010; LLIGADAS et al., 2013). The structure and reactive sites are demonstrated in Figure 2.6. The characteristics determinate how the oil can be used in the polymerization reactions or modifications for producing the monomer of interest.

Usually, vegetable oils are used to obtain a serial of polymers, as polyester, polyurethanes, polyether, and polyolefin and accomplish the synthesis of polyols and epoxies (GÜNER; YAĞCI; ERCIYES, 2006; LLIGADAS et al., 2013; ALAM et al., 2014). The most common oils used in studies are castor oil, due to the hydroxyl group presence, and soybean oils, due to the low cost and high availability. However, others oils are being investigated, as corn (FAJARDO et al., 2016), rapeseed (ZIELENIEWSKA et al., 2015), and palm oil (TAY et al., 2011). Usually, it is required a reaction, aiming the ideal polyol for the PU polymerization, as epoxidation, esterification, glycerolysis, and hydroxylation (MUTLU; MEIER, 2010; KONG et al., 2013; LLIGADAS et al., 2013; NOREEN et al., 2016).

Figure 2.6 – Demonstration of the structure of triglyceride and reactive sites.



1 Double bond
2 Monoallylic position
3 Bisallylic position
4 Ester group

Source: Adapted from Miao et al., 2014.

2.3.1 Castor oil

As mentioned, the castor oil is one of the oils that have more attractive features for performing polymerization, with six distinct fatty acids in its composition: palmitic, stearic, oleic, linoleic, linolenic and the most important, the ricinoleic (90%). The ricinoleic acid presents a hydroxyl group in its structure, enabling a series of reactions. It is well known that only two oils have fatty acids with this feature, and the other one is lesquerella oil, but there is no sales potential due to its high market price when compared to castor oil (MUTLU; MEIER, 2010; RIEGER et al., 2012).

The world-leading producer of castor oil is India, which is responsible for about 60% of seeds (between 250,000 and 350,000 tons per year), followed by China and Brazil (20% and 10%, respectively). The others 10% is produced in small regions of Africa. The industrial application of this oil is wide, such as fuel and biodiesel, soaps, waxes, and greases, to performed polymeric materials and coatings, also it possess medicinal and pharmacological properties (PATEL et al., 2016).

The application of this oil to produce polymeric materials is an important industry segment. The OH group presented in its chemical structure, allows a reaction of the oil with isocyanate to obtain PU, without requiring any modification reaction. However, since it is a secondary hydroxyl, the reaction may be sterically hindered, bringing some disadvantages in the final polymer, depending on the desired application (Vilar, 2008). As a result, some studies aiming the modification of the oil were performed.

Moghadam et al. (2016) investigated direct esterification reaction of maleic, fumaric or oxalic acids with castor oil to obtain a polyol using single step reaction without solvent, initiator or a purification process. The authors prepared polyurethane wood adhesives with good resistance to cold and hot water, and weak resistance to acid and alkali media. Ionescu et al. (2016) prepared three polyols from castor oil with high OH values using reactions of mercaptan groups with compounds having double bonds and hydroxyls. The polyols were used to obtain PU foams. Mechanical and physical properties were evaluated, and the authors concluded the possibility of application as thermal insulation in freezers, storage tanks for the chemical and food industries, building insulation and packaging, or as a wood substitute. Bresolin et al. (2017) obtained a PU foam using a green polyol with high hydroxyl value from castor oil. The oil was modified by transesterification reaction using crude glycerol and a chemical catalyst. The foam presented a high solvent resistance and crosslinking. These are some interesting examples of the importance of castor oil and how it has been used in researches to obtain PU polymer.

2.4 GLYCEROL

Glycerol, or according to IUPAC nomenclature, propane-1,2,3-triol, is composed, basically, by three carbons, two primary hydroxyls and one secondary, which is responsible for its hygroscopic character and

solubility in water. Physically, glycerol is a clear, colorless, odorless, hygroscopic, viscous and sweet taste liquid. It is derived, quantitatively, from biodiesel formation reaction (transesterification of fats/oils). As Brazil is the major producer of biodiesel, consequently, is the biggest generator of this byproduct.

The reaction for biofuel formation can be achieved using both homogeneous and heterogeneous catalyst system. However, when the glycerol is from homogeneous catalysis, high-degree purification is necessary to achieve good quality product and consequently escalated process cost (TAN; AZIZ; AROUA, 2013; GALADIMA; MURAZA, 2016; HEJNA et al., 2016). This compound can be used with and without purification. Industrially, when is used in a crude form, it is destined to burn for energy. If purified, it can be used in cosmetics, soaps, pharmaceuticals, tobacco, food and drink and paper industries, also in the production of polyglycerol esters and alkyd resins (ARDI; AROUA; HASHIM, 2015). Glycerol can be applied in some reactions, such as glycerolysis, resulting in the formation of mono- and diacylglycerols.

2.4.1 Glycerolysis reaction

Glycerolysis is one of the most important transesterification reactions, though is possible obtain mono- and diacylglycerols, which have great importance in the food and pharmaceutical industries. This reaction occurs between vegetable oil and glycerol in the presence of a catalyst (that may be chemical or biological). In this context, the biocatalysts attracted more attention during the last years, because they are environmentally friendly, present a higher conversions and milder reaction conditions are required (VALÉRIO et al., 2010; ZHONG et al., 2013; NAIK; NAIK; MOHANTY, 2014). While the chemical glycerolysis reactions need high temperatures (210-240 °C), enzymatic reactions are performed at a temperature range of 60°C-80 ºC. Through the use of high temperatures when the chemical catalyst was used, the oil can be lead to oxidation, resulting in dark color and off-flavors. Consequently, the product needs to be purified to be used in the food industry (FELTES et al., 2013).

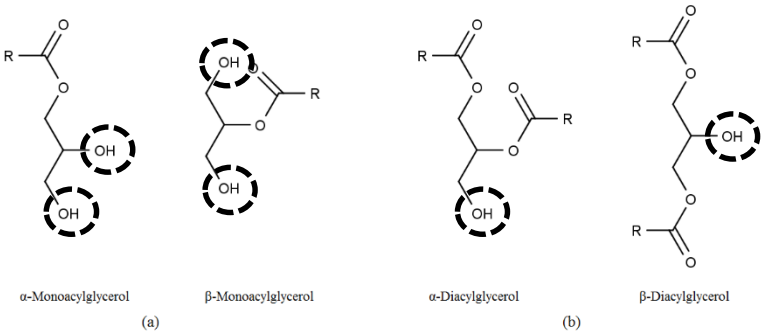
Thereby, enzymatic glycerolysis for monoacylglycerol (MAG) and diacylglycerol (DAG) production has been the subject of many studies. Initially, this reaction occurs in solvent presence system and the

studies were focused on solvent-free systems using a surfactant. Valério et al. (2010) investigated the influence of surfactant type and concentration, glycerol:oil molar ratio and enzyme concentration in the MAG and DAG production. In the study, olive oil and Novozym 435 were used as reactants under the reaction condition of 70 ºC, 600 rpm for 2 hours. The optimum reaction condition for the production of MAG and DAG was using Tween 65, and Triton X-100 was the surfactant in a concentration of 16 wt% (w/w), enzyme concentration of 9.0 wt% (w/w) and glycerol to olive oil molar ratio of 6:1. Fiametti et al. (2012) also used the commercial immobilized lipase Novozym 435 and olive oil, though investigated the reaction under the influence of ultrasound irradiation in a solvent-free system. Significant amounts of MAG and DAG were produced using 130 W of irradiation power supply in 2 hours of reaction time at 60-70 ºC and 7.5 wt% (w/w) of the enzyme. Remonatto et al. (2015) studied the lipase-catalyzed glycerolysis using Novozym 435 as the catalyst for reactions using soybean and canola oils in a free organic solvent system assisted by ultrasound. Based on experimental design, different conditions of stirring, temperature, molar ratio (oil:glycerol), ultrasound bath intensity and enzyme concentration were evaluated. The authors obtained 65 wt% of MAG and DAG conversion when the soybean oil was used with 52.8 W cm^{-1} ultrasound intensity in 180 min of reaction time, 10 wt% of enzyme concentration, 0.8:1 substrates molar ratio, 70 ºC, and 900 rpm. When canola oil was used, 75 wt% of MAG and DAG was obtained in the same conditions of stirring, enzyme concentration, molar ratio, and temperature, however the reaction time and ultrasound intensity were 120 minutes and 135 $W.cm^{-2}$, respectively. The results showed decreasing resistance to the mass transfer in the three-phase system glycerol/oil/lipase, maximizing MAG and DAG production when the ultrasound bath was used. The justification of so many studies in this area is due to the great industrial importance that these two components have.

2.4.1.1 Mono- and diacylglycerol characteristics

MAG has in its chemical structure two hydroxyls and carboxylic acid, being in this condition isomeric compound present the β-MAG and α-MAG (Figure 2.7a).

Figure 2.7 – (a) Isomeric MAG chemical structure and (b) isomeric DAG chemical structure.



Source: The Author.

The MAG has an amphiphilic profile and the free hydroxyl groups are responsible for the hydrophilic behavior and acyl grouping by hydrophobic feature. This compound is used in the food, pharmaceutical, and cosmetic industries, representing about 70% of all synthetic emulsifiers used (YANG et al., 2004; BAHMANJAH; ZHANG; DAVIS, 2012). DAG has in its chemical structure one hydroxyl and two acyl functions, it have two isomeric forms, as well as MAG. At first, the hydroxyl function is the end compound, less sterically hindered, and acyl functions are on C-1 and C-2. The isomeric form has a hydroxyl intermediate, more sterically hindered (Figure 7b) (CHRISTIE, 2013). In addition to the use in food and cosmetic industries, MAG and DAG can be used for polymerization reactions, including PU synthesis (BRESOLIN et al., 2017)

2.5 FINAL CONSIDERATIONS

Based on the above-mentioned studies from the literature, this work intends to increase the knowledge in immobilization of lipases using green polyurethane (foams and nanoparticles) as support. The immobilization of enzymes in polyurethane foam has several contributions in the literature (SILVA et al., 2013; BUSTAMANTE-VARGAS et al., 2015; NICOLETTI et al., 2015; NYARI et al., 2016; FACIN et al., 2018). However, in their absolute majority, the investigations used commercial polyols derived from petroleum sources.

When the studies were performed with nanoparticles as support (CIPOLATTI et al., 2014, 2016, CHIARADIA et al., 2016a, 2016b), the same has been observed in most of the researchers.

In this sense, this study reported the use of a biopolyol, obtained through the glycerolysis reaction between castor oil and glycerol, using lipases as catalysts. The reaction products were used for greener polyurethane polymerization and as support for lipase NS-40116 immobilization, turning the process environmentally friendly and less polluting. Also, was shown the synthesis of poly (urea-urethane) nanoparticles via miniemulsion technique using the product of glycerolysis as polyol and stabilizer with high solids contents (20 to 40%). To the best of our knowledge, until the last research done on digital platforms, we did not find any work that uses biopolyols synthesized through glycerolysis reaction to obtain polyurethane support for enzyme immobilization and poly(urea-urethane) NPs without the use of extra stabilazier.

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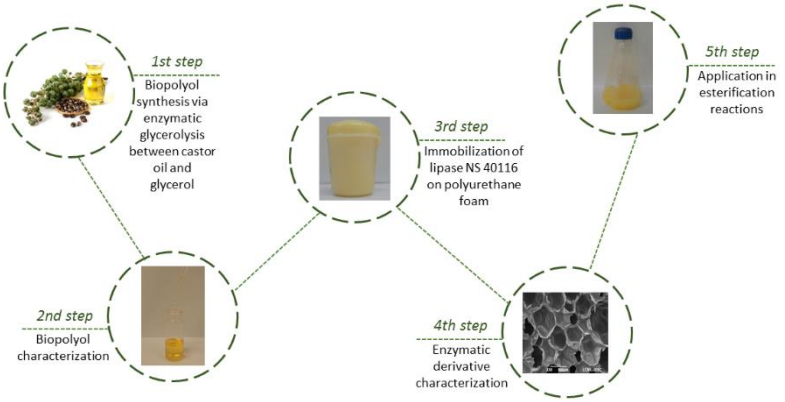
CHAPTER III

Synthesis of a green polyurethane foam from a biopolyol obtained by enzymatic glycerolysis and its use for immobilization of lipase NS-40116



- Enzymatic glycerolysis showed high conversions in mono- and diacylglycerols;
- Biopolyol was able to be used for polyurethane foam synthesis;
- Enzymatic derivative presented good stability in different medium conditions;
- Immobilized lipase proved to be capable to act as catalyst for esterification reactions.

Abstract

The use of green sources for materials synthesis has gained notoriety in recent years. This work investigated the immobilization of lipase NS-40116 in polyurethane foam using a biopolyol obtained through the enzymatic glycerolysis between castor oil and glycerol, catalyzed by the commercial lipase Novozym 435. The reaction performed to obtain the biopolyol resulted in the conversion of 64% in mono- and diacylglycerol, promoting the efficient use of the reaction product as biopolyol to obtain polyurethane foam. The enzymatic derivative with immobilized lipase NS-40116 presented apparent density of 0.19 ± 0.03 g/cm³, cell medium size of 748 ± 0.2 µm and an immobilization yield of 94±4%. Free and immobilized lipase NS-40116 were characterized in different solvents (methanol, ethanol, and propanol), temperatures (20,40, 60 and 80 ºC), pH (3, 5, 7, 9 and 11) and presence of ions Na^{+}, Mg^{++}, and Ca^{++}. The support provided higher stability to the enzyme. The enzymatic derivative was also used for esterification reactions and conversions around 66% in fatty acid methyl esters, using abdominal chicken fat as substrate were obtained in the first use, maintaining this high conversion until the fourth reuse, proving that the support obtained using environmentally friendly techniques is usable and applicable.

Keywords: Biopolyol; polyurethane foam; lipase immobilization; lipase NS-40116; biocatalysis; environmental biotechnology.

3.1 INTRODUCTION

In the last years, the search for clean technologies has encouraged the study of biocatalysts for different applications, mainly due to the characteristics as high catalytic activity, selectivity, specificity and high activity under mild environmental conditions (BARBOSA et al., 2015; CARVALHO et al., 2016; CORTEZ et al., 2017) and in these studies, one of enzyme class, the lipases, have gained notoriety due to their large applicability (KAPOOR; GUPTA, 2012; TAN et al., 2015). They are triacylglycerol ester hydrolases able to catalyze synthesis reactions of industrial importance. Lipases can be used in a free and immobilized form; in the free form, the biocatalyst consists in a liquid formulation, generally stabilized by sorbitol with a low-cost preparation associated (AGUIEIRAS; CAVALCANTI-OLIVEIRA; FREIRE, 2015). Though, the use of the lipase in the free-form shows some disadvantages, as instability in the reaction medium and reuse inability. Thus, the immobilization process becomes attractive due to the reuse possibility, more efficient recovery and often enhanced stability (SHUAI et al., 2017).

Immobilization can occur in different matrices and the polymers have been extensively investigated in recent years, for example, the polyurethane foam (PUF) (SILVA et al., 2013; BUSTAMANTE-VARGAS et al., 2015; NICOLETTI et al., 2015; NYARI et al., 2016). The PUF is a porous polymeric matrix that presents a wide range of application (JANIK; MARZEC, 2015; NOREEN et al., 2016), and the polymer has formed by repeating unit of urethane bonds in the structure, non-regularly distributed, thereby differentiating it from other (HOOD et al., 2010). The PU is obtained by the reaction between a diol (OH) and a diisocyanate (NCO) group in a polycondensation reaction (BILLIET; FOURNIER; DU PREZ, 2009). The PU characteristics depend on the polyol nature, functionality and diisocyanate, influencing parameters such as crosslink density, foam density, urea content and cell structure.

