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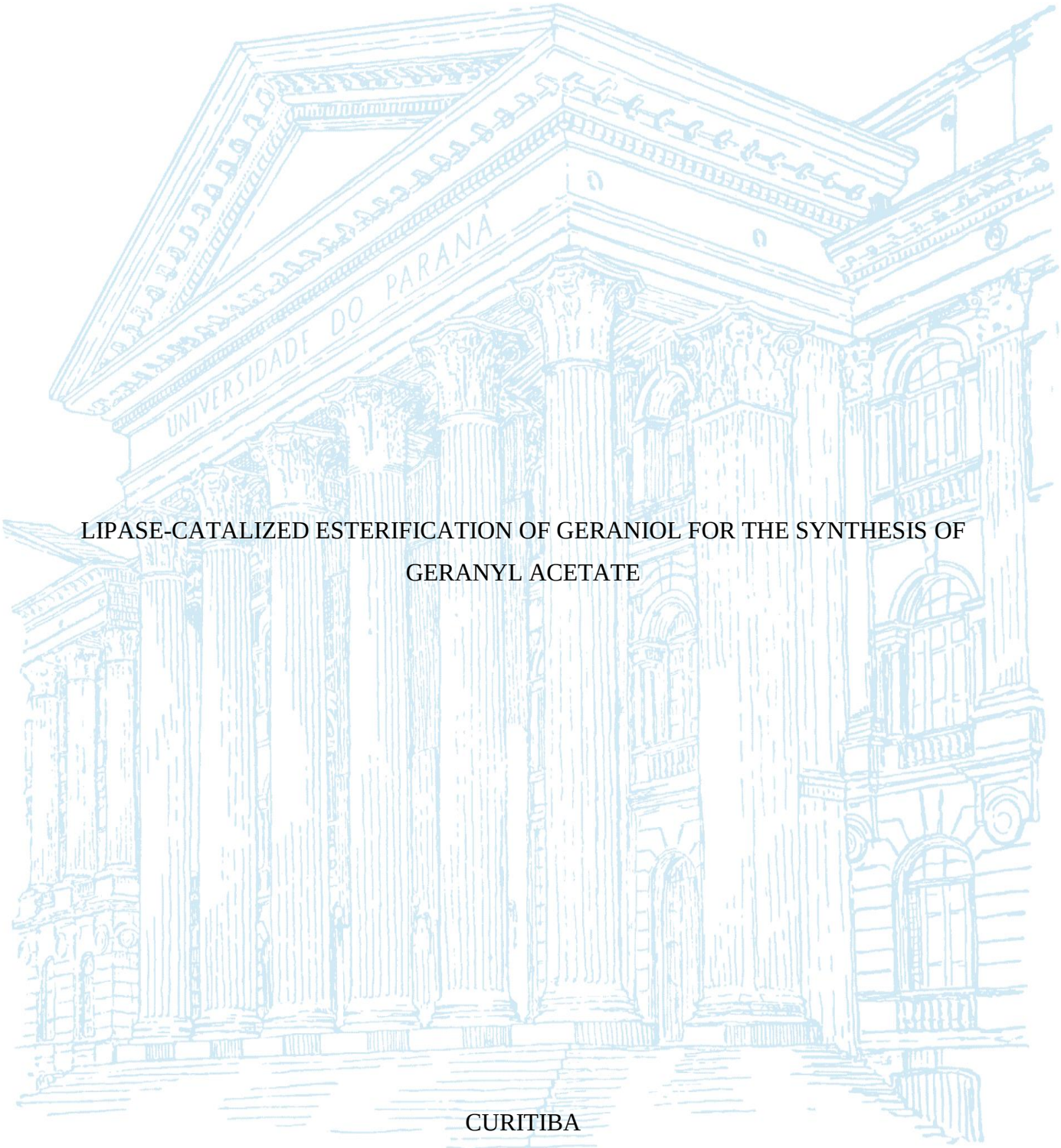
MATHEUS VON LINSINGEN TAVARES



LIPASE-CATALIZED ESTERIFICATION OF GERANIOL FOR THE SYNTHESIS OF GERANYL ACETATE

CURITIBA

2020

MATHEUS VON LINSINGEN TAVARES

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Dissertação de mestrado apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Marcos Lúcio Corazza

Coorientadora: Profa. Dra. Luciana Porto de Souza Vandenberghe

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **MATHEUS VON LINSINGEN TAVARES** intitulada: **Lipase-catalized esterification of geraniol for the synthesis of geranyl acetate**, sob orientação do Prof. Dr. MARCOS LÚCIO CORAZZA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 31 de Março de 2020.

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DEDICATÓRIA

Dedico esta dissertação a mim e a todas as pessoas que me ajudaram nessa trajetória.

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Meu muitíssimo obrigado!

Porque o verdadeiro segredo não é fazer uma limonada com os limões que a vida nos dá, mas conviver numa boa com a úlcera no estômago que vem em seguida.

Mark Manson

RESUMO

Geraniol é uma molécula bastante utilizada na indústria farmacêutica, de cosméticos, fragrâncias e flavorizantes. Porém, como apresenta certa toxicidade na forma alcoólica, ele deve ser utilizado na forma de éster, como acetato de geranila. Acetato de geranila pode ser obtido pela esterificação de geraniol com um agente doador de grupo acil, utilizando um biocatalisador e um solvente orgânico com meio reacional. Desse modo, o objetivo deste trabalho é apresentar uma análise crítica (dados da literatura e confirmação e obtenção de dados inéditos) dos principais processos para produção e apresentar os dados experimentais obtidos na síntese do acetato de geranila focando principalmente a utilização de biocatalisadores e solventes considerados "verdes". Nesse sentido, duas lipases comerciais, Lipozyme® RM IM e Novozym® 435, foram testadas em temperaturas que variaram de 40 °C a 70 °C, utilizando reatores em batelada, um Reator Tanque Agitado Descontínuo (em inglês, BSTR) e um reator de volume variável (em inglês, VVR), e diferentes solventes (n-hexano, como solvente convencional, e dióxido de carbono, como solvente alternativo). Utilizando o BSTR e n-hexano, após apenas 240 min, conversão foi 82,9% quando a Novozym® 435 foi utilizada, enquanto a esterificação utilizando Lipozyme® RM IM demorou 480 min para atingir a conversão de 74,8%. Devido a preocupações ambientas e de segurança, alternativas aos solventes orgânicos estão sendo pesquisadas. Nesse contexto, CO_2 foi empregado com solvente na reação utilizando Novozym® 435 no BSTR, resultando em uma conversão de 60,5% após 240 min de reação. Embora o resultado obtido no BSTR tenha sido satisfatório, para verificar o efeito da pressão e da quantidade de dióxido de carbono adicionado, reações foram feitas no VVR. Assim, após 60 min de reação, a melhor conversão (46,9%) foi obtida na temperatura de 65 °C, pressão de 16,0 MPa e razão molar entre reagentes e CO_2 de 0,2. A principal inovação desse trabalho é avaliar o efeito da pressão de reação independentemente da quantidade de CO_2 inserida no reator. Desse modo, os resultados apresentados podem contribuir para o entendimento do papel do dióxido de carbono, o que pode ser essencial para a obtenção de conversões maiores nas reações utilizando um solvente alternativo em consonância com o conceito de química verde.

Palavras-chave: Geraniol. Acetato de geranila. Lipozyme® RM IM. Novozym® 435. CO_2 supercrítico. Biocatálise. Esterificação verde. Equilíbrio de fases.

ABSTRACT

Geraniol has an important use in flavor and fragrance, cosmetic and pharmaceutical industries, but due to a certain degree of toxicity, it has to be used as an ester, such as geranyl acetate. Geranyl acetate can be obtained by esterification of geraniol with an acyl agent using biocatalysts and an organic solvent media. Therefore, the objective of this work is to present a critical analysis (review of literature data and validation and attainment of new data) of the main processes of production and also present the experimental data obtained in the synthesis of geranyl acetate focusing mainly in the use of biocatalyst and green solvents. In this sense, two commercial lipases, Lipozyme® RM IM and Novozym® 435, were tested at temperatures from 40 °C to 70 °C, using batch-type reactors, a Batch Stirred Tank Reactor (BSTR) and a Variable Volume Reactor (VVR), and different solvents (n-hexane and CO_2). Using the BSTR and n-hexane, after only 240 min, the conversion was 82.9% when Novozym® 435 was utilized, while the esterification using Lipozyme® RM IM took 480 min to achieve 74.8% of conversion. Due to environmental and safety concerns, alternatives for the use of organic solvents are being researched, so CO_2 was employed in the reaction using Novozym® 435 in the BSTR, resulting in a conversion of 60.5% after 240 min of reaction. Despite the good results achieved in BSTR, to assess the effects of pressure and amount of CO_2, reactions were performed using a VVR. Thus, after 60 min, the best conversion (46.9%) was achieved at 65 °C, when the pressure was 16.0 MPa and the mass ratio of reactants to CO_2 was 0.2. The main feature of this work is the evaluation of pressure independently from the amount of CO_2 in batch reactors. Therefore, the results presented can contribute to the understanding of the role of CO_2, which can be a key for the obtainment of higher conversions in such reactions using a greener solvent in consonance to the concept of green chemistry.

Keywords: Geraniol. Geranyl acetate. Lipozyme® RM IM. Novozym® 435. Supercritical CO_2. Biocatalysis. Green esterification. Phase equilibrium.

FIGURE LIST

TABLE LIST

SUMMARY

1 INTRODUCTION

Geraniol (trans-3,7-dimethylocta-2,6-dien-1-ol) is an acyclic monoterpene alcohol extracted mainly from genus Cymbopogon plants (Palmarosa – *Cymbopogon martini* and Java citronella – *Cymbopogon winterianus*). It is the primary component of palmarosa oil, citronella oil, rose oil, and other essential oils (WAN et al., 2017). Due to its pleasant rose-like aroma, geraniol is commercially important and it is frequently used in flavor and fragrance, cosmetic and pharmaceutical industries (LI et al., 2012). However, geraniol possesses a particular degree of toxicity when applied directly into products (MURCIA et al., 2018). This issue is easily overcome by utilizing an ester form of the alcohol, such as geranyl acetate, in which the organoleptic properties of geraniol are maintained, but no toxicity is conferred to the final industrial product (MURCIA et al., 2018; XIONG et al., 2014).

Geranyl esters are mostly obtained by chemical synthesis or extraction from natural sources (KARRA-CHAABOUNI et al., 1996). An industrial way to produce these esters is either by direct esterification or by transesterification using biocatalysts in organic solvents and using different acyl donors (BADGUJAR and BHANAGE, 2014; BARTLING et al., 2001; BEZBRADICA et al., 2007; CHATTERJEE and BHATTACHARYYA, 1998; CHULALAKSANANUKUL, CONDORET and COMBES, 1992; CLAON and AKOH, 1993, 1994; DAMNJANOVIĆ et al., 2012; GUPTA et al., 2013; KNEŽEVIĆ-JUGOVIĆ et al., 2008; MOLINARI, VILLA and ARAGOZZINI, 1998; SHIEH, AKOH and YEE, 1996; SHINDE and YADAV, 2014). Enzymes are feasible biocatalysts due to their versatility, chemo-, enantio-, and regio-selectivity, as well as the demand for less polluting and more environmentally friendly processes and catalysts (PELLIS et al., 2018). Since Zaks and Klibanov (1985) have discovered that enzymes as hydrolases and more specifically lipases could perform a variety of synthesis reactions in anhydrous media, many researches and industrial processes have been developed in this field (BOURKAIB et al., 2018). Nonetheless, the majority of enzymatic reactions have been performed using organic solvents.

Since organic solvents are quite harmful to both the environment and human health, supercritical fluids have received considerable attention as greener eco-friendly alternatives in enzyme-catalyzed reactions, in particular the synthesis of geranyl esters (BOURKAIB et al., 2018; COUTO et al., 2011; MARTY et al., 1992; PERES, GOMES DA SILVA and BARREIROS, 2003; VARMA and MADRAS, 2008). Supercritical fluids can be used to boost phase separation and ester purification in downstream processes of esters production, as they can be easily separated during the depressurization of the system, providing products

without polar contaminants. Moreover, their gas-like transport properties and liquid-like densities can be readily adjusted by changing process conditions, such as temperature and pressure (CHULALAKSANANUKUL, CONDORET and COMBES, 1993; ESCORSIM et al., 2015; VARMA and MADRAS, 2010; VEIGA et al., 2017). Among supercritical fluids, supercritical CO_2 ($scCO_2$) is vastly used due to characteristics, such as low toxicity, non-flammability, abundance, low-cost, relative easiness to achieve critical points and tunable physicochemical and thermodynamic properties (BOURKAIB et al., 2018; DAZA SERNA, ORREGO ALZATE and CARDONA ALZATE, 2016; VEIGA et al., 2017).

In this context, the objective of this work is to present experimental results of the lipase-catalyzed esterification of geraniol using acetic acid as acyl donor for the synthesis of geranyl acetate. This enzymatic catalysis of geraniol has already been studied by previous authors. However, in this work, the effect of pressure in the reaction conversion is evaluated independently of the amount of carbon dioxide added to the system. The temperature range was from 40 °C to 70 °C, while the pressure range was from 8.0 MPa to 16.0 MPa. Therefore, different types of enzymes (Lipozyme® RM IM and Novozym® 435) and solvents (n-hexane and $scCO_2$) were employed. First, the effects of temperature and molar proportion of reactants were studied in a Batch Stirred Tank Reactor (BSTR) using n-hexane as solvent and Lipozyme® RM IM. Further, Lipozyme® RM IM was replaced by Novozym® 435 to verify the conversion difference between these biocatalysts. Then, n-hexane was substituted by $scCO_2$, to assess the effect of the solvent. However, the change of solvent also required the use of a different reactor configuration in order to evaluate the pressure effect. Therefore, a Variable Volume Reactor (VVR) was employed to perform esterification reactions at different temperatures, pressures, and reactants to CO_2 mass ratio.

2 LITERATURE REVIEW

In this section, the theoretical referential of this work is presented. The literature review about terpenes, geranyl esters, esterification reactions, lipases, supercritical fluids, synthesis of geranyl esters in different solvents, a comparison of conversion in different reaction media, the publication through the years about the synthesis of geranyl esters, the influence of water on lipase activity and a brief description of the thermodynamic model utilized and methods of measurements of thermodynamic data are presented in the topics below.

2.1 TERPENES

Terpenes are the largest single class of compounds found in essential oils (BUCKLE, 2015). They are mostly hydrocarbons constituted of a building block of a five-carbon isoprene unit or 2-methyl-1,3-butadiene (FIGURE 1) (BLANCO and BLANCO, 2017).

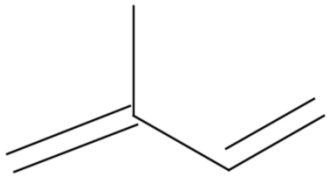

FIGURE 1 – CHEMICAL STRUCTURE OF AN ISOPRENE UNIT OR 2-METHYL-1,3-BUTADIENE
SOURCE: Retrieved from: <researchgate.net%2Ffigure%2FChemical-structure-of-isoprene_fig3_265124924&psig=AOvVaw0QLupmxO1iKSnm6rSs2Zw1&ust=1533818868467025>. Accessed: 08 August 2018.

They are formed by two or more isoprene units bound, generally, between C4 of one isoprene and C1 of another, following, usually, the special isoprene rule, which states that the isoprene molecules are bound by the “head to tail” fashion (FIGURE 2) (BLANCO and BLANCO, 2017; YADAV, YADAV and GOYAL, 2014). Their molecular formula is $(C_5H_8)n$, which “*n*” represents the number of units involved (ALDRED, BUCK and VALL, 2009).

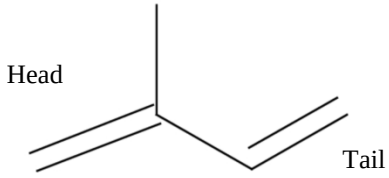

FIGURE 2 – "HEAD" AND "TAIL" POSITION IN AN ISOPRENE UNIT
SOURCE: Adapted from: <researchgate.net%2Ffigure%2FChemical-structure-of-isoprene_fig3_265124924&psig=AOvVaw0QLupmxO1iKSnm6rSs2Zw1&ust=1533818868467025>. Accessed: 08 August 2018.

Polyisoprenes can have a linear structure (acyclic), such as geraniol, or a cyclic one, like limonene (FIGURE 3) (BLANCO and BLANCO, 2017).

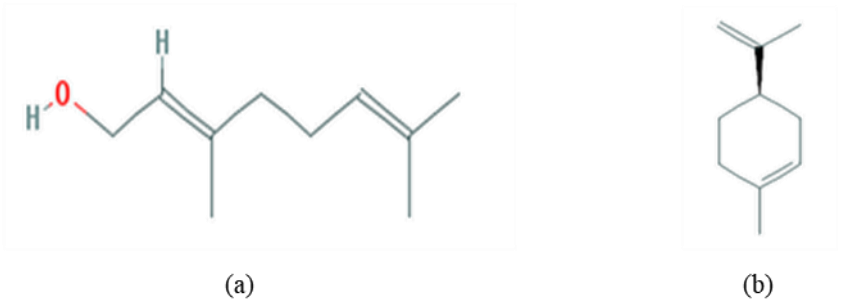

FIGURE 3 – CHEMICAL STRUCTURE OF (a) GERANIOL AND (b) LIMONENE
SOURCE: Retrieved from: <https://pubchem.ncbi.nlm.nih.gov/image>. Accessed: 08 August 2018.

Polyisoprenes are classified in accordance with the number of isoprene units: monoterpenes (2 isoprene units); sesquiterpenes (3 isoprene units); diterpenes (4 isoprene units); triterpenes (6 isoprene units); tetraterpenes (8 isoprene units) (ALDRED, BUCK and VALL, 2009). All terpenes present the suffix *–ol* (BUCKLE, 2015). When a terpene is modified to its corresponding alcohol the suffix is changed to *–oid*.