The entrapment method is the most commonly used for the enzyme immobilization in polyurethane foam, associated with the support low cost and easy immobilization technique and this method presents several advantages, such as the possibility to be obtained by renewable sources and biodegradability (MIAO et al., 2014). Furthermore, mild conditions are required for the reaction process, and the immobilization can be

performed during the matrix formation (ADLERCREUTZ, 2013; NICOLETTI et al., 2015; MEHTA et al., 2016). However, in most investigations the foam is obtained using petrochemical monomers source (NICOLETTI et al., 2015; NYARI et al., 2016), though the biopolyols synthesis through renewable sources and clean catalysts is possible.

Therefore, this work aimed to evaluate the immobilization of lipase NS-40116 (*Thermomyces lanuginosus*) in polyurethane foam, using a biopolyol obtained by enzymatic glycerolysis between castor oil and glycerol in a solvent free system, catalyzed by commercial lipase Novozym 435. Also, the stability of free and immobilized lipase in different media was studied, and, as a final goal, the application and reuse of the immobilized lipase NS-40116 as a catalyst was evaluated in reactions to obtain fatty acid methyl esters (FAME) using abdominal chicken fat as feedstock.

3.2 MATERIAL AND METHODS

3.2.1 Material

The lipases were donated by Novozymes Latin American. Castor oil (Campestre Industry), glycerol (Vetec, 99.5%), Tween 80 (Vetec, 65-80 mg (OH/g)) and Novozym 435 (*Candida antarctica B*) were used to obtain the biopolyol. N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) from Merck Millipore, n-heptanol (Sigma-Aldrich) and pyridine (Sigma-Aldrich) were used in the gas chromatograph analyses. Standards were acquired with Sigma. Methylene diphenyl diisocyanate (MDI - specflex NE 134) was donated by Dow Chemistry. ρ-nitrophenyl palmitate (Sigma, ≥ 98%) and ethanol (Sinth, 99.5%) were used as substrates to determinate the enzymatic activity. NaOH, Ca_2OH and Mg_2OH (all from Dinâmica Química, 97%) were used to determine the metals ions stability of the free and immobilized enzymes. Methanol (Sinth, 99.5%), ethanol (Sinth, 99.5%) and propanol (Dinâmica Química, 99.5%) were used to determine the stability of the lipases to organic solvents. All the chemicals were used as received. Abdominal chicken fat, donated by BRFoods, was firstly heated to separate the fatty from the residual skin and n-hexane (Lafan, 99.5%) was used as solvent in the FAME synthesis.

3.2.2 Enzyme concentration

Since water can act as a blowing agent, by the reaction with the diisocyanate group to form urea bonds, reflecting directly on the quality of the polymer, the liquid formulation of free lipase NS-40116 was concentrated using collagen membranes (11.625 m^2/g of surface area and pore diameter 21.752 Å, from Devro) in phosphate buffer solution 0.05 mmol/L (pH 7.0), for 10 cycles, during 72 hours. The content of the membranes was transferred to Petri dishes and subjected to freezing at -10 °C (Glacier Ultralow Temperature Freezer) for 24 hours. After freezing, the enzyme was lyophilized (LIOTOP Lyophilizer, Model L101) for 48 hours. The concentrated lipase NS-40116 was used in the immobilization process.

3.2.3 Biopolyol preparation and characterization

Biopolyol was obtained by enzymatic glycerolysis, according to the method previously reported by Valério et al. (2010). In a jacketed glass reactor, castor oil and glycerol in a molar ratio 1:6, 9 wt% of immobilized Novozym 435 as catalyst and 16 wt% of Tween 80 as surfactant was mixed and kept at 70 °C and 600 rpm for 3 h. After the reaction, the product was separated from the unreacted glycerol. For this, the reaction product was placed in a phase separation funnel for 24 hours, the lower part (unreacted) was discarded and the upper phase was used as biopolyol. Mono- and diacylglycerol (MAG and DAG) determination was carried out according to the ASTM D6584-00 (2014), using gas chromatography (GC, Shimadzu 2010) with an automatic on-column injector and flame ionization detector (FID). The programming of column temperature was as follows: 50 °C for 1 min, followed by increases of 15 °C/min to 180 °C, 7 °C/min to 230 °C, and 10 °C/min to 380 °C, kept for 8 min. Detector temperature was 380 °C, and nitrogen gas as the carrier (pressure 80 kPa). The hydroxyl value was calculated according to ASTM D4274-16, following the method A, where the biopolyol was diluted in a mixture of pyridine and acetic anhydride and titrated with a standard sodium hydroxide solution. The hydroxyl content is calculated from the difference in the titration of the blank and sample solutions. The pH was measured on pHmeter (AN2000 Analion). All analyses were performed in triplicate (n=3).

3.2.4 Enzyme immobilization and characterization

The concentrated lipase NS-40116 was immobilized by entrapment method through bulk polymerization in one single step during the polyurethane foam synthesis. The PUF synthesis was carried out at room temperature with mechanical stirring (3000 rpm) for 1 minute, and the molar ratio diisocyanate to biopolyol (NCO:OH) used was 1:1. Distilled water was applied as blowing agent (1 wt% in relation to the monomers mass) and 20 wt% of concentrated lipase NS-40116 was immobilized. The enzymatic derivative was stored under refrigeration for further use.

The characterization of immobilized lipase was performed by Fourier Transform Infrared Spectroscopy (FTIR) by Prestige-21 (Shimadzu). Samples were homogenized in KBr pellets and analyzed by transmittance in the region of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. The sample morphology was verified by scanning electron microscopy (SEM) analysis with field emission (SEM-JEOL JSM-6390LV). The PUF was cut and fixed with carbon tape on a stub and coated with gold. The average size analysis was carried out with the software Size 1.1 meter. The apparent density analysis was performed according to the methodology described in ASTM D1622/D 1622M-14. The samples were cut 5x5x5 mm with a vernier caliper (Starrett: 125MEB-6/150) aid. Then, it was weighed on a digital scale with a precision of 0.001 g and 220 g capacity (model AUY220- Marte). The calculated density was expressed in g/cm^3. The analyses were performed in triplicate.

3.2.5 Determination of enzymatic activity

The hydrolytic activity of enzyme was measured following Chiou & Wu (CHIOU; WU, 2004) method using ρ-nitrophenyl palmitate (ρ-NPP) ethanol solution as substrate (5%). The lipase (free, concentrated and immobilized) and the substrate were incubated for 5 minutes at 30 °C with 1 mL of phosphate buffer solution 0.05 M (pH=7). The reaction was terminated by the addition of 2 mL of NaOH solution (0.1 M) followed by centrifuging for 10 min (10,000 rpm). 100 µL of the supernatant was diluted 100-folds with distilled water. The increase in absorbance at 400 nm caused by the release of ρ-nitrophenol in the hydrolysis of ρ -NPP

was measured spectrophometrically (UV/VIS spectrophotometer Hitachi U-1900). One unit (U) of enzymatic activity was defined as the amount of enzyme which catalyzed the production of 1 μmol ρ-nitrophenol per minute under the experimental conditions. All assays were carried out in triplicate.

3.2.6 Determination of the kinetic parameters and yield of immobilization

The kinetic parameters were determined for free and immobilized lipase NS-40116 using p-nitrophenyl palmitate as substrate in different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM), enabling the determination of Michaelis-Menten constant (K_m) and the maximum specific activities (V_{max}) by Lineweaver-Burk plots. The yield of immobilization (γ) was determined using method described for Nyari and coauthors (2016) and calculated according equation 1:

$$\gamma\ (\%) = \frac{UT_{exp}}{UT_0} \times 100$$

Where γ (%): immobilization yield; UT_{exp}: is the total activity of the immobilized enzyme; UT_0: total activity of the enzyme solution offered for immobilization.

3.2.7 Stability of free and immobilized lipase NS-40116

The pH stability for the free and immobilized lipase was performed in a buffer solution at different pH (3, 5, 7, 9 and 11). The thermal stability of free and immobilized lipase NS-40116 was studied at 25, 40, 60 and 80 °C. The stability in solvent presence was performed at room temperature using methanol, ethanol and propanol. For these assays, 0.1 g of free and immobilized enzyme were placed in test tubes and left in contact with the solvents. To determine the metal ions stability of the enzyme, 0.1 g of free and immobilized enzyme were placed in contact with 1 Na^{+1}, $Ca^{+2,}$ and Mg^{+2} ions (50 mM and 100 mM). All the analyses were carried out for the period of 2, 4, 6, 24, 48, 168 and 360 hours in triplicate and 0.1g of lipase was put in contact with 1 mL of reaction

medium of interest. Concentrated lipase was subjected to the same conditions as those mentioned for 24 hours.

3.2.8 Enzymatic synthesis of FAME using the enzymatic derivative

FAME synthesis using the enzymatic derivate as catalyst was carried out in a horizontal incubator shaker (Texano, TE 424) in which a lidded 100 mL erlenmeyer flask was placed. The reactions mixture consisted of 30 g of abdominal chicken fat, 5 wt% of immobilized lipase, methanol - 3 equivalents (eqv) and 2 wt% of water. The methanol was added stepwise (1/3 eqv each time) at 0, 1 and 2 h to prevent enzyme inhibition. The reactions were performed at 30 °C for 24 hours and constant agitation of 250 rpm, based on previous studies conducted by our research group (Silva et al., 2016). To evaluate the reuse of the immobilized lipase, at the end of the reaction the enzymatic derivative was washed with hexane and submitted to a new esterification reaction. All the experiments were performed in duplicate.

The conversion into products was determined by gas chromatography analyses using a GC-2010 (Shimadzu) with flame ionization detector, using a MTX Biodiesel TG (15 m × 0.53 × 0.32 mm) as column. The operation conditions used were according Standard Method N° 14103 (European Committee for Standardization). The programming of column temperature was as follows: 150 °C for 1 min, followed by increases of 10 °C/min to 250 °C, kept for 1 min. Detector temperature was 250 °C, and nitrogen gas was used as carrier (pressure 80 kPa). All the analyses were performed in triplicate.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzymatic glycerolysis

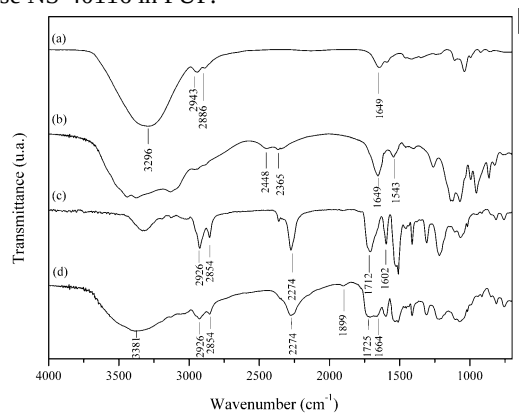
The conversion of glycerolysis reaction was calculated according to gas chromatography analysis and presented a 43.34 ± 2.32% (w/w) of MAG, 21.18 ± 2.59 (w/w) of DAG, and the unreacted TAG was 35.63 ± 4.73% (w/w). The unreacted TAG (castor oil) should not negatively affect the polymerization, since the major fatty acid of the castor oil (ricinoleic acid) has a hydroxyl groups in its chemical chain and this hydroxyl can be reacted with the NCO group, to form the urethane linkage (BRESOLIN

et al., 2017). About the hydroxyl content, the biopolyol presented value of 722 ± 5.3 mg KOH/g, which confirm the high conversion in MAG and DAG and the biopolyol pH was of 6.4, indicating a favorable condition for the lipase.

3.3.2 Free and immobilized NS-40116 lipase characterization

With regard to the assessment of the FTIR analysis (Figure 3.1) of the free liquid enzymatic extract, concentrated lipase NS-40116, polyurethane foam and enzyme immobilized on PUF.

Figure 3.1 - Fourier Transform Infrared Spectra (FTIR) of (a) free lipase NS-40116, (b) concentrated lipase NS-40116, (c) polyurethane foam and (d) immobilized lipase NS-40116 in PUF.



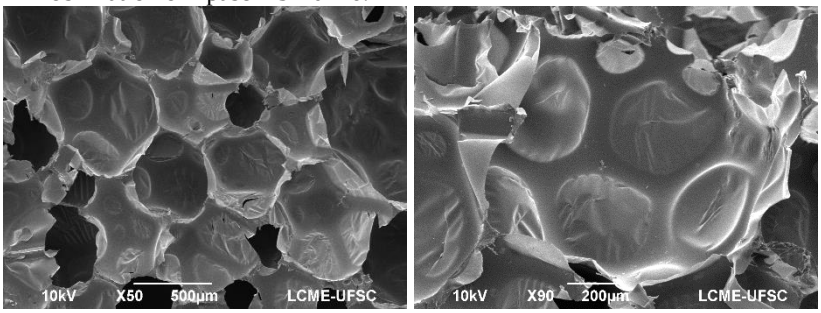
Source: The Author.

It was possible to observe the presence of a peak in 3296 cm^{-1}, probably referred to the water and sorbitol presence. In the concentrated lipase, it was observed the presence of peaks in the region of 3441 cm^{-1} and 3377 cm^{-1}, due to the O-H stretching and primary amide NH_2 asymmetric stretching, respectively. The N-H bonds showed stretching vibrations at 3132 cm^{-1}, characteristic to the amide A region. Both spectra showed a peak located at 1649 cm^{-1}, characteristic to the α-helix secondary assignment, common motif in the secondary structure of proteins. Bands centered at 1543 cm^{-1} are assignable to the amide II band

(STUART, 2006; FORESTI et al., 2010; COLLINS; LASSALLE; FERREIRA, 2011). In the PU foam it was possible to observe the presence of the asymmetric and symmetric vibration of methyl linkage at 2926 cm^{-1} and 2854 cm^{-1}, respectively. Stretching OH bond (3381 cm^{-1}) in the immobilized lipase NS-40116 indicates a possible enzyme-PU linkage region and the interaction between enzyme. The pikes present in 2274 cm^{-1} is relative the N=C=O bounds. The region at 1750-1700 cm^{-1} can be attributed to the carbonyl bonds from urethane (STUART, 2006).

The apparent density of the rigid PUF and immobilized lipase were 0.17 ± 0.04 g/cm³ and 0.19 ± 0.03 g/cm³, respectively. It is important to emphasize the water influence, used as blowing agent, to obtain of PU foams since the apparent density and the porosity of the foam are controlled by the amount of water present in the biopolyol (THIRUMAL et al., 2013; NICOLETTI et al., 2015; BRESOLIN et al., 2017). SEM analysis (Figure 3.2) showed the morphology of the enzymatic derivative, where cells with uniform size (749 ± 1 μm) were observed and the support can be considered a microcellular cell (<100 μm).

Figure 3.2 - SEM images of polyurethane foam used as support for immobilization of lipase NS-40116.



Source: The Author.

In addition to the FTIR and SEM analyses, the determination of the hydrolytic activity (Table 3.1) of lipase NS-40116 in its free, concentrated and immobilized forms presented values of 13.33 ± 1.01 U/g, 284.27 ± 18.02 U/g and 54.01 ± 5.62 U/g, respectively. The calculated immobilization yield was of 94 ± 4 %, showing the high efficiency of the applied process. The kinetic constants were determined using ρ-NPP as substrate in a range of substrate concentrations (0.5, 1.0,

1.5, 2.0, 2.5 and 3.0 mM) by Lineweaver–Burk plots. Results presented in Table 1 show the values for K_m and V_{max}. A variation of the values after immobilization is observed. Several reasons can explain this event, however, probably this occurs due to the formation of a tridimensional network, since the high crosslink of the polymer can difficult the interaction between immobilized enzyme with the substrate, due to mobility reduction (ANSARI; HUSAIN, 2012).

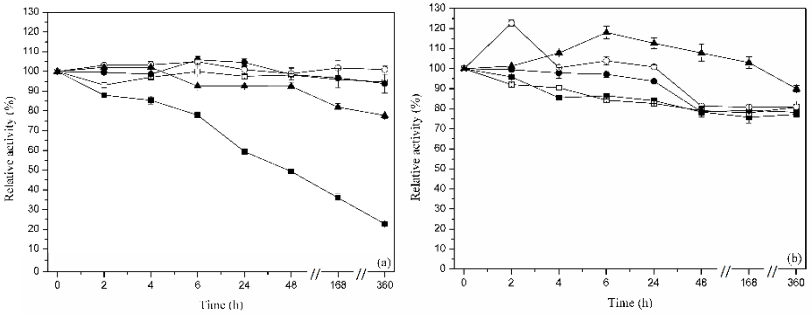
Table 3.1 - Enzymatic activity and kinetic parameters of free and immobilized lipase NS-40116.