Some other examples of terpenes (FIGURE 4) are: myrcene (acyclic monoterpenoids); menthol, α-terpineol (monocyclic monoterpenoid); farnesol, zingiberene (sesquiterpenes); retinol [vitamin A] (diterpenoid).

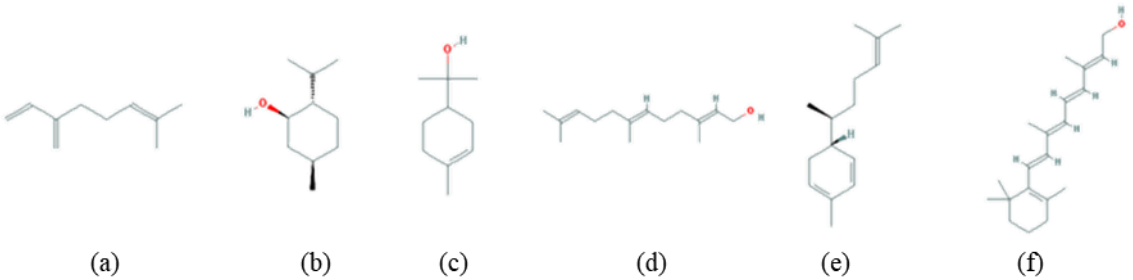

FIGURE 4 – CHEMICAL STRUCTURE OF (a) MYRCENE, (b) MENTHOL, (c) α-TERPINEOL, (d) FARNESOL, (e) ZINGIBERENE AND (f) RETINOL
SOURCE: Retrieved from: <https://pubchem.ncbi.nlm.nih.gov/image>. Accessed: 10 August 2018.

Geraniol (trans-3,7-dimethylocta-2,6-dien-1-ol) (FIGURE 5) is an acyclic monoterpene alcohol extracted mainly from plants from the genus Cymbopogon, such as Palmarosa (*Cymbopogon martini*) and Java citronella (*Cymbopogon winterianus*). It is the primary component of palmarosa oil, citronella oil, rose oil and other essential oils (WAN et al., 2017).

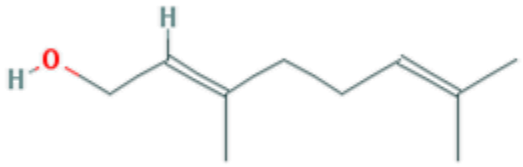

FIGURE 5 – CHEMICAL STRUCTURE OF GERANIOL
SOURCE: Retrieved from: < https://pubchem.ncbi.nlm.nih.gov/image/imagefly.cgi?cid=637566>. Accessed: 03 August 2018.

The molecular formula of this monoterpenoid is $C_{10}H_{18}O$ and its physical-chemical properties are shown in the table below (TABLE 1):

TABLE 1 – PHYSICAL-CHEMICAL PROPERTIES OF GERANIOL

Property	Value	Reference
Molecular Weight	154.25 $g.moL^{-1}$	SIGMA-ALDRICH (2014)
Boiling Point	230 °C	LARRAÑAGA et al. (2016)
Melting Point	-15 °C	HAYNES (2014)
Critical Temperature	421 °C	CHEMEO (2018)
Critical Pressure	2545.61 kPa	CHEMEO (2018)
Flash Point	108 °C (closed cup)	SIGMA-ALDRICH (2014)
Vapor Pressure	~0.2 mmHg at 20 °C	SIGMA-ALDRICH (2014)
Relative Density	0.879 g/cm^3 at 20 °C	SIGMA-ALDRICH (2014)
Water Solubility	0.1 g/L at 25 °C	SIGMA-ALDRICH (2014)
Partition coefficient	log P: 2.5 at 25 °C	SIGMA-ALDRICH (2014)
Color	Colorless to pale yellow liquid oil	LEWIS (2012)
Odor	Sweet rose odor	O'NEIL (2013)

Due to its pleasant rose-like aroma, geraniol is frequently used in the flavor and fragrance, cosmetic and pharmaceutical industry (LI et al., 2012).

According to Chen and Viljoen (2010), a survey of consumer products in Europe revealed that geraniol is present in 76% of investigated deodorants on the European market and is a component of 41% of domestic and household products and 33% of cosmetic formulation based on natural ingredients.

Geraniol shows various biochemical and pharmacological properties, an effective plant-based insect repellent and a potential antimicrobial agent (BARD et al., 1988;

BARNARD and XUE, 2004; CHEN and VILJOEN, 2010). It also exerts *in vitro* and *in vivo* antitumor activity against hepatoma and melanoma cells and murine leukemia (BURKE et al., 1997; YU, ANDERSON e ELSON, 1995; YU, HILDEBRANDT and ELSON, 1995).

This monoterpenoid is known to be derived from geranyl diphosphate (GPP) by associated synthases based on a common ionization-dependent reaction mechanism (BOHLMANN, MEYER-GAUEN and CROTEAU, 1998; TEMPLETON, 1969). Essentially, the geraniol biosynthesis is via the mevalonate route, however, in some plants geraniol can be synthesized via the non-mevalonate route (LUAN and WÜST, 2002).

Geraniol is constructed by the Isoprene Rule, following, therefore, the "head to tail" fashion (TEMPLETON, 1969). As can be seen in FIGURE 6, geraniol derives from geranyl pyrophosphate, which is synthesized from the binding of dimethylallyl pyrophosphate and isopentenyl pyrophosphate. The linkage occurs between C1 of isopentenyl pyrophosphate (head position) and C4 of dimethylallyl pyrophosphate (tail position), evidencing the Isoprene Rule.

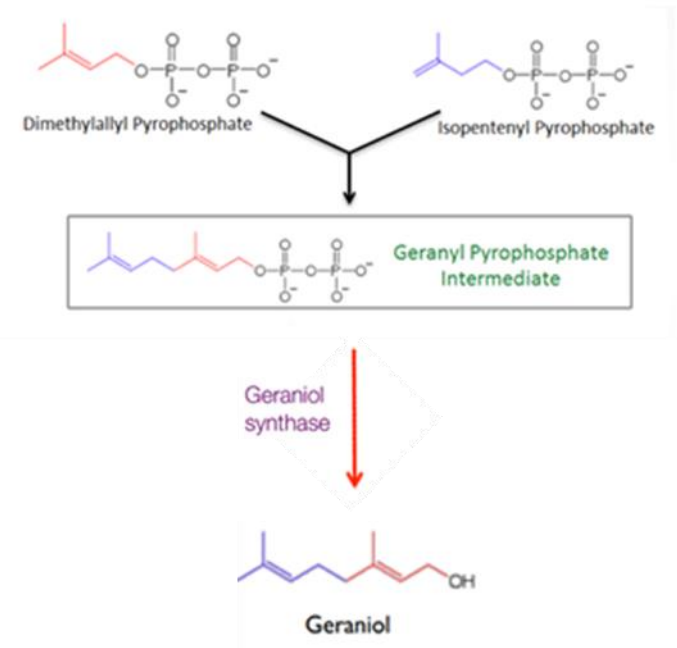

FIGURE 6 – BIOSYNTHESIS OF GERANIOL
SOURCE: Adapted from: <http://2014.igem.org/Team:UGA-Georgia/Geraniol>. Accessed: 10 August 2018.

2.2 GERANYL ESTERS

Esters of terpene alcohols are considered to be natural flavors due to their organoleptic properties and vast applications in edible flavors, in foods, fragrances, cosmetics, pharmaceuticals and home care products (BADGUJAR and BHANAGE, 2014).

Short-chain monoterpene esters contribute to the pleasant smell of a variety of essential oils (BOURKAIB et al., 2018). These esters are vastly used in cosmetic, pharmaceutics, perfumes and food industry as flavoring agents and fragrance compounds and due to their biological activities, such as antioxidant, antibacterial and anti-inflammatory activities (BEZBRADICA et al., 2007; CHATTERJEE and BHATTACHARYYA, 1998; CLAON and AKOH, 1993; ELGNDI et al., 2017; HODAJ-ÇELIKU et al., 2017; "Report Overview in : BCC Research Chemical Report, Global Markets for Flavors and Fragrances, Report ID : CHM034E", 2018; TRUSEK-HOLOWNIA and NOWORYTA, 2007; VARMA and MADRAS, 2010; ZANETTI et al., 2017).

Terpene esters, like geranyl esters, are essentially obtained by chemical synthesis or extraction from natural sources (KARRA-CHAABOUNI et al., 1996). Another way to obtain these esters is by direct esterification or by transesterification reactions using biocatalysts in organic solvents (CHATTERJEE and BHATTACHARYYA, 1998), in supercritical fluids or solvent-free systems.

In industrial scale, aromatic terpene esters are presently synthesized in a nonspecific chemical process resulting in low yields and low-quality products (CLAON and AKOH, 1994; KARRA-CHAABOUNI et al., 1996). In addition, the chemical process requires high costs for additional separation and purification steps (IKEDA and KUROKAWA, 2001).