	Enzymatic activity (U/g)	K_m (mM)	V_{max} (mmol/min/mL)	V_{max}/K_m
Free enzyme	13.33 ± 1.01	0.29	2.88	9.60
Concentrated enzyme	284.27 ± 18.02	1.29	14.06	4.49
Immobilized enzyme	54.01 ± 5.62	0.31	1.40	10.94

3.3.3 Stability of free and immobilized NS-40116 lipase

The lipase NS-40116 had a better behaviour in alkaline pH, as can be observed in Figure 3.3a. In acids pH, the free lipase had reduction of 80% inrelative activity after 360 hours.

Figure 3.3 - Effect of pH medium on the hydrolytic activities of the (a) free and (b) immobilized NS-40116 lipase stability submitted to different pH at room temperature (■ pH 3; □pH 5; ● pH 7; ○ pH 9; ▲ pH 11).



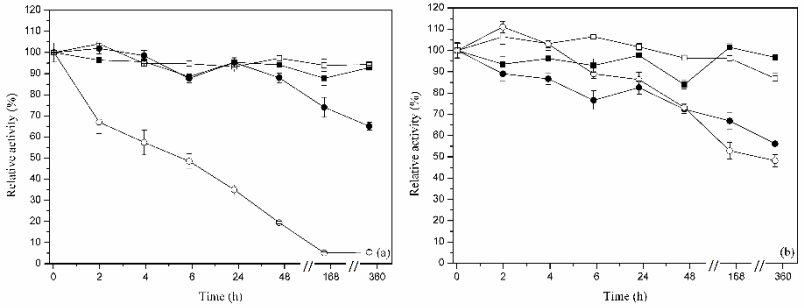
Source: The Author.

On the other hand, the same tendency was not observed for the enzyme immobilized in PUF (Figure 3.3b), which had a decrease of approximately 22%, probably due to the enzyme conformational changes undergoes when reacted with the support and the NH_2 could generate less inhibition. Also, it has also been possible to change the charge of a certain active or catalytic site of the enzyme, altering the conformation of the protein. In addition, the immobilized enzyme presents an increase of the relative activity in alkaline pH, being that in the first two hours at pH 9 the enzymatic activity increases 25% in relation to the initial and then presents a decrease, remaining with 100% activity up to 24 hours.

Higher enzymatic activity in pH 11 was also observed for immobilized lipase. After 6 hours, there was an increase of 20% in enzymatic activity in relation to the initial one, and after 360 hours the activity remained in 95%. In general, at extremely acidic pH, immobilized enzyme maintains higher activity when compared to the free enzyme. The free lipase presented the optimum pH at 7 and the immobilized presented the optimum pH at 9.

The free and immobilized enzyme was also evaluated for enzymatic activity at different temperatures (20, 40, 60 and 80 °C) (Figures 3.4a and 3.4b).

Figure 3.4 - (a) Free and (b) immobilized lipase NS-40116 stability submitted to different temperatures (■ 20°C, □ 40°C, ● 60°C and ○ 80 °C).



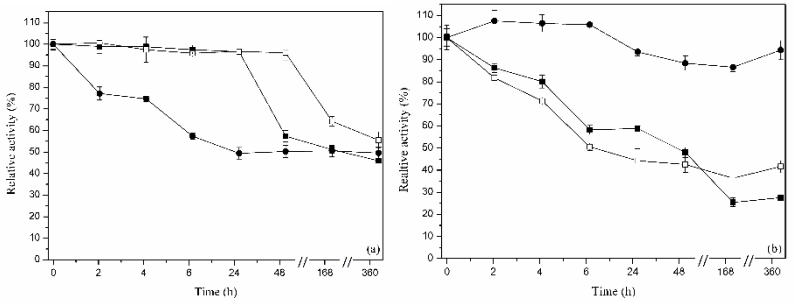
Source: The Author.

Similar results of the free and immobilized derivative were observed at 20 and 40 ºC, donot showing a significant lipase activity losses during 360 hours. When the lipase activity at 80 °C was evaluated, it was possible to note a decrease of the initial enzymatic activity, mainly

in the free enzyme, that presented the half-life of the activity remained 6 hours at 80 ºC and in 168 hours was denatured. For the immobilized derivative, a decrease of about 75% in the activity was observed. However, the enzyme half-life was between 24-48 hours, promoting a longer time of lipase used when high temperatures are required in reactional media. Evaluating the results presented by Nicoletti et al. (2015), the entrapment method for lipase immobilization presented better temperature stability when compared to the techniques evaluated by the authors (physical and ionic adsorption), keeping the enzyme less exposed to environmental interactions.

The evaluation of relative activity of free and immobilized lipase NS-40116 in the presence of solvents showed higher stability when compared to the immobilized derivative (Figures 3.5a and 3.5b).

Figure 3.5 - Stability of the free (b) and immobilized (b) lipase NS-40116 in the presence of ■ methanol, □ ethanol, and ● propanol.



Source: The Author.

When the evaluation was performed in methanol medium, free lipase presented a decrease of 50% in relative activity after 48 hours, while immobilized enzyme the activity decreased this percent after 6 hours. In contact with ethanol, free lipase presented greater resistance when compared with the immobilized, being the decrease of the activity was only verified in 168 hours for the free lipase, when immobilized lipase presented losses around 40% in the first 24 hours. The activity losses of the immobilized lipase against the ethanol can occur due to reversible changes in protein structure or irreversible inactivation due the immobilization process (ABDULLA; RAVINDRA, 2013; SECUNDO, 2013). The presence of propanol in the medium was also evaluated, and

it was possible to observe a decrease of 25% in the relative enzymatic activity after 2 hours. Free lipase showed activity reaching 50% in 24 hours, while for the immobilized enzyme after 360 hours, the enzyme still had around 95% of the activity compared to the initial values.

Due to this difference in the behavior on solvents presence, concentrated lipase NS-40116 was subjected to evaluation enzymatic activity after 24 hours of contact. The results are shown in Table 3.2. It was possible to observe similar behaviors of the concentrated lipase and enzymatic derivative submitted in the same conditions, indicating that the loss of activity to the presence of the solvents does not occur due to immobilization process and some stability is conferred to the enzyme when it is in concentrated form.

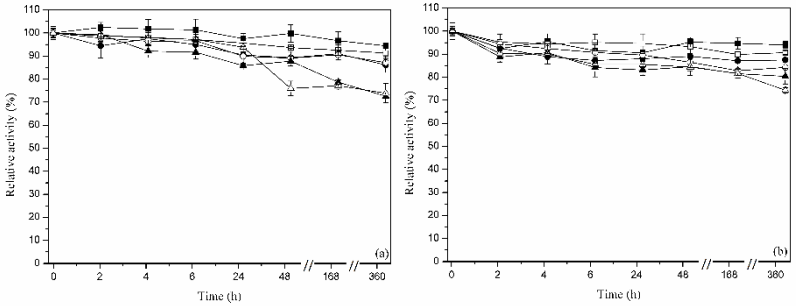
Table 3.2 - Effect of different reaction medium on the concentrated lipase NS-40116 activity.

Reaction medium contact[a]	**Lipase NS-40116 relative activity (%)[b,c]**
Buffer solution pH 3	88.64 ± 2.01
Buffer solution pH 5	86.45 ± 3.47
Buffer solution pH 7	88.04 ± 1.67
Buffer solution pH 9	101.74 ± 1.52
Buffer solution pH 11	99.47 ± 1.41
20 °C	96.85 ± 1.48
40 °C	98.96 ± 1.61
60 °C	79.51 ±1.34
80 °C	66.03 ± 2.41
Methanol	71.41 ± 2.38
Ethanol	67.39 ± 2.54
Propanol	94.57 ± 1.57
Ions Mg^{++} 50 mM	97.21 ± 3.03
Ions Mg^{++} 100 mM	96.04 ± 1.43
Ions Ca^{++} 50 mM	101.86 ± 2.49
Ions Ca^{++} 100 mM	97.31 ± 2.44
Ions Na^{+} 50 mM	98.45 ± 4.06
Ions Na^{+} 100 mM	100.93 ± 1.62

[a] all the reactions were carried out at room temperature, except for the temperature evaluation.
[b] Hydrolytic activity measurements were performed in triplicate analyses (n=3).
[c] Correlation according to the values obtained in the time 0 and after 24 hours of contact.

The study of the effect of the ions presence on the enzymatic activity is shown in Figures 3.6a and 3.6b. The Mg^{+2} ions at 50 mM stimulate the activity of the lipase slightly in the first 6 hours of contact. The Na^{+1} ion at 100 mM showed a decrease of 25% of the relative activity, while at 50 mM the effect of a decrease in the activity was observed after 168 hours of contact, leading to believe this ion at high concentrations may cause the inhibition of enzymatic activity. The Ca^{+2} is associated with the increase of the lipases activity (GAUR; GUPTA; KHARE, 2008; PATEL; NAMBIAR; MADAMWAR, 2014), but in this case not presented a significant change in the enzymatic activity in the free and immobilized form.

Figure 3.6 - Effect of ions presence in enzymatic activity for (a) free and (b) immobilized lipase NS-40116 (Mg^{++} ■ 50 mM and □ 100 mM; Ca^{++} ● 50 mM and ○ 100 mM; Na^{+} ▲ 50 mM and Δ 100 mM).



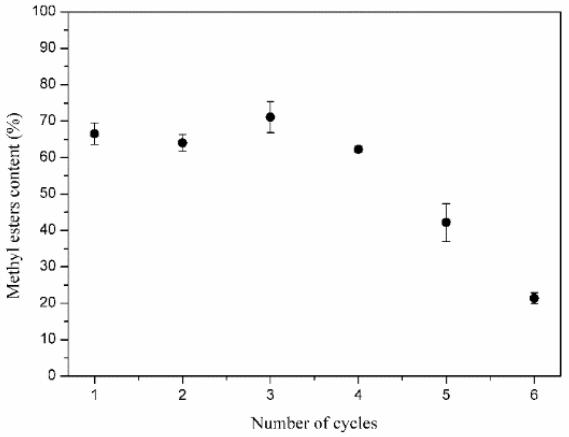
Source: The Author.

3.3.4 Enzymatic synthesis of FAME

In order to explore the catalytic potential of the lipase NS-40116 immobilized derivative, transesterification reactions were conducted based in method previously described by Silva et al. (2016). Abdominal chicken fat and methanol were used as substrates. The results expressed in Fig. 7 show a FAME conversion of 66 ± 3% in the first cyclbe using of lipase derivative, remaining constant until the fourth cycle (considering the statistical error). The conversion on the fifth experiment cycle had a low conversion (42 ± 5%) and in the sixth was 21± 1% since usually it is considered that an enzyme can be reused until 50% of initial activity (NYARI et al., 2016). With this, it was determined that the possibility of

reuse is for up to four reactional cycles. FAME synthesis reaction using chicken fat was reported by Matta et al. (2011) using basic catalytic (NaOH). The methanol/fatty molar ratio of 6:1 was used, with about 0.8% (w/w) KOH catalyst to the fatty. The authors achieved a conversion rate of around 76%. Gameiro and coauthors (2015) reached conversions around 97% using chicken fat as a raw material in the production of biodiesel using a supercritical continuous reactor, at pressures of 250 bar, using Lipozyme RM IM (*R. miehei* lipase immobilized on an ion exchange resin) as catalyst. Silva et al. (2016) use the free lipase NS-40116 to FAME synthesis using abdominal chicken fat as feedstock and performed the kinetic of reaction. After 20 hours of reaction, the authors obtained conversions around 92% using 0.7% (w/w) of lipase, 2% (w/w) of water and 1.2 eqv. of methanol.

Figure 3.7 - Reuse of immobilized lipase NS-40116 in polyurethane foam via entrapment in the synthesis of acid methyl esters using abdominal chicken fat as substrate.



Source: The Author.

Although methanol was used in FAME synthesis, since the method was based on the literature (SILVA et al., 2016), and immobilized lipase presented significant losses in its activity in the presence of this solvent, it not prevents the first four syntheses from occurring with high conversions, probably due to the three-part addition. Evaluating the works in the literature, the results obtained in our research showed not only the

catalytic capacity of the enzymatic derivative, but also a high possibility of its reuse since most of the investigations using these substrates applied basic/acid catalysts and high temperatures in the reactional medium (MATA et al., 2011; RAMALHO et al., 2011; HERNÁNDEZ-CRUZ et al., 2017).

3.4 CONCLUSIONS

In this study, it was obtained a biopolyol enzymatically catalyzed and after, this biopolyol was submitted to polymerization and the polyurethane foam obtained was used as support for immobilization of NS-40116 lipase. The yield of immobilization was around 94%, showing that the support was efficient for this purpose. The free enzyme and the enzymatic derivative immobilized on polyurethane foam were subjected to stability tests in different media. It was possible to observe that the support allowed stability to lipase NS-40116 at higher temperatures and acid and basic pH values. The ions presence did not affect the free and immobilized lipase. However, in the presence of solvents, free lipase was more tolerant to methanol and ethanol, whereas the immobilized enzyme showed to be more tolerant to propanol. Therefore, the results provide relevant information to the means the reactional medium lipase NS-40116 can be applied. Also, the enzymatic derivative was applied as catalyst for esterification reaction using abdominal chicken fat as substrate, being able to present conversion rates around 65% in the first four cycles of use, whit this, was possible to observe that the enzymatic derivative present high catalytic power and is susceptible of applications of commercial interest. approached this, we successfully proposed a new support for the immobilization obtained from renewable sources, promoting the possibility of using the immobilized enzyme in hardness conditions.

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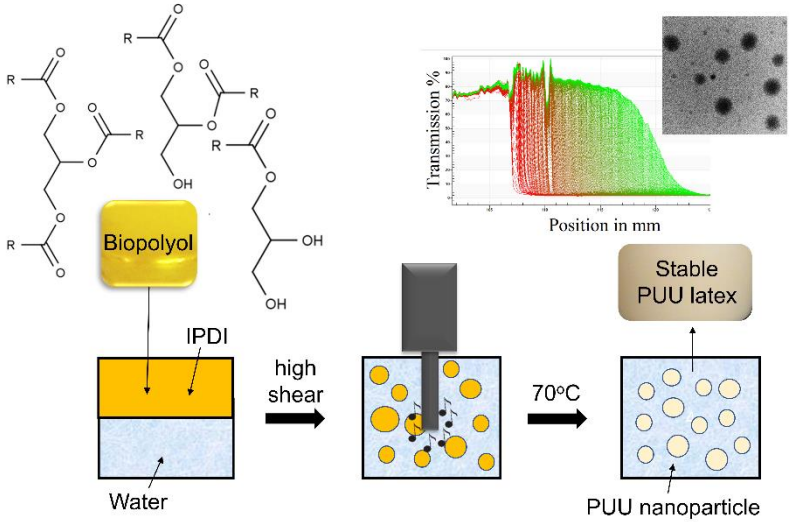
CHAPTER IV

Poly(urea-urethane) nanoparticles using mono- and diacylglycerol from glycerolysis of castor oil as biopolyol and stabilizer



- Biopolyol used in Chapter III;
- Mono- and diacylglycerols used as biopolyol and stabilizer;
- PUU latex by miniemulsion polymerization without additional surfactant;
- Stable PUU latex with solids content up to 40 wt%.