Although chemical synthesis may be cheaper, the enzymatic synthesis of terpene esters can be labeled natural and yields higher quality products (POSORSKE, 1984; RIZZI et al., 1992; SHIEH, AKOH and YEE, 1996). However, enzymatic esterification is usually challenged by high substrate concentration because of the inhibitory effects of acid, alcohol or both reactants (DAMNJANOVIĆ et al., 2012).

2.3 ESTERIFICATION REACTIONS

The esterification reaction is a reversible process in which free-fatty acids are converted into esters through acid (Fisher esterification) or enzymatic catalysis, producing water as a side product (FIGURE 7) (SANTOS, 2018).

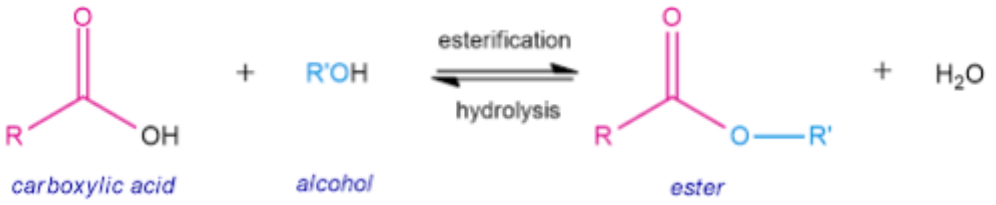

FIGURE 7 – ESTERIFICATION REACTION
SOURCE: Retrieved from: <https://www.chemistryscore.com/esterification/>. Accessed: 25 June 2019

Enzymatic reactions yield natural products, exhibit higher selectivity, mild conditions of operation and facilitate the downstream process, reducing its costs (DAMNJANOVIĆ et al., 2012; GUPTA et al., 2013; KNEŽEVIĆ-JUGOVIĆ et al., 2008).

The synthesis of geranyl acetate by enzymatic esterification of geraniol will be expanded in section 2.6.

2.4 LIPASES

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of acylglycerides to fatty acids, glycerol, monoacylglycerols, diacylglycerols, over an oil-water interface (GANDHI, 1997; GEOFFRY and ACHUR, 2018; TREICHEL et al., 2010). They can recognize a vast amount of substrates and are able to catalyze different reactions (GUPTA et al., 2013). Although their relative substrate promiscuity, lipases are substrate-specific and commonly possess elevated stereo-, enantio-, and regioselectivity (GEOFFRY and ACHUR, 2018; HASAN, SHAH and HAMEED, 2006; JAVED et al., 2018; TREICHEL et al., 2010).

Most lipases hold optimal activity and stability in the range of pH of 6.0 and 8.0 and in temperature varying from 30 °C to 40 °C, although these values may vary fairly depending on their source (PASCOAL et al., 2018). Lipases' active site is commonly portrayed as a triad of serine, histidine and an acid residue, which can be aspartate or glutamate (PASCOAL et al., 2018). Their catalytic mechanism involves four steps: (1) serine deprotonation mediated by histidine and aspartate; (2) attack of the serine hydroxyl group to the carbonyl carbon from the substrate, resulting in an intermediate; (3) transfer of the acyl group to the lipase and discharge by the water nucleophilic attack; (4) regeneration of the catalytic site (PASCOAL et al., 2018).

As lipases bear great catalytic activity and stability in organic solvents, as has been settled by Zaks and Klibanov (1985), they are able to catalyze a large number of

heterogeneous reactions both in water-soluble media and organic media (HASAN, SHAH and HAMEED, 2006; PASCOAL et al., 2018; TAN et al., 2010). Besides their hydrolytic activity, lipases can also perform synthesis, as esterification and transesterification, when in a low-water content medium.

Therefore, lipases are the enzymes most frequently used for the production of geranyl esters in an organic solvent, supercritical fluids, and free-solvent systems in reactions of esterification and transesterification (PERES, GOMES DA SILVA and BARREIROS, 2003). The most common lipase utilized is lipase B from *Candida antarctica* as it is a versatile and robust enzyme (DAMNJANOVIĆ et al., 2012; PERES, GOMES DA SILVA and BARREIROS, 2003).

Lipases in the free form are regularly unstable and demonstrate low activity in organic solvents or harsh environments, like high temperatures and extreme pH (GUPTA et al., 2013). In homogenous media, lipases possess a small lid or flap partly sheltering the active site, which is dislocated when a small amount of lipophilic reactant is present in the reaction (GUPTA et al., 2013). Only then the active center becomes available, as the hydrophobic residues surrounding the active site interact with the hydrophobic internal face of the enzyme, hence the lipase sticks to hydrophobic surfaces unmasking the active site, a mechanism called interfacial activation of lipase (GUPTA et al., 2013).

Enzyme use in its free form is economically inviable in industrial operations as it lacks reusability, it is difficult to recover from the reaction and it shows low stability (DAMNJANOVIĆ et al., 2012). These problems can be overcome by using the enzyme immobilized on a support (DAMNJANOVIĆ et al., 2012).

2.5 SUPERCRITICAL FLUIDS

A supercritical state is reached when the temperature and the pressure, in which a fluid is exposed, are above their critical values (T_c and p_c, respectively). FIGURE 8 depicts a schematic pressure-temperature diagram for a pure component (BRUNNER, 2005), where the critical region is highlighted.

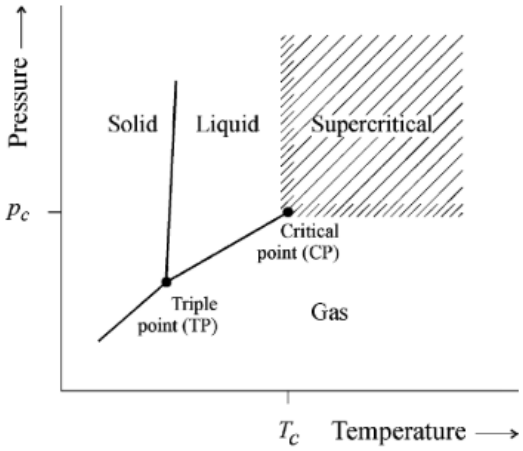

FIGURE 8 – PHASE DIAGRAM FOR A PURE COMPONENT
SOURCE: BRUNNER (1994)

Supercritical fluids (SCFs) are vastly utilized as carriers for mass transfer, reaction media and solvents in many applications, as they possess liquid-like density and solvating properties and gas-like transport properties, which can be easily tuned by changing the process conditions, such as temperature and pressure (BRUNNER, 2005; KNEZ et al., 2014; SCHÜTZ, 2007; VEIGA et al., 2017).

These fluids, especially CO_2, affect the properties of components with which they are mixed, as they are capable of reducing the viscosity of condensed phases and the surface tension of liquids, depending on the quantity of dissolved supercritical fluid, making the manipulation of the mixture easier and enhancing mass and heat transfers (BRUNNER, 2010).

Among supercritical fluids, supercritical CO_2 is majorly used, due to its desirable characteristics: non-toxic, non-flammable, inert, abundant, low-cost, relative ease to achieve critical points (T_c = 31.04 °C and p_c = 7.38 MPa) and tunable physicochemical and thermodynamic properties (BOURKAIB et al., 2018; DAZA SERNA, ORREGO ALZATE and CARDONA ALZATE, 2016; VEIGA et al., 2017).

Because of their properties, the processes in which SFCs are employed are sustainable, environmentally friendly and cost-efficient (KNEZ et al., 2014). One of the advantages of using SCFs as solvents in reactions is the ease in product recovery, as the SCF is released simply by depressurizing the system, diminishing downstream costs. The gas can be recovered, recycled and reused without many purification steps (KNEZ et al., 2014).

2.6 SYNTHESIS OF GERANYL ESTERS

In the following topics, the literature review about the synthesis of geranyl esters in different reaction media (free-solvent system, organic solvents and supercritical fluids as solvents) are presented.

2.6.1 Synthesis of geranyl esters in free-solvent system

The biosynthesis of terpenes esters extensively occurs in a system involving an organic solvent (CHATTERJEE and BHATTACHARYYA, 1998). As these esters are mainly applied as flavoring agents in the food industry, it is appealing to get rid of organic solvents even during ester synthesis (CHATTERJEE and BHATTACHARYYA, 1998).

Their elimination, aside from the "green label" attributed to the product, reduces environmental hazard, facilitates and cheapens the downstream process, which reduces production cost (BEZBRADICA et al., 2007; CHATTERJEE and BHATTACHARYYA, 1998; KARRA-CHAABOUNI et al., 1996).

When reagents alone provide enough homogeneity for the reaction mixture, regarding the interfacial area to sustain the efficiency of the enzyme, solvent-free synthesis is a suitable option for the esterification of geraniol and organic acids (XAVIER MALCATA et al., 1990).

Temperature is a very important parameter in solvent-free reactions as it may enhance miscibility and improve the diffusion process of reactants, diminishing mass transfer limitations, benefiting interactions between the lipase and substrates (PAROUL et al., 2010).

Thus, Chatterjee and Bhattacharyya (1998) investigated the synthesis of terpene esters in a solvent-free system using immobilized lipase from *Rhizomucor miehei* (Lipozyme® IM) as a catalyst. The reaction was conducted in 50 mL Erlenmeyer flasks with equimolar quantities of terpene alcohols and fatty acids, 10% (wt./wt.) of the weight of the alcohol of Lipozyme® IM, at a temperature from 55 to 60 °C under vacuum of 10 mmHg. After 360 min of esterification, the optimum molar conversion of 96 to 99% was achieved.

Karra-Chaabouni et al. (1996) also studied the synthesis of geranyl esters in a solvent-free system. The esterification occurred in capped tubes with equimolar amounts of geraniol and propionic or valeric acid, 10% (wt./wt.) of the alcohol of crude lipase (esterase 30 000) as biocatalyst, at 37 °C and 250 RPM, achieving a conversion yield of 85% after 96 h of reaction.

Paroul et al. (2010) obtained a conversion yield of 98% in 30 min of reaction between geraniol and propionic acid (3:1, alcohol to acid) using 10% (wt./wt.) of Novozym® 435 as biocatalyst, at a temperature of 50 °C and 150 RPM.

Ferraz et al. (2015), using a non-commercial immobilized lipase from *Penicillium crustosum*, reacted geraniol and propionic acid (3:1, alcohol to acid) for 360 min at 150 RPM in 50 mL Erlenmeyer flasks. The optimal conditions, which led to a conversion of 53.28%, were temperature of 60 °C and enzyme concentration of 50% (wt./wt.).

2.6.2 Synthesis of geranyl esters in organic solvents

Many substrates of esterification reactions have low miscibility in water, therefore, the addition of organic solvents sometimes is necessary (PAROUL et al., 2010).

Molinari, Villa and Aragozzini (1998) investigated the esterification between equimolar quantities of geraniol and acetic acid in n-heptane with whole microorganisms. The reaction was conducted in 10 mL screw-capped tubes. When using *Rhizopus delemar* as biocatalyst at a concentration of 30 $g.L^{-1}$ and at 55 °C, molar conversion of 54% was achieved after 24 h of reaction (MOLINARI, VILLA and ARAGOZZINI, 1998).

Bezbradica et al. (2007) tested the esterification of geraniol with n-butanoic acid using porcine pancreatic lipase and Lipozyme® in isooctane and in a solvent-free system at 45 °C and 150 RPM in stoppered flasks. In the solvent-free system, the final molar conversion was achieved faster than in isooctane (ca. 15 h after the start of reaction for the solvent-free system and around 40 h for the reaction in isooctane). Yet, molar conversion without the organic solvent did not overpass 20%, while in isooctane the conversion yield was 90% after 70-hour reaction using the porcine pancreatic lipase (BEZBRADICA et al., 2007).

These authors attribute minor final conversion yield in the solvent-free system to the damage in the water layer of the enzyme caused by a high concentration of polar substrates when no solvent was used, as the main reason to use organic solvents is to maintain the vicinal water layer, which confers stability to the enzyme (BEZBRADICA et al., 2007).

Damnjanović et al. (2012) also studied the esterification reaction between geraniol and butyric acid in isooctane using, however, immobilized *Candida rugosa* lipase (CRL) in batch system and in fluid bed reactor (FBR). The flask with the reagents and the biocatalyst was incubated at 150 RPM for 48 h, the mean residence time was 3.1 min in the FBR (DAMNJANOVIĆ et al., 2012).

In the batch system, a conversion yield of more than 99.9% was reached after 48 h when temperature ranged from 25 to 30 °C, water concentration was 3.6% (v/v) and mole ratio of alcohol to acid was 0.4 (DAMNJANOVIĆ et al., 2012). This led to low volumetric productivity of 5.2 mmol.$(L.h)^{-1}$, while performing the esterification gave volumetric productivity of 26.7 mmol.$(L.h)^{-1}$ as a molar conversion of 53.3% was reached after 120 min of reaction at a flow rate of 10.0 $mL.min^{-1}$ at 35 °C with the mole ratio of alcohol to acid of 0.5 (DAMNJANOVIĆ et al., 2012).

Knežević-Jugović et al. (2008) studied the synthesis of geranyl butyrate with immobilized *Candida rugosa* lipase (CRL) in isooctane in screw-capped flasks with an agitation speed of 150 RPM. A conversion yield of 94.3% was reached after 48 h of reaction conducted at 25 °C with enzyme concentration of 3 $g.mL^{-1}$ and mole ratio of alcohol to acid of 2:3 (KNEŽEVIĆ-JUGOVIĆ et al., 2008).

Claon and Akoh (1993) reacted equimolar amounts of geraniol and low-mass fatty esters using immobilized lipase from *Candida rugosa* (SP) and *Mucor miehei* (IM) as biocatalyst (10% (wt./wt.)) and n-hexane solvent in screw-capped tubes. The esterification was conducted at 30 °C at 200 RPM for 24 h (CLAON and AKOH, 1993). As result, the conversion yield of geranyl acetate using immobilized enzyme from *C. rugosa* was around 90%, of geranyl butyroate was ca. 98%, of geranyl caproate was near 95% and of geranyl capryloate was approximately 88% (CLAON and AKOH, 1993). When lipase from *M. miehei* was used the conversion yield of geranyl acetate, geranyl butyroate, geranyl caproate and geranyl capryloate was around 30%, 96%, 86% and 90%, respectively.

Claon and Akoh (1993) have stated that lipase from *M. miehei* had had more affinity for fatty esters with longer chain fatty esters (butyric, caprylic and caproic acid), with results similar to those obtained with *C. rugosa* lipase. These authors also concluded that the best order to add the reactants was the following: solvent, geraniol, fatty acid and for last the enzyme (CLAON and AKOH, 1993). One hypothesis that may explain this order of addition is that the inhibitory effect of terpene alcohols and/or acetic acid on the lipase during the reaction may be minimized when that order is used (CLAON and AKOH, 1993).

Chulalaksananukul, Condoret and Combes (1992) studied the esterification of geraniol and several short-chain length fatty esters in n-hexane. They have concluded that as the chain length was increased up to a 5-carbon molecule (propyl acetate), the molar conversion increased (CHULALAKSANANUKUL, CONDORET and COMBES, 1992). Above that chain size, the yield of reaction was not improved due to steric hindrance effects

in the active size of the lipase (CHULALAKSANANUKUL, CONDORET and COMBES, 1992).

Therefore, the synthesis of geranyl acetate was conducted with 30 mM of geraniol and 600 mM of propyl acetate with 50 mg of immobilized enzyme from *Mucor miehei* with n-hexane in glass tubes at 40 °C and stirred magnetically stirred (CHULALAKSANANUKUL, CONDORET and COMBES, 1992). After the 3-day reaction, these authors have obtained the conversion yield of 85% (CHULALAKSANANUKUL, CONDORET and COMBES, 1992).

These authors have inferred that when agitation speed was above 200 RPM, mass transfer limitations were not significant as the initial velocity in the tubes agitated above 200 RPM was very similar (CHULALAKSANANUKUL, CONDORET and COMBES, 1992).

Shinde and Yadav (2014) investigated the synthesis of geranyl cinnamate in n-heptane under microwave irradiation. They reacted 0.5 mM of ethyl cinnamate with 1.0 mM of geraniol, therefore a mole ratio of 2:1 (alcohol to acid), in a 50 mL glass reactor with Novozym® 435 as biocatalyst with constant microwave radiation, conducted at 60 °C at 300 RPM for 15 min with a conversion yield of 83% (SHINDE and YADAV, 2014).

The authors above have stated that microwave irradiation improves the rate of reaction as microwave energy heats molecules much faster enhancing collision between reactants, due to its ability to instantaneously heat substances above the normal bulk temperature (SHINDE and YADAV, 2014). Besides that, microwave irradiation changes enzyme conformation, which may have led to an easy approach of the substrates to the catalytic site of the enzyme (SHINDE and YADAV, 2014). A 4.2-fold increase in the initial rate of reaction was achieved under microwave irradiation when compared to a conventional heating reaction (SHINDE and YADAV, 2014).

Gupta et al. (2013) examined geranyl acetate synthesis via transesterification in screw-capped glass tubes wit geraniol and vinyl acetate using immobilized lipase from *Thermomyces lanuginosus* and hexane as solvent. The transesterification was mainly conducted at 35° at 200 RPM for 24 h (GUPTA et al., 2013). When equimolar quantities of reagents were used, a conversion yield of 90% was achieved (GUPTA et al., 2013). Yet, when the mole ratio was 1:5 (alcohol to ester), the conversion yield was 95% (GUPTA et al., 2013). The authors have pointed out that increasing the vinyl acetate concentration, the conversion yield improves, however, caution must be taken not to overcrowd the enzyme if too much vinyl acetate is added to the medium (GUPTA et al., 2013).