Abstract

Vegetable oils as renewable raw materials for polymer synthesis are considered a clean and environmentally friendly alternative to substitute partially, and to some extent totally, petroleum-based monomers. In this work, enzymatic glycerolysis of castor oil with Novozym 435 (C. antarctica lipase B) as catalyst was performed in a solvent-free system at 70 °C. The glycerolysis product was used as biopolyol and stabilizer for the synthesis of poly(urea-urethane) (PUU) nanoparticles via miniemulsion polymerization. Results showed that the PUU particles size increased with solids content with average particle diameters ranging from 167 to 282 nm when the solids content was increased from 20 to 40 wt%. All lattices were stable towards sedimentation, although the latex with lower particle size exhibited higher stability. Thereby, this work showed a green route to produce stable PUU lattices from renewable resources without the use of any additional surfactant.

Keywords: Enzymatic glycerolysis; castor oil; poly(urea-urethane) nanoparticles; biopolyol; miniemulsion polymerization.

4.1 INTRODUCTION

Vegetable oils as renewable raw materials for polymer synthesis are considered a clean, low-cost and environmentally friendly alternative to substitute partially, and to some extent totally, petroleum-based monomers (XIA; LAROCK, 2010; GANDINI; LACERDA, 2015; ZHANG et al., 2017). The vegetable oils are mostly composed of triacylglycerols of different fatty acid chains length, number of unsaturation, and presence or absence of a hydroxyl group. Their chemical structure allows the modification by transesterification, epoxidation, and hydrolysis reactions (LLIGADAS et al., 2013; MIAO et al., 2014).

Glycerol can be obtained as a by-product of biodiesel production and can be employed to synthetize monoacylglycerols (MAG) and diacylglycerols (DAG) (VERSTRINGE et al., 2014; REMONATTO et al., 2015) by a transesterification reaction with a vegetable oil using chemical or enzymatic catalysts. The chemical catalyst requires high reaction temperatures (~ 225 °C) whereas the enzymatic reactions proceed at much lower temperatures, around 70 °C, being considered an environmentally friendly alternative (FELTES et al., 2013).

The monoacylglycerol, MAG, has in its chemical structure two hydroxyl groups and a fatty acid chain, while the diacylglycerol, DAG, has in its chemical structure one hydroxyl group and two fatty acid chains. Due to their amphiphilic characteristic, MAGs and DAGs are widely used as stabilizers in different industrial applications. More recently, it was shown that the product of the glycerolysis of castor oil could be used as biopolyol to obtain polyurethane foams (BRESOLIN et al., 2017).

The polyurethane is obtained by a polyaddition reaction between diisocyanate and polyol molecules forming urethane linkages between the monomeric units. It is considered a versatile polymer, as its properties could be tuned by changing the diisocyanate and the polyol and find different applications as foams, resins and coatings (ENGELS et al., 2013; MEHRAVAR et al., 2017; ZHAO et al., 2018). In the presence of water, it is possible to observe the formation of urea linkages, due to the hydrolysis of isocyanate (NCO) group, resulting in carbamic acid, which forms an amine that reacts with another isocyanate and form an urea linkage, resulting in a poly(urea-urethane) (PUU) polymer chain (GAUDIN; SINTES-ZYDOWICZ, 2011; CALLE et al., 2016).

PUU nanoparticles can be obtained by miniemulsion polymerization (TIARKS; LANDFESTER; ANTONIETTI, 2001; RIX et al., 2015; VALÉRIO et al., 2015). This technique is performed by the dispersion of monomer droplets, that act as the main polymerization locus, in an aqueous medium and results in polymer particles with diameters around 50-500 nm. For this, it is necessary a high shear mechanical force such as an ultrasonic disperser or high-pressure homogenizer (LANDFESTER; WEISS, 2010). A co-stabilizer, usually a very hydrophobic compound, should be added to prevent monomer diffusional degradation (Ostwald Ripening). Examples of co-stabilizers are hexadecane or a triacylglycerol, as Crodamol (ASUA, 2002; LANDFESTER, 2003). The surfactant, ionic or non-ionic, prevents the coalescence of monomer droplets and polymer particles during the polymerization reaction and increases the shelf-life of the polymer latex (ASUA, 2002; SCHORK et al., 2005). Studies about PUU nanoparticles have emerged in the last years, due to a wide variety of potential applications in physical, chemical, biological and health sciences and other interdisciplinary fields of science and engineering (VALÉRIO et al., 2014; BOUR et al., 2015; MORRAL-RUÍZ et al., 2015; CHIARADIA et al., 2016).

In this work, the use of the product of castor oil enzymatic glycerolysis as biopolyol and stabilizer to obtain stable PUU nanoparticles by miniemulsion polymerization was investigated. To the best of our knowledge this is the first work that reports the use of MAG and DAG as biopolyol and stabilizer showing a green route to produce stable PUU lattices from renewable resources without the use of any additional surfactant.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Commercial immobilized lipase (Novozym 435, from *Candida antarctica*, immobilized on a microporous anionic resin) was kindly donated by Novozymes and used as catalyst for glycerolysis reaction. Castor oil (Campestre), glycerol P.A. (Vetec, 98%), and Tween 80 surfactant (Vetec, 65 - 80 hydroxyl number) were also used in the glycerolysis reactions. N-Methyl-N-(trimethylsilyl) trifluoroacetamide

(MSTFA) from Merck Millipore, n-heptanol (Sigma-Aldrich) and pyridine (Sigma-Aldrich) was used in the gas chromatograph. Deuterated chloroform anhydrous ($CDCl_3$) from Sigma-Aldrich was used as solvent to the ^{1}H-NMR analysis. For the calculation of the estimated HLB index, span 85 (kindly donated by Croda) and tween 80 (Synth) were used. Isophorone diisocyanate (IPDI, 99%, Mw 222 g/mol) from Sigma-Aldrich was used as diisocyanate monomer. Tetrahydrofuran (THF), from Neon (99.5%), was used for insoluble fraction determination. All chemicals were used as received.

4.2.2 Biopolyol preparation and characterization

Biopolyol was obtained by enzymatic glycerolysis, according to Valério et al. (2010), as described in the previous chapter (Chapter III). The biopolyol was characterized by gas chromatograph to quantify MAG and DAG contents obtained from enzymatic glycerolysis. The conversion was determined according to the ASTM D6584-13 (2014), using a gas chromatograph (GC, Shimadzu 2010) with an automatic on-column injector and flame ionization detector (FID). The programming of column temperature was as follows: 50 °C for 1 min, followed by increases of 15 °C/min to 180 °C, 7 °C/min to 230 °C, and 10 °C/min to 380 °C, kept for 8 min. Detector temperature was 380 °C, and nitrogen gas as the carrier (pressure 80 kPa). The product of glycerolysis reaction was also submitted to nuclear magnetic resonance (^{1}H-NMR) in $CDCL_3$ on Bruker AVANCE DPX spectrometer operating at 200MHz. Chemical shifts (δ) are reported in parts per million related to the internal standard tetramethylsilane (δ=0.00ppm). The hydroxyl value was calculated according to ASTM D 1957-86 method (2001), the acid index was evaluated using titration method, according to NBR 11115 ((2014) and pH was measured on pHmeter (AN2000 Analion). HLB was measured according to Griffin (1949) direct methodology using Span 65 (HLB 1.6) and Tween 80 (HLB 15) as surfactant standards. The product of glycerolysis did not undergo any purification step. All analyses were performed in triplicate (n=3).

4.2.3 Poly(urea-urethane) nanoparticles synthesis and characterization

PUU nanoparticles were prepared by miniemulsion polymerization. The solids content was investigated in a range of 20, 30 and 40% and the molar ratio NCO:OH was fixed in 1.5:1 in all reactions. The biopolyol and diisocyanate were left to react for 15 minutes at 40 °C under stirring at 300 rpm to promote the urethane groups formation. The water phase was added, and the dispersion was performed using an ultrasonic probe (Fisher - Scientific - Ultrasonic Dismembrator 500, 400 W), at 70% of amplitude for 3 minutes (15 s pulse on and 5 s pulse off) in an ice bath to temperature control. After that, the glass jacketed reactor was connected to a thermostatic bath at 70 °C under magnetic stirring (350 rpm) for 3 hours.

Fourier transform infrared (FTIR) spectrum of poly(urethane-urea) were obtained with Shimadzu IR Prestige 21, with samples prepared in KBr pellet technique. The samples were analysed by transmittance mode in the region between 4000-550 cm^{-1} with a resolution of 2 cm^{-1} and 32 scans. Dynamic light scattering (DLS, Zetasizer Nano S, from Malvern) was used to measure the intensity average diameter of PUU nanoparticles. Samples were prepared by lattices dilution (1:10) at 25 °C and were evaluated in triplicate (n=3). Zeta potential data was recorded on a Stabino Particle Charge Mapping at room temperature. Sample solutions used to study the pH dependence of the potential zeta were prepared adjusting to the desired pH with 0.1 M NaOH and 0.1 M HCl solutions. The insoluble fraction of PUU latex was evaluated according to the solvent-extraction methodology described in ASTM D 2765-01 (2001) where 250 mg of dry polymer was soaked in tetrahydrofuran (Neon, 99.5%) and refluxed with boiling THF at atmospheric pressure for 24 h in a soxhlet apparatus. After that, the remaining insoluble fraction was dried in a vacuum oven to remove the residual solvent. The insoluble fraction of the PUU nanoparticles was calculated by weight of the dried insoluble part to the weight of the initial sample. The morphology of PUU nanoparticles was evaluated by Transmission Electron Microscopy with field emission (TEM 100kV – JEM 1011).

The shelf life prediction was determined by the settling velocity using analytical centrifugal analyzer (LUMiSizer, L.U.M. GmbH, Berlin, Germany), working at 3025 rpm for 8.2 hours at 23°C, using a synthetic

cell (model 110-131xx, optical path of 2 mm), which measured the transmission variations of particles suspensions under centrifugation against time. LUMiSizer allows measurement of time and space-resolved transmission extinction profiles under analytical centrifugation via settling velocity analysis, while the sample was being centrifuged. The shelf life prediction was calculated based on previous report by Lerche and Sobisch (2011).

4.3 RESULTS AND DISCUSSION

4.3.1 Enzymatic glycerolysis

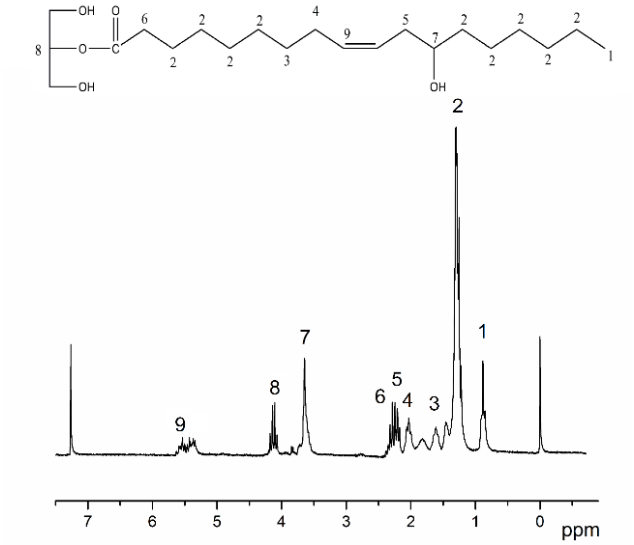
From enzymatic glycerolysis reaction using castor oil and glycerol as a substrate, the conversion calculated based on the initial amount of TAG (castor oil) was 43.34 ± 2.32% (w/w) in MAG and 21.18 ± 2.59 (w/w) in DAG and unreacted TAG was 35.63 ± 4.73% (w/w) The content of MAG and DAG obtained in the present work in solvent-free system is similar to those reported in the literature for different reaction systems. For example, Naik et al. (2014) obtained high conversation, about 70%, for the glycerolysis of sunflower oil in *t*-butanol using 15% of lipase CalB. Remonatto et al. (2015) in a solvent-free system assisted by ultrasound and 10 wt% of lipase CalB obtained 65 wt% of MAG and DAG when soybean oil was used as substrate and 75 wt% of MAG and DAG when canola oil was used.

The high conversion in MAG and DAG was confirmed by NMR analyses (Figure 4.1). The ratio between fatty acid and hydroxyl groups was evaluated using the areas of the methylene proton attached to hydroxyl (–CH–OH) (∂ = 3.6 ppm) and the methyl protons were at ∂ = 0.9 ppm. The estimated ratio was 2.9 moles of hydroxyl per 1 mole of fatty acid allowing high amounts of hydroxyl group to react with the NCO group from the diisocyanate, to carry out the polymerization reaction of polyurethane.

The glycerolysis product had a calculated HLB value of 3.2 considering that a generic monoacylglycerol HLB value is approximately 3.5 (ZHA et al., 2014) and a generic diacylglycerol HLB value is 2.8 (SHIMADA; OHASHI, 2003), being the biopolyol a mixture between MAG, DAG and TAG, this value is within this range. The acid number of biopolyol was found to be relatively low (1.7 ± 0.3 mg KOH/g) and the

biopolyol pH was 6.4, indicating no hydrolytic degradation of the fatty acid esters during glycerolysis. The hydroxyl content of 722.1 ± 5.3 mg KOH/g corroborated the GC and ^{1}H-NMR results showing high conversion in MAG and DAG.

Figure 4.1 - ^{1}H-NMR spectra of the glycerolysis reaction product**.**



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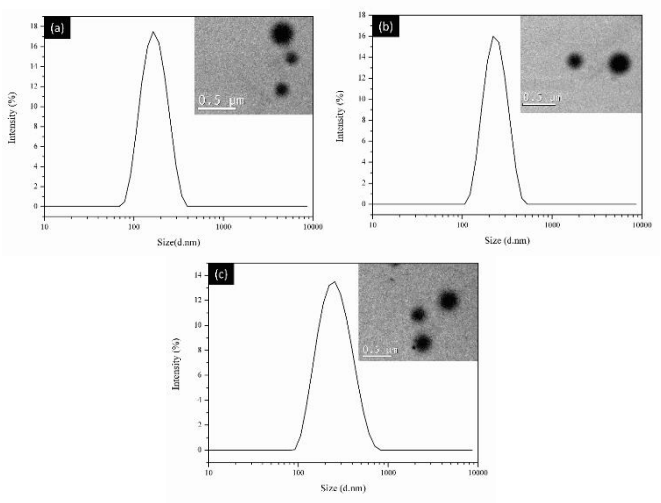
4.3.2 Poly(urea-urethane) nanoparticles

The nanoparticles were analyzed by DLS to determine the intensity mean particle diameter in order to evaluate the influence of biopolyol and the solids content in the formation of the PUU nanoparticles. Figure 4.2 shows particle size distribution obtained by DLS and TEM images of PUU nanoparticles. All the nanoparticles were spherical, and it was not observed the presence of aggregates by TEM or DLS measurements, indicating a good colloidal stability.

Figure 4.2 - Dynamic light scattering (DLS) data and transmission electronic microscopy images of the nanoparticles obtained using (a) 20%, (b) 30% and (c) 40% of solids content.



Source: The Author.

According to the results observed in Table 4.1, when the polymerization reaction was performed with 20% of solids content, PUU nanoparticles with an average size of 167.3 ± 3.6 nm were obtained. Increasing the solids content to 30% and 40%, the average size increased to 215.1 ± 2.1 nm and 281.7 ± 2.4 nm, respectively. It is important to emphasize that no additional surfactant was added to the reaction medium when increasing the solids content. The average size increased because higher solids content led to a higher shock frequency, however, after the sonication step, the nanoparticles remained stable, being the final nanoparticles average size (D_p) equal to the initial droplet average size (D_d) for all formulation (LANDFESTER, 2003). Similar average particles diameter, around 215 nm, were found when PUU nanoparticles were synthetized by miniemulsion polymerization of IPDI, castor oil and poly(ethylene glycol) with 15 wt% of solids content and 20 wt% (related to the monomers) of tween 80 as surfactant (VALÉRIO et al., 2014). Stable PUU lattices with diameters around 200–300 nm were obtained for high solids content dispersions, up to 50% (RIX et al., 2015) for the

miniemulsion reactions containing IPDI and butanediol and propanediol esters were stabilized using the anionic surfactant sodium dodecyl sulfate.