Badgujar and Bhanage (2014) scrutinized the transesterification of geraniol and vinyl acetate too. These authors reacted the substrates with a mole ratio of 1:4 (alcohol to ester)

with immobilized lipase from *Pseudomonas cepacia* at 55 °C at 140 RPM in a 10 mL glass vessel using n-hexane as solvent achieving, after 360 min of reaction, a molar conversion of 99% (BADGUJAR and BHANAGE, 2014).

Shieh, Akoh and Yee (1996) investigated the esterification of geraniol and tributyrin in n-hexane at 200 RPM with Lipase AY as biocatalyst. When the reaction was conducted with a mole ratio of 2:1 (alcohol to tributyrin) at 45 °C with 30% and 10% (wt./wt.) in terms of geraniol of lipase and of water, respectively, the conversion yield was 85.56% (SHIEH, AKOH and YEE, 1996).

At last, Bartling et al. (2001) studied the geranyl acetate synthesis in n-hexane using geraniol and acetic acid as substrates and Novozym® 435 as biocatalyst. A 94.0% conversion yield was reached when the temperature of the reaction was set to 30 °C and almost 100% was achieved when water was removed from the reaction with membrane pervaporation (BARTLING et al., 2001).

2.6.3 Synthesis of geranyl esters in supercritical fluid

Supercritical fluids exhibit some advantages over organic solvents: can be easily eliminated without leaving residues (CHULALAKSANANUKUL, CONDORET and COMBES, 1993; COUTO et al., 2011); are readily recyclable under pressurized forms, being able to recycle it after adequate re-pressurization and heating (BOURKAIB et al., 2018); deliver “green labeled” products as there is no trace of organic solvents (BOURKAIB et al., 2018); their properties, like viscosity, density, diffusivity, dielectric constant can be efficiently tuned by adjusting pressure and/or temperature conditions, promoting mass transfer (BOURKAIB et al., 2018; COUTO et al., 2011); easy recovery of product and enzyme just by depressurization (VARMA and MADRAS, 2010), diminishing downstream complexity and costs. Therefore, they are a feasible environmentally friendlier option over organic solvents.

Besides those characteristics, supercritical carbon dioxide is not flammable, non-toxic and cheap and its critical point is rather effortlessly reached and appropriate with mild process conditions in which enzymes are employed as its critical pressure is 7.38 MPa and its critical temperature is 31.04 °C (BOURKAIB et al., 2018).

Bourkaib et al. (2018) when synthesizing geranyl acetate using Lipozyme® 435 in supercritical CO_2 obtained steady-state conditions after 15 min in a 1.0 mL/6.5 cm column of a PBR and after 20 min in a 5 mL/32.5 cm column of a PBR, in which the conditions of operation were 55 °C and 20.0 MPa. This behavior can be explained as the column is

elongated, more time is needed to reactants impregnate at the packed bed, therefore, the steady-state condition takes longer to be established (BOURKAIB et al., 2018).

As the longer column was 5 times longer, 5 times more enzyme was used, however, the conversion rate achieved in the short column was 45% and 73% for the longer one (BOURKAIB et al., 2018). This indicates that no linear proportionality between conversion and enzyme amount occurred. Full conversion could not be obtained as water accumulation may have occurred in the PBR (BOURKAIB et al., 2018).

Bourkaib et al. (2018) observed that as temperature increases, the conversion also rose. They concluded too that at a certain temperature, pressure had a negative impact on conversion rates (BOURKAIB et al., 2018). This can be explained due to loss of enzyme stability at high pressure and increase of fluid viscosity, reducing mass transfer, as pressure is incremented at constant temperature. The best molar conversion of 73.8% was obtained at 65 °C, 15.0 MPa in 30 min of reaction (BOURKAIB et al., 2018).

These authors have stated that a temperature above 65 °C or pressure below 1.5 MPa, supercritical CO_2 might not be sufficiently dense to solubilize all the reactants (BOURKAIB et al., 2018).

The enzyme showed high stability with an average conversion of 58% (55 °C, 15.0 MPa) and had a decay of only 5% after 22 h of reaction (BOURKAIB et al., 2018), indicating that this temperature was high enough to slowly interfere in enzyme activity.

When two parallel 1 mL PBRs were operating, the conversion rate was close to 73%, a result similar to the obtained using one long PBR (BOURKAIB et al., 2018). However, when the parallel PBRs are utilized, less enzyme is used (1500 mg of enzyme in the long PBR and 600 mg in the parallel one) (BOURKAIB et al., 2018). On the other hand, the conversion was not doubled when compared to just one PBR, which could be explained by the slower impregnation of reactants as the flow rate was divided by two.

Bourkaib et al. (2018) have also observed that the higher the residence time, the higher the molar conversion. At 36.5 s of residence time, at 20.0 MPa and 55 °C, using one PBR or parallel PBRs, the conversion rate was 83% (BOURKAIB et al., 2018). This could be explained by the fact that when residence time is longer, there is more contact between substrates and the enzyme, enhancing ester production.

The authors achieved at 65 °C and 15.0 MPa a molar conversion of 98% when using only one column of PBR (BOURKAIB et al., 2018).

Couto et al. (2011), when reacting with Novozym® 435 an equimolar mixture of geraniol and acetic acid in supercritical fluid in a BSTR for 32 h under pressure of 10.0 MPa

and at 35 °C reached a molar conversion of 73% using $scCO_2$ and 98% when scEthane was used.

When conducting the same reaction in the same conditions, except for the time of reaction, but in a PBR, the maximum conversion was achieved around 96% and ca. 86% in scEthane and in $scCO_2$, respectively with a flow rate of 0.25 $mL.min^{-1}$ (COUTO et al., 2011).

This behavior could be explained as the difference in water solubility in these two solvents. Solvents in which water is less soluble favor the esterification (VALIVETY et al., 1991). As ethane is less polar and can dissolve approximately five times less water than CO_2 (COUTO et al., 2011), the reaction was favored towards the synthesis of geranyl acetate.

Peres, Gomes da Silva and Barreiros (2003) obtained a molar conversion of 98% in scEthane and 73% in $scCO_2$ after 10 h of reaction of an equimolar mixture of geraniol with acetic acid using Novozym® as catalyst in a 14.0 mL vessel at 14.0 MPa and 40 °C.

These authors have proposed that $scCO_2$ could be less supportive for enzyme functions than alkanes due to its acid-basic effects (CARVALHO, SAMPAIO and BARREIROS, 2000; HARPER and BARREIROS, 2002; KAMAT, BECKMAN and RUSSELL, 1995; MARTY et al., 1992) and possible formation of carbamates, which could diminish enzyme stability and activity.

Varma and Madras (2010), using 7.0 mL batch reactors, reacted equimolar quantities of geraniol and butyric esters with Novozym® 435 in supercritical dioxide carbon for 30 min at 21.3 MPa and 50 °C achieving a rate of conversion of 47%.

Chulalaksananukul, Condoret and Combes (1993), on the other hand, only reached a 30% yield after 72 h of reaction between geraniol and propyl acetate, using immobilized lipase from *Mucor miehei*, in supercritical CO2 at 14.0 MPa and 40 °C in a 7.5 mL vessel.

These authors have suggested two hypotheses to explain the low yield: a side reaction, which had led to acetic acid formation; or an intrinsic effect of the solvent on the equilibrium state (CHULALAKSANANUKUL, CONDORET and COMBES, 1993). As acetic acid was not found in the reaction, they have discarded the first hypothesis. Therefore, the authors have concluded that solvent interfered in the reaction, possibly inhibiting the enzyme (CHULALAKSANANUKUL, CONDORET and COMBES, 1993).

As it can be noticed from the literature about the synthesis of geranyl acetate in supercritical fluid, there is a lack of information about the phase behavior of the reaction system. The phase behavior is crucial to understand the experimental results as the reaction rate and conversion are severely affected by the number of phases in the system and their

physicochemical properties. Therefore, there is a need for this kind of information to comprehend better these esterification reactions.

2.7 COMPARISON OF CONVERSION YIELD IN DIFFERENT SOLVENTS

As can be seen in TABLE 2, different conversion yields were obtained using organic solvents, supercritical fluids or just the reactants (solvent-free system). The enzyme utilized and the process conditions interfere with the results obtained, which may explain the variety of conversion yields.

TABLE 2 – BIOSYNTHESIS OF GERANYL ESTERS OVERVIEW (CONTINUE)

Ester	Acyl Donor	Molar Ratio*	Biocatalyst**	Solvent	Temperature	Pressure	Time	Agitation	Reactor	Conversion	Reference
Geranyl cinnamate	Cinnamic acid	2:1	Novozym 435® (CALB)	n-Hexane	65 °C	-	0.4 h.	300 RPM	BSTR	88 ± 2%	SHINDE and YADAV (2014)
Geranyl butyrate	Butyric acid	1.5:1	*C. rugosa* Lipase[1]	n-Heptane	40 °C	-	3.5-4 h	200 RPM	BSTR	91%	WANG et al. (2018)
Geranyl butyrate	Butyric acid	1.5:1	*C. rugosa* Lipase[2]	n-Heptane	40 °C	-	4.7 h	-	CFBR	77%	WANG et al. (2018)
Geranyl butyrate	Butyric acid	1:2.5	*C. rugosa* Lipase[3]	Isooctane	28 °C	-	48 h	150 RPM	BSTR	≥ 99.9%	DAMNJANOVIĆ et al. (2012)
Geranyl butyrate	Butyric acid	1:2	*C. rugosa* Lipase[3]	Isooctane	35 °C	-	10 h	-	FBR	78.9%	DAMNJANOVIĆ et al. (2012)
Geranyl butyrate	Butyl butyrate	1:1	*C. antarctica*[4]	$scCO_2$	50 °C	21.3 MPa	26 h	-	BSTR	47%	VARMA and MADRAS (2010)
Geranyl acetate	Acetic acid	1:1	Novozym 435® (CALB)	$scCO_2$	40 °C	10.0 MPa	8 h	-	PBR	86%	COUTO et al. (2011)
Geranyl acetate	Acetic acid	1:1	Novozym 435® (CALB)	scEthane	35 °C	10.0 MPa	6 h	-	PBR	96%	COUTO et al. (2011)
Geranyl acetate	Vinyl acetate	1:5	*T. lanuginosus* Lipase[5]	n-Hexane	45 °C	-	24 h	200 RPM	BSTR	95%	GUPTA et al. (2013)
Geranyl acetate	Acetic acid	1:1	*C. antarctica* Lipase[6]	n-Hexane	30 °C	-	24 h	200 RPM	BSTR	90%	CLAON and AKOH (1993)
Geranyl butyrate	Butyric acid	1:1	*C. antarctica* Lipase[6]	n-Hexane	30 °C	-	24 h	200 RPM	BSTR	98%	CLAON and AKOH (1993)
Geranyl caproate	Caproic acid	1:1	*C. antarctica* Lipase[6]	n-Hexane	30 °C	-	24 h	200 RPM	BSTR	92%	CLAON and AKOH (1993)
Geranyl caprylate	Caprylic acid	1:1	*C. antarctica* Lipase[6]	n-Hexane	30 °C	-	24 h	200 RPM	BSTR	87%	CLAON and AKOH (1993)
Geranyl acetate	Propyl acetate	1:3	Lipozyme®	$scCO_2$	40 °C	14.0 MPa	72 h	-	BSTR	30%	CHULALAKSANANUKUL, CONDORET and COMBES (1993)
Geranyl acetate	Propyl acetate	1:3	Lipozyme®	n-Hexane	40 °C	-	72 h	200 RPM	BSTR	85%	CHULALAKSANANUKUL, CONDORET and COMBES (1992)
Geranyl acetate	Vinyl acetate	1:4	*P. cepacia* Lipase[7]	n-Hexane	55 °C	-	3 h	140 RPM	BSTR	99,7%	BADGUJAR and BHANAGE (2014)
Geranyl acetate	Acetic acid	1:1	Novozym 435® (CALB)	$scCO_2$	40 °C	10.0 MPa	10 h	-	BSTR	73%	PERES, GOMES DA SILVA and BARREIROS (2003)
Geranyl acetate	Acetic acid	1:1	Novozym 435® (CALB)	scEthane	40 °C	10.0 MPa	10 h	-	BSTR	98%	PERES, GOMES DA SILVA and BARREIROS (2003)
Geranyl acetate	Acetic acid	1:1	*R. delemar*[8]	n-Heptane	55 °C	-	240 h	-	BSTR	54%	MOLINARI, VILLA and ARAGOZZINI (1998)
Geranyl acetate	Acetic acid	1:1	Novozym 435® (CALB)	n-Hexane	30 °C	-	12 h	-	BSTR	> 99%	BARTLING et al. (2001)
Geranyl butyrate	Butyric acid	1:1	Esterase 30000 from *M. miehei*	Solvent-free	37 °C	-	150 h	-	BSTR	85%	KARRA-CHAABOUNI et al. (1996)
Geranyl propionate	Propionic acid	5:1	Lipase extract from *P.crustosum*[9]	Solvent-free	60 °C	-	6 h	150 RPM	BSTR	53%	FERRAZ et al. (2015)

TABLE 2 – BIOSYNTHESIS OF GERANYL ESTERS OVERVIEW (CONCLUSION)

Geranyl butyrate	Tributyrin	1:0.5	Lipase AY from *C. rugose*	n-Hexane	45 °C	-	9 h	200 RPM	BSTR	85.6%	SHIEH, AKOH and YEE (1996)
Geranyl butyrate	Butyric acid	1:1	Porcine Pancreatic Lipase	Isooctane	45 °C	-	70 h	150 RPM	BSTR	90%	BEZBRADICA et al. (2007)
Geranyl acetate	Acetic acid	1:1	Lipozyme 435 ® (CALB)[10]	$scCO_2$	65 °C	15.0 MPa	27 s (Rt)	-	PBR	98%	BOURKAIB et al. (2018)
Geranyl butyrate	Butyric acid	3:2	Lipase from *C. rugosa*[11]	Isooctane	25 °C	-	48 h	150 RPM	BSTR	94.3%	KNEŽEVIĆ-JUGOVIĆ et al. (2008)
Geranyl caproate	Caproic acid	1:1	Lipozyme IM[12]	Solvent-free	58 °C	-	6 h	-	BSTR	95%	CHATTERJEE and BHATTACHARYYA, (1998)
Geranyl caprylate	Caprylic acid	1:10	Lipozyme IM[12]	Solvent-free	58 °C	-	6 h	-	BSTR	96%	CHATTERJEE and BHATTACHARYYA (1998)
Geranyl propionate	Propionic acid	3:1	Novozym 435® (CALB)[12]	Solvent-free	50 °C	-	0.5 h	150 RPM	BSTR	98%	PAROUL et al. (2010)

BSTR: Batch Stirred Tank Reactor; CFTR: Circulating Fluidized Bed Reactor; FBR: Fluidized Bed Reactor; PBR: Packed Bed Reactor; scCO2: Supercritical CO_2; scEthane: Supercritical Ethane; Rt: Residence time *Alcohol/Acyl donor **Immobilized on:

1 Macroporous Adsorptive Resin DA201-V
2 Macroporous Ion Exchange Resin D151
3 Sepabeads® EC-EP
4 Macroporous Acrylic Resin
5 Polyacrylonitrile Nanofibers Membrane
6 Acrylic Resin
7 Polymer film of PLA (polylactic acid), CH (chitosan) and PVA (polyvinyl acetate)
8 Whole microorganism (lyophilized)
9 Sodium alginate
10 Macroporous poly (methyl methacrylate-co-divinylbenzene)
11 Sepabeads® EC-EP
12 Macroporous Anionic Resin

2.8 PUBLICATIONS THROUGH THE YEARS

In the present decade, growth in the number of publications regarding geranyl esters synthesis is observed, as it can be seen below (FIGURE 9):

FIGURE 9 – GERANYL ESTERS SYNTHESIS PUBLICATIONS THROUGH THE YEARS

This tendency corroborates the scope of investigation of future projects, as a growing interest in the subject is detected.

2.9 INFLUENCE OF WATER ON LIPASE ACTIVITY

Water, in the medium, influences the thermodynamic balance of the chemical reaction, in which it favors the hydrolysis of the ester, inhibiting esterification (KARRA-CHAABOUNI et al., 1996). Also, water plays an important role in enzyme stability and activity, as a certain amount of water in the micro-layer surrounding the enzyme is necessary for keeping the active form of the lipase as it maintains the conformational flexibility of the enzyme (BADGUJAR and BHANAGE, 2014; BEZBRADICA et al., 2007). However, exceeding this amount of water (critical amount of water), the thickness of the water layer around the enzyme is increased, leading to an undesirable gain in enzyme flexibility and eventually denaturation (DAMNJANOVIĆ et al., 2012).

Water in the reaction can have many sources: the air space in the reactor, dissolved in reactants and the solvent, adsorbed on the equipment, from enzyme preparation, from chemical reaction and deliberate addition (BARTLING et al., 2001).

Water activity is the most adequate parameter to correlate enzymatic activity (COUTO et al., 2011; LIMA, PYLE and ASENJO, 1995). Water content influences hydration

of the enzyme, therefore affects protein conformation and optimal activity (CHULALAKSANANUKUL, CONDORET and COMBES, 1993).

The water content of the solid support is suggested to be the most important aspect for enzyme activity (CHULALAKSANANUKUL, CONDORET and COMBES, 1993) and water activity (Aw) is the most convenient parameter for correlating enzymatic activity in non-aqueous media (HALLING, 1994).

The best method to get good Aw control in organic media is the circulation of saturated salt solutions that can exchange water with the reaction mixture through a semipermeable membrane (WEHTJE et al., 1993, 1997). In supercritical fluids, the strategy to control the amount of water in the reaction is to set the initial hydration level of the enzyme prior to the start of reaction (COUTO et al., 2011).