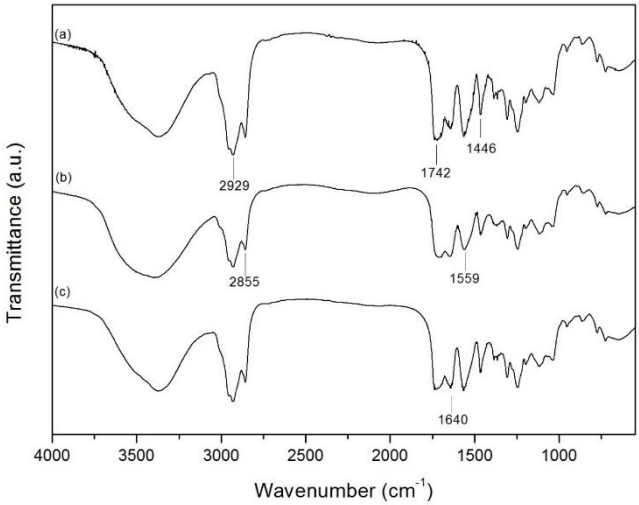
Table 4.1 - Characterization of the PUU nanoparticles obtained using the biopolyol from enzymatic glycerolysis.

Solids content (%)	**D_d (nm)***	**D_p (nm)**	**Urethane/ urea ratio**	**Insoluble fraction (%)**
20	162.3 ± 2.7	167.3 ± 3.6	1.22	63
30	213.4 ± 3.3	215.1 ± 2.1	1.36	61
40	279.6 ± 3.6	281.7± 2.4	1.32	66

*D_d – average droplet diameter

FTIR spectra of PUU nanoparticles obtained with different solids content are presented in Figure 4.3. Bands with peak location at 2929 cm^{-1} and 2855 cm^{-1} showed the presence of symmetric and asymmetric C–H_3 vibration stretching, respectively (GU et al., 2015). The peak located at 1742 cm^{-1} is characteristic of N–H urethane group and in 1640 cm^{-1} is referent to urea group (WANG et al., 2013). The absorption band at 1559 cm^{-1} occurs due to C–N stretching and at 1446 cm^{-1} characteristic to methylene groups was also observed, confirming polymer synthesis. The urethane/urea ratio was determined using the respective peak areas – urethane at 1750 – 1740 cm^{-1} and urea at 1600 –1640 cm^{-1} – and the results were listed in Table 1. Similar urethane/ur ea ratios were found in all experiments with different solids content. It is important to point out that the biopolyol and the diisocyanate were allowed to react for 15 min at 40 °C without the presence of water to promote the formation of oligomers with just urethane linkages. After the addition of water during the miniemulsification step there was the formation of not only urethane but also urea linkages in the polymer chain due to side reactions of isocyanate groups with water (GAUDIN; SINTES-ZYDOWICZ, 2011).

Figure 4.3 - FTIR characterization of PUU nanoparticles obtained using biopolyol from enzymatic glycerolysis via miniemulsion polymerization with (a) 20% of solids content, (b) 30% of solids content and (c) 40% of solids content.



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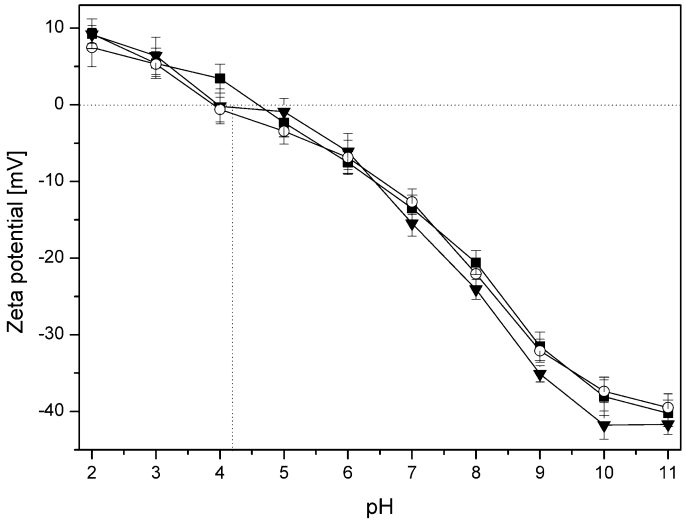
As calculated previously, the biopolyol presented an average value of 2.9 hydroxyls per molecule. If all the hydroxyls had reacted with the diisocyanate, the polymer would be fully crosslinked. However, due to steric hindrance and decreasing mobility of growing polymer chains, not all hydroxyls were reacted. Besides that, part of the diisocyanate was consumed by the reaction with water to form urea linkages. The insoluble fraction of the PUU nanoparticles in THF were similar for all samples (Table 4.1) with values around 0.63. It means that approximately 63 wt% of the polymer chains were crosslinked. It is important to emphasize that the NCO:OH molar ratio was the same in all reactions, consequently, the insoluble fraction remained constant.

The effect of pH on the zeta potential of PUU nanoparticles was investigated in a pH range of 2–11. The NPs presented similar behaviour. In Figure 4.4 it is possible to observe that zeta potential was around 10 for pH 2 and around -40 for pH 11. The isoelectric point of the PUU nanoparticles was around pH 4.2, similar to the pKa of the carboxylic acid. After polymerization, the pH of the latex was around 6 and at this

pH the zeta potential was approximately -7, increasing the colloidal stability of the latex when compared to the isoelectric point.

Figure 4.4 - Influence of the pH on zeta potential of PUU nanoparticles with 20% of solids content (■), 30% de of solids content (▼) and 40% of solids content (o).



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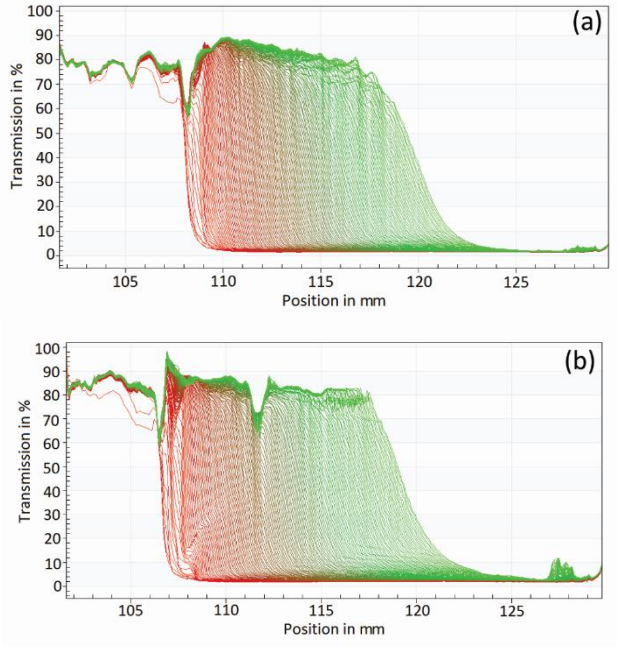
The stability of the nanoparticles was provided mainly by steric stabilization since MAG and DAG are non-ionic and the zeta potential at pH 6, as mentioned above, was relatively low. To compare the settling velocity of the latex produced with different solids content, all lattices were diluted to 20 wt% in solids and the shelf life was predicted using an analytical centrifugal analyzer. The results exhibited in Table 4.2 and Figure 4.5 showed that the latex with PUU particles with lower diameter obtained with 20 wt% of solids content were more stable presenting lower sedimentation velocity of 0.2374 µm/s. The shelf life, based on sedimentation, was 0.69 mm per month, while the latex with larger particle size obtained with 40 wt% of solids content presented a sedimentation velocity of 0.4429 µm/s and the shelf life was of 1.29 mm per month. These results are in accordance to literature, where smaller particles are more stable towards settling. Nevertheless, it is important to

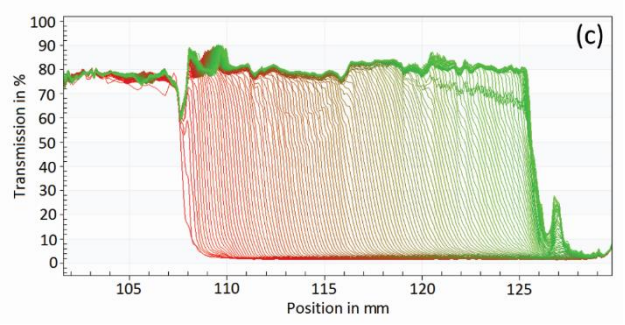
point out that those sedimentation velocities are very low indicating that all PUU lattices are colloidally stable (CHIU et al., 2012).

Table 4.2. Storage stability (shelf life), at a relative centrifugal force of 900 (1 x *g*), of PUU nanoparticles obtained at different solids content.

Solids content (%)	Sedimentation velocity V (μm/s)	Standard deviation (μm/s)	Shelf life (mm/month)
20	0.2374	0.1317	0.69
30	0.3517	0.1208	1.03
40	0.4429	0.0806	1.29

Figure 4.5 – Evolution of transmission profiles of the poly(urea-urethane) nanoparticles lattices produced with the biopolyol obtained via enzymatic glycerolysis with (a) 20% of solids content, (b) 30% de of solids content and (c) 40% of solids content.





Source: The Author.

4.4 CONCLUSIONS

In summary, stable PUU nanoparticles were successfully synthetized using a biopolyol obtained from the glycerolysis of castor oil catalysed by Novozym 435. The glycerolysis converted most part of castor oil in mono- and diacylglycerol (64.52±2.45% (w/w)) resulting in a biopolyol with 2.9 hydroxyls per molecule. The large amount of MAG and DAG provided steric stabilization for PUU nanoparticles obtained by miniemulsion polymerization and at the same time they acted as polyol for PUU synthesis. The initial solids content affected the dispersion of the organic phase and as result, the PUU particle size increased with solids content with average particle diameters ranging from 167 to 282 nm when the solids content increased from 20 to 40 wt%. All lattices were stable towards sedimentation, although the latex with lower size exhibited a higher stability. Thereby, this work showed a green route to produce stable PUU lattices from renewable resources without the use of any additional surfactant, which can be applied in different fields in the future.

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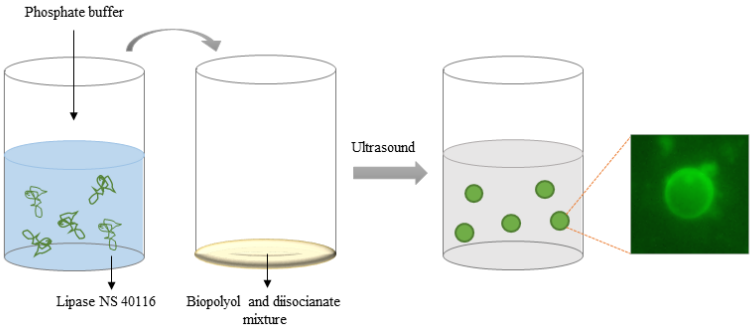
CHAPTER V

Immobilization of lipase NS-40116 in poly(urea-urethane) nanoparticles using a biopolyol from enzymatic glycerolysis



- Lipase NS-40116 was able to catalyze the glycerolysis reaction;
- Glycerolysis product was able to be used as biopolyol and surfactant;
- Stable nanoparticles and enzymatic derivatives were successfully obtained;
- Lipase NS-40116 was immobilized on the nanoparticles surface.

Abstract

In this work, the ability of the free lipase NS-40116 as a catalyst for enzymatic glycerolysis reaction in a solvent-free system was evaluated under conditions: the same conditions describe in Chapter III and IV. The glycerolysis product was submitted to ^{1}H NMR analysis, indicating the presence of 2.3 mol of hydroxyl in relation to 1 mol of fatty acid. The product of glycerolysis was used as stabilizer and biopolyol for the poly(urea-urethane) nanoparticles (NPs) synthesis via miniemusion technique, without the use of extra surfactant in the system and stable nanoparticles with diameter size of 19 nm were obtained. After, was study the fomation of NPs in the presence of concentrated lipase NS-40116, aming the lipase immobilization and stable enzymatic derivative with diameter size around 231 nm was obtained. The enzymatic activity was determined by titration using olive oil and arabic gum as substrate and the immobilization was confirmed by morphological analysis using transmission electron microscopy and fluorescence microscopy.

Keywords: Glycerolysis, poly(urea-urethane), nanoimmobilization, lipase NS-40116, biocatalysis.

5.1 INTRODUCTION

Lipases are triacylglycerol ester hydrolases and have gained greater prominence over the years, mainly in catalyzing synthesis reactions of industrial importance and they have been the subject of investigations in the academic area (DE MIRANDA; MIRANDA; DE SOUZA, 2015; SALIHU; ALAM, 2015). The wide range of reactions performed for these proteins includes epoxidation, esterification, transesterification, i.g, and consequently, could be applied in the production of biofuels, perfumes, cosmetics, foods and flavors developments (JAVED et al., 2018).

Even carring out a large number of reactions and all these of desirable characteristics, the lipases can present operational disadvantages when used in free form, as the shelf life, incompatible in high temperatures, extreme values of pH and solvent presence, and by their recovery and reusability (ADLERCREUTZ, 2013; CIPOLATTI et al., 2014a; SHUAI et al., 2017). Enzyme immobilization is one of the strategies to overcome these problems.

The enzymes immobilization is an important research field, promoting greater usability and applications of these proteins. Studies evaluating the immobilization of lipases using different techniques and supports have been carried out in the last years. As the used techniques, the adsorption, covalent binding, entrapment and cross-linking is the most common (AHMAD; SARDAR, 2015). However, when the supports are discussed is possible to observe a large number of options, as zeolites (MACARIO et al., 2007), polymers (FANG et al., 2011), clays minerals (AN et al., 2015), metal-organic framework (MEHTA et al., 2016) and a wide range of nanomaterials (CIPOLATTI et al., 2014a).

The nanomaterials for lipase immobilization have great advantages since these materials present a larger specific surface area, less diffusion limitation, high mechanical strength, lower mass transfer resistance, homogeneous coating and well-defined particles (AHMAD; SARDAR, 2015; SHUAI et al., 2017). Among them, poly(urea-urethane) nanoparticles have gained attention as a subject of several investigations (CIPOLATTI et al., 2014b, 2015, CHIARADIA et al., 2016a, 2016b).

Therefore, the aim of this work was to use the free lipase NS-40116, a liquid formulation of a modified *Thermomyces lanuginosus* (TLL), as a catalyst for the glycerolysis reaction of castor oil and then use

the product of this reaction (mono- and diacylglycerol) as a biopolyol, due to the hydroxyl linkage, for the synthesis of PUU nanoparticles. This lipase was chosen because it has a lower cost when compared to the Novozyme 435. After, the study of lipase NS-40116 immobilization on the nanoparticles surface using miniemulsion technique was performed.

5.2 MATERIALS AND METHODS

5.2.1 Biopolyol production and characterization

The biopolyols were synthesized by enzymatic glycerolysis between castor oil and commercial. The lipase NS-40116 was used as a catalyst, with the purpose of reducing the cost of biopolyol production. The biopolyol synthesis was performed in a jacketed glass reactor using castor oil and glycerol in a molar ratio of 1:6, as substrates, and 16 wt% Tween 80 as surfactant. 1 wt% of lipase NS-40116 and 1 wt% of distilled water was added to the reaction system. This amount of water was added to the reaction medium, since this lipase presents higher conversions in water presence, as reported by Facin and co-authors (2018).

The product of glycerolysis reaction was submitted to nuclear magnetic resonance (^{1}H NMR) in $CDCL_3$ on Bruker AVANCE DPX spectrometer operating at 400MHz. Chemical shifts (δ) are reported in parts per million related to the internal standard tetramethylsilane (δ=0.00ppm). The relation between fatty acid and hydroxyl groups was evaluated using the areas of the methylene proton attached to hydroxyl (– CH–OH) (∂ = 3.6 ppm) and the methyl protons were at ∂ = 0.9 ppm.

The sample had the physicochemical parameters evaluated, aiming the polymerization reactions. The hydroxyl value of the biopolyol was measured according to ASTM D 4274 (2016) using test method A and pH was measured on pHmeter (AN2000 Analion). The HLB value determination of the biopolyol was performed by the direct method, based on Griffin (1949), which visually compares the behavior of surfactants with known HLB patterns. The product of glycerolysis did not undergo any purification step. All analyses were performed in triplicate (n=3), except the HLB determination.

5.2.2 Enzyme concentration

To perform lipase immobilization, the free enzyme NS-40116 was concentrated using collagen membranes (Devro). The liquid formulation of free lipase NS-40116 was concentrated using collagen membranes (11.625 m²/g of surface area and pore diameter 21.752 Å, from Devro) in phosphate buffer solution 0.05 mmol/L (pH 7.0), for 10 cycles, during 72 hours. The content of the membranes was transferred to Petri dishes and subjected to freezing at -10 °C (Glacier Ultralow Temperature Freezer) for 24 hours. After freezing, the enzyme was lyophilized (LIOTOP Lyophilizer, Model L101) for 48 hours. The concentrated lipase NS-40116 was used in the immobilization process.

5.2.3 Synthesis of poly(urea-urethane) nanoparticles and enzymatic derivate

The PUU NPs were obtained via miniemulsion technique. The reactions were performed using molar ratio NCO:OH 1.5:1 and 15% of solids content, intending to maintain low the viscosity of the latex. The NCO source was the isophorone diisocyanate (IPDI, 99%, M_w 222 g/mol) from Sigma-Aldrich. The miniemulsion was prepared by sonication (Fisher-Scientific – Ultrasonic Dismembrator 500, 400 W) with 70% of amplitude and 15" ON/5" OFF pulses, for 3 minutes. To perform the enzymatic derivative immobilization in the nanoparticles, the aqueous phase was formed by concentrated lipase NS-40116 (0.3 g) diluted in a 0.05 M phosphate buffer pH 7.0 (20 mL). The sonication conditions used were the same as mentioned previously.

5.2.4 Characterization of poly(urea-urethane) nanoparticles and enzymatic derivative

The average particle size was analyzed by Dynamic Light Scattering (DLS, Zetasizer Nano S, from Malvern). The latex obtained was diluted in distilled water (1:15), and the final values were expressed as the mean of three measures. Morphological characterization of polymeric nanoparticles was evaluated by Transmission Electron Microscopy (TEM) in a device JEOL, JEM-1011 model, with a maximum acceleration voltage of 100 kV and a magnification range of 50 to 600,000

times. Samples for analysis were diluted to 0.05% of solids, and a drop (10 µL) was placed on the carbon-coated copper grid and dried at room temperature. The immobilization of enzyme NS-40116 was verified by a fluorescence optical microscope (Olympus, BX41 model) with capture images coupled with a digital color camera. The dye methylene blue was used as a target. The FTIR analysis was performed in a prestige-21 (Shimadzu). Samples were embedded in KBr pellets and analyzed by transmittance in the region 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans.

5.2.5 Enzymatic activity assays

As the p-NPP method is quantified by spectrophotometry, it is not considered suitable for the analysis of the miniemulsion samples, due to their coloration. In this way, the tests performed to investigate the influence of sonication time on the enzymatic activity of NS-40116 during the miniemulsion were made by the method of hydrolysis of olive oil, according previously described by Soares et al. (1999) using an olive oil emulsion, which was prepared as follows: 50 mL of olive oil (Bunge) and 50 mL of a solution containing 7% arabic gum (Vetec, ultra-high purity). The reaction mixture containing 5 mL of an olive oil emulsion, 4 mL of 0.05 M phosphate buffer (pH 7.0) and 1 mL of the enzyme suspension (15 $mg \cdot mL^{-1}$) was incubated at 37 °C for 4 minutes with magnetic stirring. Immediately after incubation, the emulsion was disrupted by the addition of 10 mL of acetone-ethanol-water (1:1:1 v/v), and the liberated free fatty acids were titrated with 50 mM KOH (Dinâmica Química, 99%) in phenolphthalein presence. The calculation of enzymatic activity was performed accord to Equation 1, and one unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acids per minute. All assays were carried out in triplicate.

$$Hydrolytic\ activity\ \left(\frac{\mu mol}{g.min}\right) = \frac{(V_A - V_B).M.10^6}{t.m} \qquad (1)$$

Where: V_A = KOH volume using in the titration of the sample, V_B = KOH volume using in blank titration, M = KOH molarity, t = time of reaction in minutes, m = mass of enzyme (g).

The yield of immobilization (γ) was determined using method described in Chapter III.

5.2.6 Determination of the kinetic parameters

The kinetic constants were determined for free concentrated and immobilized NS-40116 using the substrate in different concentrations (282, 564, 846, 1128, 1410 and 1692 mM of fatty acid), enabling the determination of Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) by Lineweaver–Burk plots. The mass of concentrated lipase used was 15 mg, and for the assay of the enzymatic derivative, 1 mL of latex was used (considering a homogeneous solution, the amount of enzyme in this volume is 15 $mg \cdot mL^{-1}$).

5.3 RESULTS AND DISCUSSION

5.3.1 Biopolyol characterization

In order to perform the glycerolysis reactions, the amount of lipase NS-40116 required was evaluated according to the enzymatic activity of the liquid formulation in relation to commercial lipase Novozym 435, since this enzyme is used in many investigations aiming the enzymatic glycerolysis (VALÉRIO et al., 2010; CAMINO FELTES et al., 2012; REMONATTO et al., 2015). According to the results, the lipase NS-40116 liquid formulation presented activity superior 9 times to Novozym 435, thereby 1% of the liquid formulation is equivalent to 9% of Novozym 435, which was the optimal condition for Valério et al. (2010), justifying the amount of lipase used in this work.

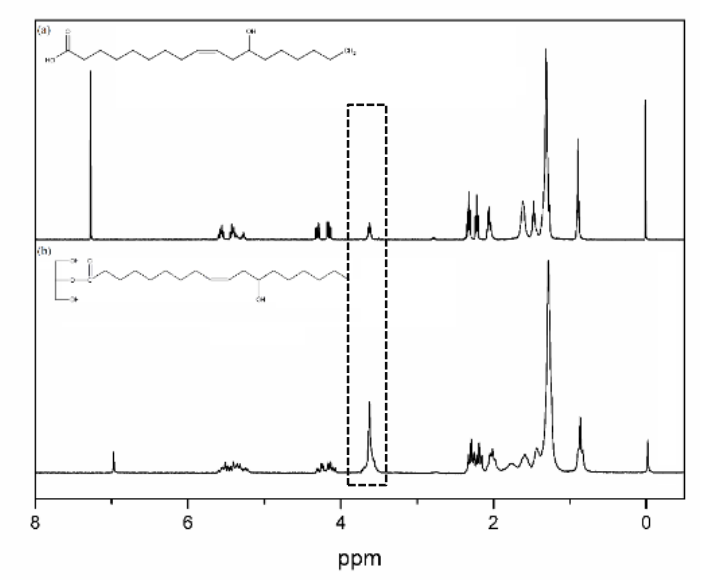
The physicochemical parameters of the obtained biopolyol is presented in Table 5.1. The biopolyol HLB index was 4.7, and the hydroxyl content was of 569.49±6.2 mg KOH/g. The values, when comparte to the obtained in Chapter IV (biopolyol produced with the lipase Novozym 435), indicate a decrease in MAG and DAG conversion.

Table 5.1 – Physicochemical parameters for the biopolyol obtained by enzymatic glycerolysis of castor oil.

Parameters	Value
Acid value (mg KOH/g)	3.0 ± 0.2
Hydroxyl number (mg NaOH/g)	569.49±6.2
HLB	3.9
pH	6.6

The product of the glycerolysis reaction presented a hydroxyl group content of 2.3, in relation to the fatty acid mols, corroborating with the other characterization results of biopolyol. In Figure 5.1 it is possible to observe a significant increase in the hydroxyl group (∂ =3.6 ppm) when comparing the castor oil and the glycerolysis product spectra. The presence of this peak in the castor oil is because the oil has about 90% of ricinoleic acid in the composition, which had a hydroxyl group in its chain. When submitted to the glycerolysis reaction, the number of hydroxyl groups have an increase and due to the overlap of the peaks, the relative area also increased.

Figure 5.1 - ^{1}H NMR (400 MHz) spectrum in $CDCl_3$ of the (a) castor oil and the (b) glycerolysis product.



Source: The Author.

5.3.2 Poly(urea-urethane) nanoparticles synthesis

PUU nanoparticles were prepared by step miniemulsion polymerization, with 15% (3 g) of solids content and molar ratio diisocyanate to hydroxyl (NCO:OH) of 1.5:1. The NPs were synthesized

without the presence of extra surfactant, using only the product of the glycerolysis to avoid coalescence. The average size (D_p) of the PUU nanoparticles using the biopolyol previously synthesized was 199 ± 3 nm with a polydispersity index (PdI) of 0.19 ± 0.01. The enzymatic derivative presented an increase in the D_p value (231 ± 1 nm) and PdI of 0.21 ± 0.01, being this increase associated with the enzyme immobilization, similar to values found in the literature. Cipolatti et al. (2014) immobilized the lipase TLL in PEGylated poly(urea-urethane) nanoparticles by miniemulsion using sodium dodecyl sulfate (SDS) as surfactant and the authors found values around 161 nm and 169 nm, for the nanoparticle without lipase and for the enzymatic derivative, respectively, using an ultrasound intensity of 70% for 180 seconds. Chiaradia and coauthors (2016) studied the immobilization of *Candida antarctica* Lipase B on magnetic poly(urea-urethane) nanoparticles using the same surfactant and same sonication intensity and time as the previously mentioned research. The D_p of the nanoparticles, when 30 wt% of magnetite was present in the system, was around 241 nm.

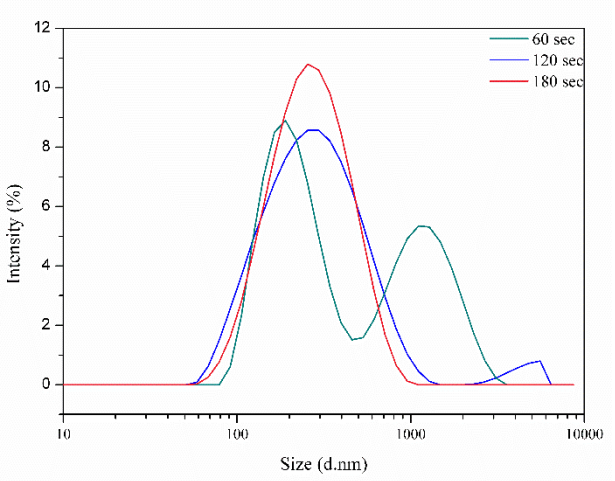
The effect of sonication time on enzymatic activity was also evaluated, to observe the behavior of the enzyme when subjected to high shear rates and immobilization (Table 5.2). According to the results, the initial activity for the concentrated lipase NS-40116 was 29084 ± 3803 U/g. It was observed that the enzymatic activity decreased with the sonication and immobilization, showing a negatively affect the enzyme when it submmited to immobilization. However, the sonication itself, without the presence of the monomers for the polymer formation, showed the same effect, although less pronounced, since it shows a much less significant decrease in the activity of the enzyme. As immobilization has a number of advantages, and the activity test was performed with a specific substrate, immobilization of the enzyme was performed using the sonication time of 180 seconds. This condition was adopted in the production of NPs due the major stabilty in the reaction, since the reactions using 60 and 120 seconds presented unstable system and in the end of the reaction (3 h) was observed the formation of two ou more populations with different z-avarega size (D_p) (Figure 5.2).

Table 5.2 – Effect of sonication time in the D_p, PdI and enzymatic activity in PUU nanoparticle with 10% of the enzyme.

Time (sec)	Enzymatic activity (U/g)	D_p (nm)	PdI
60*	25399 ± 1103	--	--
120*	21255 ± 1056	--	--
180*	15457 ± 1585	--	--
60	16542 ± 1426	303 ± 16	0.43 ± 0.01
120	15542 ± 1840	240 ± 1	0.27 ± 0.01
180	13542 ± 1317	231 ± 1	0.19 ± 0.01

*Enzyme submitted to sonication force in the system containing PBS buffer solution and enzyme (without monomer).

Figure 5.2 - Intensity particle size distributions (measured by DLS) of enzymatic derivate of lipase NS-40116 in different times of sonication.



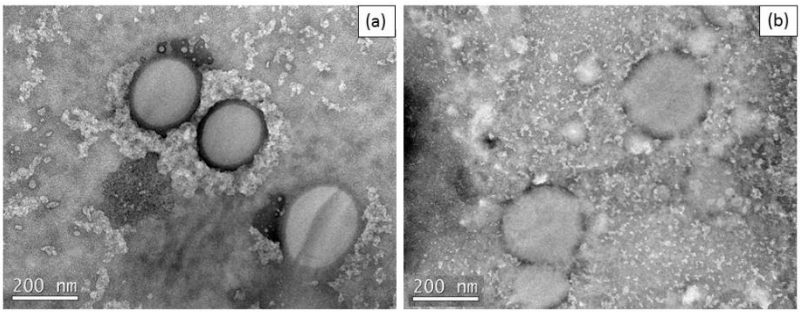
Source: The Author.

The yield of immobilization was around 52%, showing that the support was can be more efficient for this purpose. Cipolatti et al. (2014) studied the immobilization of lipase TLL in PEGylated poly(urea-urethane) nanoparticles using 10 wt % of crodamol, and the immobilization yield was of 82.4%. Based on this, in the future some strategies can be performed for this index increases, such as the incorporation of crodamol into the synthesis of the enzymatic derivative.

However, as this synthesis was performed without the use of stabilizer the result of immobilization yield is higher than the expected.

With the analysis of transmission electron microscope (Figure 5.3) it is possible to observe the nanoparticles and the immobilized lipase NS-40116 immobilized on the PUU nanoparticles surface. The morphology of PUU nanoparticle without the enzyme (Figure 5.2a) is different when compared to the PUU nanoparticle which served as a support for the enzyme NS-40116 (Figure 5.2b), mainly on the surface of the material where the enzyme is present.

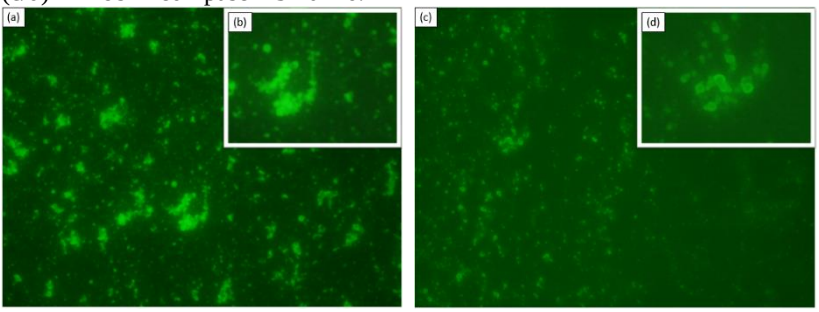
Figure 5.3 – Transmission electron microscopy images of (a) poly(urea-urethane) nanoparticles and (b) enzymatic derivative of lipase NS- 4016 immobilized on poly(urea-urethane) nanoparticles surface.



Source: The Author.

The images obtained using fluorescence optical microscopy (Figure 5.3) confirmed the linkage between the surface and the enzyme. It is possible to verify the formation of strong luminescence around the support due to the amount of enzyme present on the surface of the polymeric material, forming an enzymatic halo. The same is not observed in nanoparticles obtained without the presence of lipase. According to Cipolatti and collaborators (2014) the linkage between the enzyme and the PUU can occur due to the amino groups present in the lipase and in the NCO groups from IPDI, explaining the existence of such connection on the nanoparticles surface.

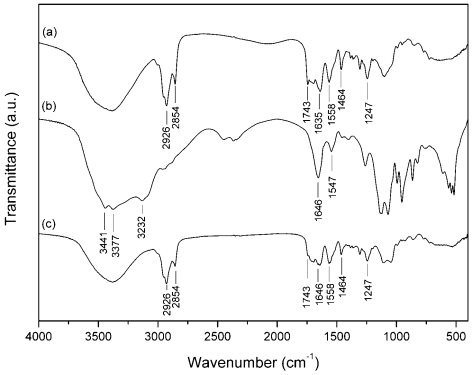
Figure 5.4 - Fluorescence optical microscopy of (a/b) PUU nanoparticles and (c/d) immobilized lipase NS 40116.



Source: The Author.

The free lipase NS-40116, PUU nanoparticles and enzymatic derivative were also characterized using FTIR analysis. Figure 5.4a corresponds to the regular PUU nanoparticle and it is possible to observe the presence of bands in 2926 cm^{-1} and 2854 cm^{-1} relatives to the presence of $–CH_2–$ asymmetric and symmetric stretching, respectively. The signal at 1464 cm^{-1} is relative to the C–H scissoring vibrations. The band in 1740 cm^{-1} is relative to the C=O stretching of urethane groups, 1558cm^{-1} represents the aromatic groups present in the material and the C–O stretching present a characteristic band in 1247 cm^{-1}. Also, the band centered in 1635 cm^{-1} is associated urea carbonyl stretching vibrations. The investigation of the free concentrated lipase NS-40116 spectra (Figure 5.4b) permitted to observe the peak in the region of 3441 cm^{-1} due to O–H stretching. Signals in the range of 3377 cm^{-1} and 3132 cm were results of primary amide $–NH_2–$ asymmetric stretching and N–H from amide A, respectively. Bands centered at around 1543 cm^{-1} are assignable to the amide II and the signal in 1649 cm^{-1} characteristic for the α-helix secondary assignment, a common motif in the secondary structure of proteins. The PUU nanoparticle with the lipase NS-40116 (Figure 5.4c) shows typical bands of PUU above mentioned, besides the band in 1646 cm^{-1}, characteristic of the α-helix secondary assignment of the enzyme, which is an evidence of the protein interaction with the polymeric material.

Figure 5.5 – FTIR spectra of the analyzed materials: (a) regular PUU nanoparticles, (b) free concentrated lipase NS 40116 and (c) lipase NS 40116 immobilized in PUU nanoparticles.



Source: The Author.

Results presented in Table 5.3 show the kinetic constants for the free lipase NS-40116 and enzymatic derivative immobilized in the PUU nanoparticles. Lower K_m and V_{max} values for immobilized lipase in relation the free enzyme were observed. This effect can occur possibly due to the formation of very small polymer particles causing agglomeration, which may increase the interaction between immobilized enzyme with the substrate (CIPOLATTI et al., 2015).

Table 5.3 – Kinetic constants values calculated through hydrolytic activity as a function of substrate concentration for the concentrated and immobilized lipase NS-40116.

Lipase	**K_m (mM)**	**V_{max} (U/g)**
Free concentrated NS 40116	708.5	$82{,}25 \times 10^4$
Immobilized NS 40116	218.0	$3{,}92 \times 10^3$

5.4 CONCLUSIONS

In this chapter, it was reported for the first time the glycerolysis reaction using the lipase NS-40116 as catalyst. The reaction resulted in a product with high hydroxyl presence (2.6 mol in relation to the fatty acid mol) proving the high formation of mono- and diacylglycerol. From the product of glycerolysis was obtained spherical poly(urea-urethane)

nanoparticles with z-average size of 199 nm, without the use of extra stabilizer. After, the study aiming the immobilization of lipase NS-40116 on the nanoparticles surface, and the assays showed the real possibility of obtainment these enzymatic derivative using the same technique explored to NPs synthesis. The enzymatic derivative presented a particle size around 231 nm, and compared to current literature, that uses an extra surfactant with the same technique, presented similar values, not only in size but also in kinetic constants behavior. Thereby, in summary, was possible to obtain enzymatic derivatives using a polyol produced from renewable sources and with the low-cost catalyst.

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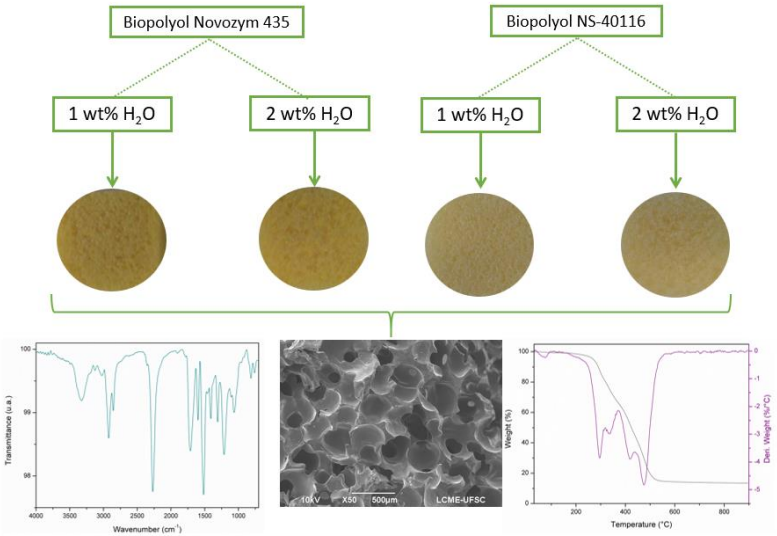
CHAPTER VI

Properties of rigid polyurethane foams obtained from biopolyols produced by enzymatic glycerolysis



- Enzymatic glycerolysis showed high conversions in mono- and diacylglycerols (biopolyol);
- Glycerolysis products were able to be used as biopolyol for polyurethane foams polymerization;
- The different biopolyols showed different foams characteristics;
- The foams presented higher resistance to compressive strength.

Abstract

Vegetable oils and their derivatives have been widely used for the production of various polymers including polyols and polyurethanes. In this work, the synthesis of biopolyols derived from castor oil and glycerol was performed by enzymatic glycerolysis with the lipases Novozym 435 and NS-40116 (*Thermomyces lanuginosus*) as catalysts at 70 °C. The biopolyol obtained using the lipase Novozym 435 led to predominantly MAG formation, whereas the biopolyol produced using lipase NS-40116 resulted in higher DAG content due to the lipases selectivity. Both biopolyols were employed for the synthesis of polyurethane foams (PUF) by bulk polymerization using polymeric methylene diphenyl diisocyanate (pPMDI papi-27) at molar ratio NCO:OH 1:1 with distilled water as blowing agent. The foams were evaluated by apparent density, degree of crosslinking, Fourier Transform Infrared Spectroscopy, morphology, thermogravimetric analysis and compressive strength. The results showed that both biopolyols provided PU foams polymerization, however the biopolyol it presents a higher amount of MAG (from Novozyme 435) has higher resistance than compression than the biopolyol it presents a higher amount of DAG, probably due to the higher hydroxyls availability to react with the diisocyanate, which aided in the formation of foams with uniform cellular structure.

Keywords: Glycerolysis, polyurethane foam, biopolyol, Novozym 435, lipase NS-40116.

6.1 INTRODUCTION

The use of renewable sources to obtain polymers has emerged in the last years due to the high cost of petrochemical sources, and high demand of polymers by society and environmental concerns (LLIGADAS et al., 2013; MIAO et al., 2014; SHARMIN et al., 2015; ZHANG et al., 2017a). In this scenario, polyurethane (PU) has been a polymer of great interest in this investigation field. PU is formed by the reaction between a polyol, containing hydroxyl groups, and an isocyanate, containing NCO groups, being the polyol from renewable sources. The possibility to obtain a polyol from greener process is the goal of various researchers (NOREEN et al., 2016; LI; BOUZIDI; NARINE, 2017), since the vegetable oils allows a possibility of structural modifications through reactions, e.g.: epoxidation, transesterification, hydrogenation, among others (ZHANG et al., 2017b).

Glycerolysis is an example of transesterification reaction, present in a range of studies aiming the biopolyols production, since this reaction allows the formation of mono- and diacylglycerols (MAG and DAG), which are basically hydroxylated emulsifiers widely used in the food industry and they can be used with high efficiency as biopolyol due to the presence of the hydroxyl groups (TANAKA; HIROSE; HATAKEYAMA, 2008; ABDUL HALIM; SOLOI; MAJID, 2015; BRESOLIN et al., 2017; DA SILVA; CARDOZO; PETZHOLD, 2018). The monoacylglycerol has in its chemical structure two hydroxyl groups and a carboxylic acid and diacylglycerol has in its chemical structure one hydroxyl group and two acyl groups. In the isomeric condition, the compound have β-monoacylglycerol and α-monoacylglycerol and β-diacylglycerol and α-diacylglycerol (VEREECKEN et al., 2009; CHRISTIE, 2013, 2014).

When it is desired to obtain polymers using the products of vegetable oil reactions, chemical catalysts are most commonly used (TANAKA; HIROSE; HATAKEYAMA, 2008; LI et al., 2014), generating a series of undesired factors, such as, the need of high energy expenditure and possibility of product degradation, due to high reactional temperatures. In this sense, the enzymatic catalyst is an interesting alternative, promoting high conversion rates using mild reaction conditions (KALTENBACH; TOKURIKI, 2014; PELLIS et al., 2018). Also, it is explicit and concise in the literature the use of enzymatic

catalysis in this type of reaction, being the lipases widely reported as ideal to perform this function (VALÉRIO et al., 2010; SOLAESA et al., 2016).

Therefore, the goal of this chapter was obtainment of polyurethane foams (PUFs) using the biopolyols produce in the previously chapter, following an environmentally friendly process. For this purpose, castor oil, a non-edible oil which presents a natural hydroxyl in the composition, were used with glycerol for enzymatic glycerolysis reaction in a solvent-free system catalyzed by the lipases Novozym 435 and NS-40116. The foams were produce by bulk technique and properly characterized.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

Commercial immobilized lipase Novozym 435 (CalB, from *Candida antarctica*, immobilized on a macroporous anionic resin) and lipase NS- 40116 (liquid formulation of *Thermomyces lanuginosus*) were kindly donated by Novozymes and used as catalysts for glycerolysis reaction. Castor oil (donated by Campestre, Mw 928 g/mol), glycerol P.A. (Vetec, 98%), and Tween 80 (Vetec, 65 - 80 hydroxyl number) were also used in the glycerolysis reactions. Deuterated chloroform was used as solvent to the ^{13}C NMR analysis. All chemicals were used as received.

The biopolyols were produce according method describe in the chapters III and V The polyurethane foams formation were performed using polymeric methylene diphenyl diisocyanate (pMDI – papi-27; 31.4% of NCO reactive groups) and different percent of blowing agent (distillate water).

6.2.2 Biopolyols characterization

The biopolyols were synthesized by enzymatic glycerolysis as already described in previous chapters. MAG and DAG conversions were evaluated using ^{13}C NMR analysis on Bruker AVANCE DPX spectrometer operating at 200 MHz. Chemical shifts (δ) are reported in parts per million related to the internal standard tetramethylsilane (δ=0.00ppm). The hydroxyl value was calculated according to ASTM D 1957-86 (2001); the water content was calculated using a coulometric

Karl Fischer titration (HI 904), and pH was measured on pHmeter (AN2000 Analion). All analyses were performed in triplicate (n=3).

6.2.3 Polyurethane foams production and characterization

To obtain polyurethane foams, the biopolyol without purification, diisocyanate and blowing agent were added to a beaker at room temperature (~ 23 °C) and mixed vigorously (3000 rpm) for 90 seconds. After that, the mechanical stirrer was removed, and the PUF expansion started. The formulations used are presented in Table 6.1 and the molar ratio NCO:OH was fixed in 1:1 for all reactions. The amount of diisocyanate was based on biopolyols total hydroxyl content and the blowing agent percent did not take into account the water contained in the biopolyol.

Table 6.1 - Polyurethane foams formulation.

	Ingredients			
	Biopolyol 1 (wt %)	**Biopolyol 2 (wt %)**	**pMDI (wt %)**	**Blowing agent (wt %)**
PUF 1	37.5	–	61.5	1
PUF 2	37.1	–	60.9	2
PUF 3	–	41.7	57.3	1
PUF 4	–	41.3	56.7	2

The PUFs were characterized by Fourier Transform Infrared Spectroscopy (FTIR) using Cary 600 Series Spectrometer (Agilent Technologies) equipped with ZnSe crystal. The analysis was performed by transmittance in the region of 4000-700 cm^{-1} (resolution of 4 cm^{-1}) and 32 scans. The apparent density analysis was performed according to the method described in ASTM D 1622/D 1622M-14 (2004). The samples were cut 6x6x6 mm with a vernier caliper (Starrett: 125MEB-6/150) aid, and the foam was weighed on a digital scale with a precision of 0.001 g and 220 g capacity (model AUY220 - Marte). The sample morphology was verified by scanning electron microscopy (SEM) analysis with field emission (SEM-JEOL JSM-6390LV). Thermogravimetric analysis (TGA) were performed with a STA 449 F3 Jupiter. Samples of approximately 10 mg were heated from 30 to 650 °C with a heating rate

of 10 °C min^{-1}. Compressive strength tests of PUFs were performed on a TA HD plus texture analyzer (Stable Micro Systems) using cylindrical specimens (35 mm in diameter), speed of 1 mm/min and maximum deformation of 1 % based on standard ASTM D 1621 (2016).

6.3 RESULTS AND DISCUSSION

6.3.1 Biopolyols characterization

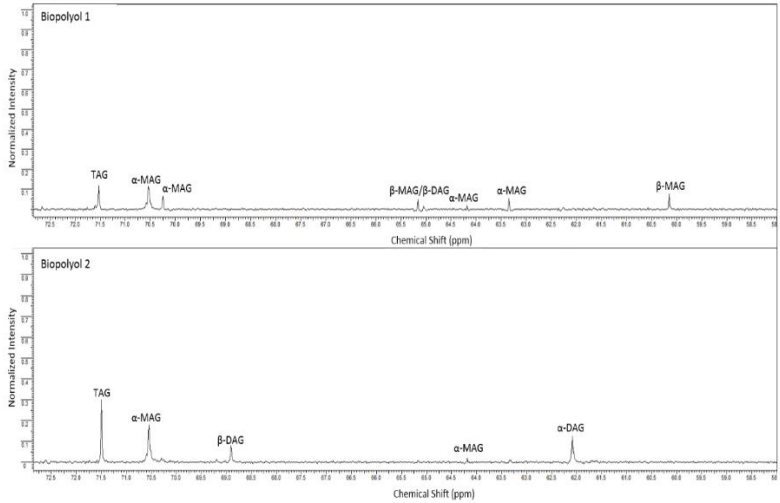
The ^{13}C NMR spectra of the biopolyols are presented in Figure 6.1. It is possible to observe the formation of α and β mono- and diacylglycerols in both syntheses. The regioselectivity of the lipases used in the reaction of the biopolyol was evidenced. The biopolyol synthesized with the lipase Novozym 435 (biopolyol 1) presented a high conversion in monoacylglycerol, as expected, since it is the most common lipase applied in this type of reaction; this behavior has already been widely explored (VALÉRIO et al., 2010; NAIK; NAIK; MOHANTY, 2014; REMONATTO et al., 2015). The biopolyol 2 synthesis, catalyzed by the lipase NS-40116, presented a lower conversion in MAG, being efficient in diacylglycerol obtainment. The biopolyols characterization is shown in Table 6.2.

Table 6.2 – Biopolyols characterization.

Parameters	**Sample**	
	Biopolyol 1	**Biopolyol 2**
Hydroxyl number (mg NaOH/g)	722.1 ± 5.3	569.49 ± 6.2
Water content (wt %)	0.35 ± 0.08	0.45 ± 0.03
pH	6.4	6.6
Functionality (mol)	2.9	2.3
MAG content (%)	43.2	25.5
DAG content (%)	20.4	37.4
TAG content (%)	36.4	37.1

For the is possible observed that biopolyol 1 presented higher hydroxyl content than biopolyol 2, due the higher conversion in MAG, and this triggers greater functionality, which will influence in the formation of the foam, mainly in the morphology and consequently in the mechanical properties.

Figure 6.1 - ^{13}C NMR spectrum of a mixture the biopolyols synthetized using enzymatic glycerolysis.



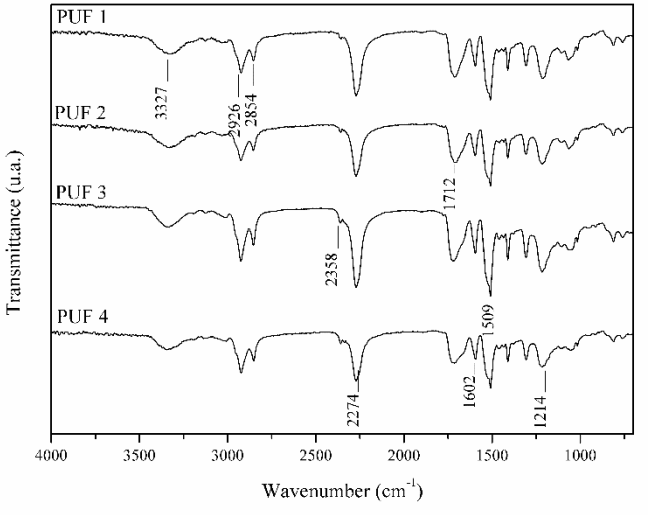
Source: The Author.

This result is very interesting since this reaction has never been explored with the lipase NS-40116. Thus, the catalytic capacity of this lipase to be applied for the acylglycerols formation has been proved.

6.3.2 Polyurethane foams characterization

The PUF obtained were characterized using FTIR analysis (Figure 6.2). All samples presented the same peaks, which is characteristic of polyurethane foam. FTIR spectra show a peak at 3327 cm^{-1} characteristic for the vibrations of the N–H groups present in urethane groups (ZIELENIEWSKA et al., 2015). The stretching observed in the region of 2926 and 2854 cm^{-1} indicates the presence of asymmetric and symmetric vibration of CH_3, respectively. In 2274 cm^{-1} it is possible to observe a peak refered to the stretching vibrations of –NCO. The peaks at 1712 cm^{-1} and 1602 cm^{-1} are characteristic of carbonyl bonds from urethane. The stretching present in 1509 cm^{-1} and 1214 cm^{-1} corresponds to the presence of C–N bonds (STUART, 2004).

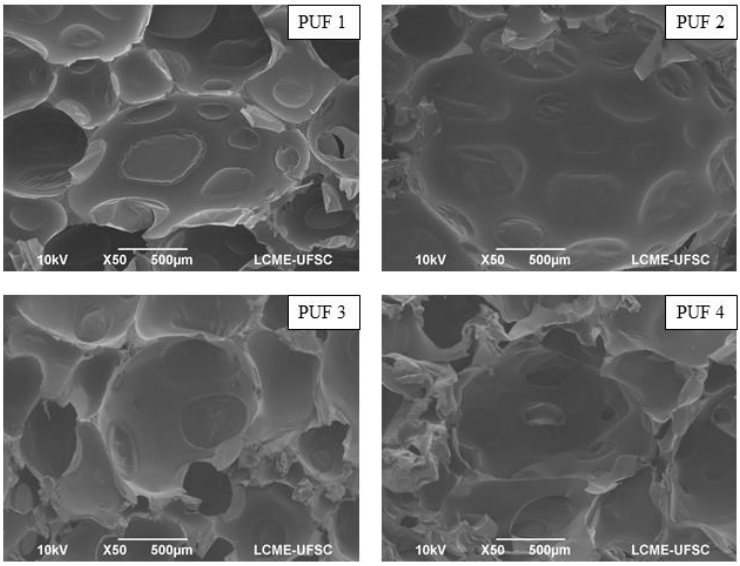
Figure 6.2 – FTIR spectra of polyurethane foams obtained using the biopolyols from renewable sources.



Source: The Author.

The foams morphology was verified using a SEM and are shown in Figure 6.3. It was possible to observe differences between the samples, according to the monomers and blowing agent quantities used in the polymerization. The foams obtained using the biopolyol 1 presented a well-defined Plateau borders and uniforms cells. The blowing agent concentration showed differences in foams, being PUF 1 cells smaller when compared with PUF 2. The reactions performed using the biopolyol 2 presented significant differences when compared to the polymerizations using the biopolyol 1. Cells are smaller in size than both PUF 3 and PUF 4, however it is possible to observe that the PUF 4 formulation does not present a well-defined cell appearance. Several events may explain this, but probably, the cell formation system collapsed due to water quantity, in combination with the diisocyanate and biopolyol functionality and reactivity (LIM; KIM; KIM, 2008; ZHANG; JUNE; LONG, 2012).

Figure 6.3 – Polyurethane foams morphology.



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In Table 6.3 it is possible to observe the foams properties and the morphology is related to the results obtained. All the foams presented a high degree of crosslinking independent of the biopolyol used, showing strong linkages between the NCO bonds, present in the diisocyanate and the hydroxyls groups, present in the biopolyol, form a three-dimensional network (LI; LUO; HU, 2015); however, this results did not guarantee the formation of cells, which this will accentuate the characteristics of the foams, such as, compression force and apparent density.

Several previous works have shown that the response of the foam in compression is governed by the size and shape of the elementary cells (Buzzi et al., 2008). The apparent density presented different values according to the biopolyol and percent of blowing agent used in the polymerization. This event can be explained by the increase in the reaction kinetic rate, when in water presence, due to the increase of the temperature caused between water and pMDI reaction (CHOE et al., 2004). Also, the size of the Plateau borders influence in this aspect, and was observed in the analysis of morphology the differences between the foams obtained, include cell size and Plateau borders. When biopolyol 2

was used as monomer, it was possible to observe differences between the samples in this characterization. This can occur due to the diisocyanate and biopolyol reactivity, since the kinetics will be faster according to the monomers functionality (LIM; KIM; KIM, 2008; DWORAKOWSKA et al., 2014).

Table 6.3 - Polyurethane foams properties.

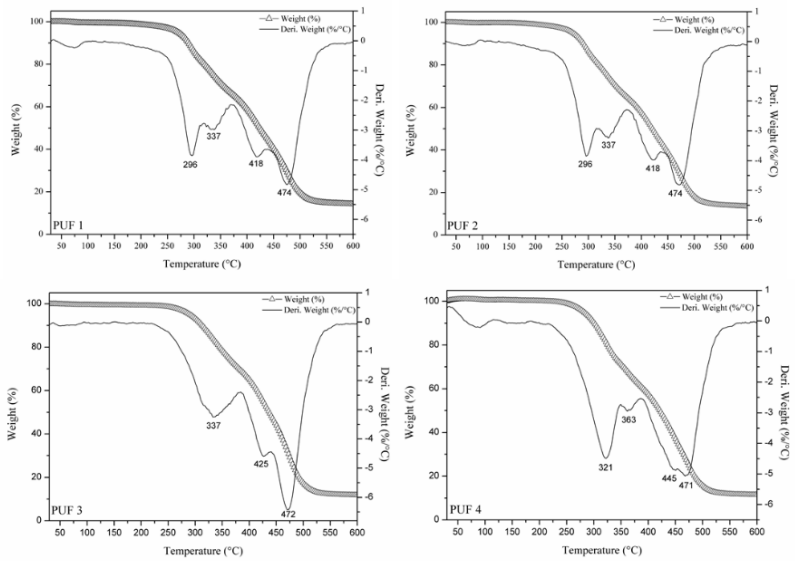
Biopolyol 1		**PUF 1**	**PUF 2**
	Degree of crosslinking (%)	95.7 ± 0.3	97.8 ± 0.4
	Apparent density (g/cm³)	0.091 ± 0.006	0.079 ± 0.004
	Compression strength (MPa)	0.075 ± 0.011	0.068 ± 0.008
Biopolyol 2		**PUF 3**	**PUF 4**
	Degree of crosslinking (%)	97.2 ± 0.8	97.7 ± 0.6
	Apparent density (g/cm³)	0.138 ± 0.008	0.115 ± 0.005
	Compression strength (MPa)	0.036 ± 0.009	0.042 ± 0.001

The compression strength is closely related to the morphology and to the dimensional stability of cell foams (LIM; KIM; KIM, 2008). The mechanical tests showed the compressive strength of the foams obtained using the biopolyol 1 and pMDI with 1 and 2% water (PUF 1 and PUF 2) presented higher resistance than the other polymers. The foams PUF 3 and PUF 4 presented lower resistance, probably due to low cell formation.

The thermogravimetric analysis are present in Figure 6.4. DTG curves of polyurethane foams prepared using both biopolyols are shown in Figure 6.4 and reveal that the thermal decomposition of rigid PU foams in nitrogen was a multistage process. In the range of 200 – 430 °C the events were attributed to the decomposition of urethane and urea linkages, which involves dissociations to isocyanate and the alcohol, amines and olefins or to secondary amines (LI et al., 2014; STIRNA et al., 2013). It is observed that all foams were thermally stable up to 200 °C. The first stage of weight loss, around 229 °C, referred to the degradation of –C–H linkage and PU segments. In the second stage, observed in 330 °C was due to the degradation of C=O and N–H linkages. In the last stage, around

470 °C, occurred the complete degradation of the polymer, being the temperature range of 440 – 490 °C associated with clearance of carbon–carbon bonds (LI; BOUZIDI; NARINE, 2017).

Figure 6.4 - Thermogravimetric analyses of rigid polyurethane foams obtained from the biopolyol 1 and biopolyol 2 with CO:OH molar ratio 1:1 (heating rate: 10 °C min^{-1}; atmosphere: N_2).



6.4 CONCLUSIONS

In this chapter, it was possible to evaluate the formation of polyurethane foams using different formulations. Initially, enzymatic glycerolysis reactions were performed using two different catalysts (lipase Novozym 435 and lipase NS-40116 - biopolyol 1 and biopolyol 2, respectively), where it was observed a higher regioselectivity for the formation of monoacylglycerol when Novozym 435 was used and the when lipase NS-40116 was applied as catalyst, the formation of diacylglycerol was more pronounced. Afterward, polymerizations were performed varying the blowing agent amounts. It was possible to record that the samples using biopolyol 1 presented higher compressive strength and lower apparent density than the foams obtained using the biopolyol

2, due the morphology of the cell formed. This chapter also showed that it is possible to develop polyurethane foams using enzymatic reactions, which is relevant when the aim is the search of environmentally friendly alternatives, since it is possible to obtain biopolyols using mild temperatures.

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CHAPTER VII

7. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORKS

7.1 General conclusions

In this work it was possible to synthetize polyurethane foam and polyurethane nanoparticles using the product of enzymatic glycerolysis as biopolyol (mono-, diacylglycerol and unreacted triacylglycerol – MAG, DAG and TAG). Also, enzymatic derivatives of lipase NS-40116 using the polymerics supports were obtained.

Initially, the enzymatic glycerolysis was carried out with the lipase Novozym 435 showed a high conversion rate in MAG and DAG, allowing the use as biopolyols in the PU foams synthesis using bulk polymerization. After, reactions aiming the lipase NS-40116 immobilization via entrapment technique were performed. The free and immobilized lipase NS-40116 were characterized in relation to the stability in different reactional medium and immobilization showed advantages regarding the stability in propanol, temperature and acid pH medium. Thus, the enzymatic derivative was applied as catalyst for fatty acid methyl esters production using abdominal chicken fat as feedstock. Conversions around 65% in the first four cycles of use were obtained, and it was possible to observe that the enzymatic derivative presented high catalytic power and it is susceptible for applications of commercial interest.

The same biopolyol was applied in the synthesis of poly(urea-urethane) nanoparticles using miniemulsion polymerization. To the best of our knowledge, the product of glycerolysis has never been used as biopolyols and stabilizer in miniemulsion without the use of additional surfactant. The reactions were performed using 20, 30 and 40% of solids content and average particle diameters ranging from 167 to 282 nm were obtained. The large amount of MAG and DAG provided steric stabilization for PUU nanoparticles obtained by miniemulsion polymerization and at the same time acted as polyol for PUU synthesis.

With the purpose of reducing the cost of the biopolyols production, in the second stage, the enzymatic glycerolysis was performed using the low-cost free lipase NS-40116 as catalyst. The reaction was carried out using the same conditions applied before, however, the amount of lipase was significantly decreased to 1%, due to the high activity as compared

to lipase Novozym 435. Also, 1 wt% of water was added in the system. After, the product of glycerolysis was used for poly(urea-urethane) nanoparticles synthesis and they were was used as support for lipase NS-40116 immobilization using adsorption technique. Stable nanoparticles with average particle diameters around 199 nm for the polymer and 231 nm for the enzymatic derivative were obtained. There was a loss in enzymatic activity due to enzyme immobilization and an immobilization yield of 52 %, although this yield is low, it is important empathized that this technique, without the use of surfactant for enzyme immobilization, had never been used to the best of our knowledge.

After, the two biopolyols obtained previously were evaluated and compared in the synthesis of polyurethane foams using the two biopolyols obtained in the work and different percentage of blowing agent (distilled water) was applied in the polymerizations. It was found that both biopolyols can produce high resistance compressive strength polyurethane foams. Also, it has been observed that in these foams the compressive strength increases when using the biopolyol with higher hydroxyl content, probably due to the formation of more uniform cells.

Hence, the results obtained in this work lead to the conclusion that, although still little explored for this purpose, the synthesis of biopolyols can be an efficient environmentally friendly alternative to produce polymers and supports for lipases immobilization.

7.2 SUGGESTIONS FOR FUTURE WORKS

The application of lipase NS-40116 immobilized on the surface of the poly(urea-urethane) nanoparticles should be investigated. Also, due to the lack of studies that use the miniemulsion, without the use of additional surfactant in the synthesis of polymeric materials it would be interesting to immobilize other lipases as well as other classes of enzymes, such as a work whose objective is to immobilize cellulase in poly(urea-urethane) nanoparticles, something that has never been done.

There are few works in the literature using the lipase NS-40116. It would be interesting to use the enzymatic derivatives obtained in this work to produce aromatic esters, which have high commercial value, as well as to explore other supports to immobilize this lipase.

7.3 RELATED SCIENCE PUBLICATION

Submissions in scientific journals and conferences as first author:

2017 – Immobilization of Lipase NS 40116 in Polyurethane Foam – XXI Simpósio Nacional de Bioprocessos – Award for best poster presentation. Aracaju/Brazil.

2018 – Polyurethane foam based on a green polyol from non-edible oil (*Ricinus communis*)– 5th French Brazilian Meeting on Polymers – Poster presentation. Florianópolis/Brazil.

2018 – A green polyol as support for the immobilization of lipase NS 40116 in polyurethane foam – 10th International Conference on Fiber and Polymer Biotechnology – Oral presentation. Balneário Camboriu/Brazil.

2018 – Enzymatic glycerolysis for a biopolyol production and its application as support for lipase immobilization – XII Seminário Brasileiro de Tecnologia Enzimática – Will occur in September at Florianópolis/Brazil.

2018 - Evaluation of the enzymatic glycerolysis products in the polymerization of poly(urea-urethane) nanoparticles – XII Seminário Brasileiro de Tecnologia Enzimática – Will occur in September at Florianópolis/Brazil.

2018 – Synthesis of a green polyurethane foam from a biopolyol obtained by enzymatic glycerolysis and its use for immobilization of lipase NS-40116. Submitted to Bioprocess and Biosystems Engineering.

2018 – Poly(urea-urethane) nanoparticles using mono- and diacylglycerol from glycerolysis of castor oil as biopolyol and stabilizer. Submitted to European Polymer Journal.

Published paper in journal as coauthor:

2018 – Improving reuse cycles of *Thermomyces lanuginosus* lipase (NS-40116) by immobilization in flexible polyurethane - Bruno R. Facin,

Alexsandra Valério, **Daniela Bresolin**, Giselle Centenaro, Débora de Oliveira & J. Vladimir Oliveira.