Partial elimination of water produced has led to significant increases in esterification conversion. The addition of molecular sieves along the reaction might be a useful strategy from controlling the water content in the medium (KNEŽEVIĆ-JUGOVIĆ et al., 2008). Many researches have employed this method to remove water from the reaction. Couto et al. (2011) mixed the enzyme with molecular sieves to set the water activity (Aw), which has led to some improvement. MOLINARI, VILLA and ARAGOZZINI (1998) used molecular sieves in the esterification of geraniol with acetic acid and obtained an improvement of 3.5% on reaction yield. Damnjanović et al. (2012) used molecular sieves as well in the esterification reaction of geraniol and butyric acid achieving high molar conversions and good volume productivity (>99.9% in batch system after 48 h reaction and 26.7 mm/(L.h)). Knežević-Jugović et al. (2008) added molecular sieves too in the esterification of geranyl butyrate after 16 h of reaction and have obtained a molar conversion of 94.3%. Claon and Akoh (1993) also inserted molecular sieves in the medium too to prevent water accumulation, reaching a yield of ca. 98% for geranyl butyroate.

Other techniques can be applied to remove water from the reaction. Bartling et al. (2001) used membrane pervaporation to control water in the reaction and have obtained 100% of conversion and an increase of 150% on initial rate when compared to the reaction without water removal. Chatterjee and Bhattacharyya (1998) conducted the esterification of under vacuum of 10 mmHg to remove water synthesized during the reaction. Peres, Gomes da Silva and Barreiros (2003) used pair of salt hydrates to control water activity in the esterification and obtained a molar conversion of 98% in the esterification of geraniol and acetic acid in scEthane. Trusek-Holownia and Noworyta (2007) used a membrane to separate the enzymatic

reactor from a column filled with molecular sieves. Wehtje et al. (1997) used a mixture of immobilized enzyme and molecular sieves in a Pecked Bed Reactor (PBR).

Bourkaib et al. (2018) mixed the enzyme with a well-dried desiccant and attained a mole conversion of geraniol and acetic acid to geranyl acetate of 97%. However, Bourkaib et al. (2018) reminded that the use of a desiccant may not be a good approach due to its requirement to change it once it is saturated with water. Hence, they suggested that when a PBR is used, an optimal length of the column should be determined. It may be long enough to the synthesis occur, but not too long so there is water accumulation which could diminish the esterification reaction. Couto et al. (2011) imply that water from the reaction mixture can be removed by the CO_2 stream. Therefore, humidity must be low enough so it can remove water from the system.

2.10 EQUATIONS OF STATE

Since the van der Waals equation of state development in 1873 (VAN DER WALLS, 1873), many modifications in equation of state were made to better represent and predict phase equilibrium and thermodynamic properties of components of industrial interest. A landmark in these modifications is the equation of state proposed by Redlich and Kwong (1949) which have been later modified by Peng and Robinson (1976). The first proposal of the Peng-Robinson equation of state (PENG and ROBINSON, 1976) is shown in Eq (1):

$$P = \frac{RT}{v-b} - \frac{a(T)}{v(v-b)+b(b-v)} \tag{1}$$

where, P is the absolute pressure of the system, T is the absolute temperature and v the molar volume. To calculate the coefficients a and b, the van der Waals quadratic mixing rules can be applied:

$$a = \sum_{i=1}^{n}\sum_{j=1}^{n} x_i x_j a_{ij} \tag{2}$$

$$b = \sum_{i=1}^{n}\sum_{j=1}^{n} x_i x_j b_{ij} \tag{3}$$

The parameters a_{ij} and b_{ij} are related to the attraction energy and repulsive energy, respectively, between components i and j of the mixture (GIACOMIN-JUNIOR, 2020). Different combination rules have been proposed. However, the first approach introduced is still vastly used mainly due to its simplicity:

$$a_{ij} = \sqrt{a_i \cdot a_j} \cdot (1 - k_{ij}) \tag{4}$$

$$b_{ij} = 0.5 \cdot (b_i + b_j)(1 - l_{ij}) \tag{5}$$

where, k_{ij} and l_{ij} are binary interaction parameters, usually obtained by regression of these models using data. The parameters a_i and b_i can be defined employing the critic point condition – first and second pressure derivative in relation to specific volume equal to zero, from which is obtained the following equations (PENG and ROBINSON, 1976):

$$b_i = 0.07780 \cdot \frac{RT_{c_i}}{P_{c_i}} \tag{6}$$

$$a_i(T) = a_i \cdot a_i \tag{7}$$

$$a_i = 0.45724 \cdot \frac{R^2 T_{c_i}^2}{P_{c_i}} \tag{8}$$

$$\alpha_i^{0.5} = 1 + m_i(1 - T_{r_i}^{0.5}) \tag{9}$$

$$m_i = 0.37464 + 1.54226 \cdot \omega_i - 0.26992 \cdot \omega^2 \tag{10}$$

where, ω is the acentric factor, the subscript *c* represents the critical properties, the subscript *r* the reduced properties (T/T_c) and α the alpha function. Further, Peng and Robinson (1978) extended the correlation for the parameter *m* with an acentric factor dependent-condition: for ω less than or equal to 0.491, Eq (10) is employed; if it is greater than 0.491, Eq (11) is utilized.

$$m = 0.379642 + 1.48503 \cdot \omega - 0.164423 \cdot \omega^2 + 0.016666 \cdot \omega^3 \tag{11}$$

2.11 EXPERIMENTAL METHODS FOR PHASE EQUILIBRIA AT HIGH PRESSURES

The experimental methods for phase equilibria at high pressures can be divided into two main groups: the analytical methods and the synthetic methods (DOHRN, FONSECA and PEPER, 2012). The main difference between these groups lays in how the composition of the system is determined. In the analytical methods, the system composition is determined empirically during the experiment: usually, a small aliquot of the system in equilibrium is withdrawn and its composition is determined. When the system is highly dependent on its composition, these methods are not adequate as they might dislocate the equilibrium of the system. On the other hand, in the synthetic methods, the system composition is previously determined (before the beginning of the experiment). However, these methods require smaller equilibrium cells and stricter temperature and pressure control. Besides, to the synthetic methods provide reliable data, phase transition must be measured either visually or employing other property, such as density. (DOHRN, PEPER and FONSECA, 2010)

The analytical methods can be divided into two groups regarding whether an aliquot of the system is withdrawn or not. The one in which sampling is performed is categorized into three groups: isothermal, isobaric and isothermal and isobaric. On the other hand, the one without sampling is categorized in spectroscopic, gravimetric and others. Still, the synthetic methods can be divided into two groups regarding whether phase transition occurs or not. The one in which phase transition happens is categorized into two groups: visual and nonvisual. In the one without phase transition is categorized in isothermal, isobaric and others. (DOHRN, FONSECA and PEPER, 2012)

Regarding the analytical methods, the thermal is the most employed, representing near 55% of the experiments in which the analytical method is used. Nonetheless, respecting the synthetic method, the visual method is the most used (almost 63% of the experiments in which the synthetic method is applied. (GIACOMIN-JUNIOR, 2020).

2.12 GENERAL CONSIDERATIONS

The literature review presented a general insight into the state-of-art of the synthesis of geranyl acetate. Different biocatalysts, reactors and solvents have been employed. However, there is a lack of study about the phase behavior during the synthesis of geranyl

acetate employing supercritical fluids as an alternative solvent, especially supercritical carbon dioxide. Therefore, phase behavior studies in the synthesis of geranyl acetate using supercritical fluids are demanded, as it affects the portioning of reactants and, consequently, reaction rate and conversion. Thus, this work aspires to provide data about the synthesis of geranyl acetate along with the phase behavior of the reaction system, providing relevant knowledge to fill this literature gap.

3 OBJECTIVES

The objectives of this dissertation can be divided into two categories: the main objective and specific ones.

3.1 MAIN OBJECTIVE

The main objective of this work is to evaluate the esterification reaction between geraniol and acetic acid using enzymatic biocatalysis focused on green chemistry approach.

3.2 SPECIFIC OBJECTIVES

The specific objectives of this work are:

- Evaluate different temperatures and molar ratios between reactants, different biocatalysts and biocatalyst concentrations in the esterification of geraniol and acetic acid using n-hexane as solvent; and the esterification of geraniol and acetic acid using supercritical carbon dioxide as an alternative solvent;
- Compare the esterification in different types of solvent: organic (n-hexane) and supercritical fluid ($scCO_2$);
- Evaluate the esterification in a Variable Volume Reactor (VVR) to assess the effect of pressure independently of the amount of CO_2 inserted in the system and compare the esterification in different types of batch reactors: a Batch Stirred Tank Reactor (BSTR) and a Variable Volume Reactor (VVR);
- Investigate the effect of temperature, pressure and reactants to CO_2 mass ratio in the esterification of geraniol and acetic acid using $scCO_2$ as solvent in the VVR by performing a 2^3 factorial experimental design with triplicate in the central point;
- Determine the best condition for the esterification of geraniol and acetic acid and evaluate the reaction in the best condition;
- Analyze the phase equilibrium data for the binary system of CO_2 and geraniol and for the ternary systems englobing CO_2, geraniol and acetic acid or ethyl acetate.

A block diagram indicating the experimental strategy and its sequence is presented in FIGURE 10:

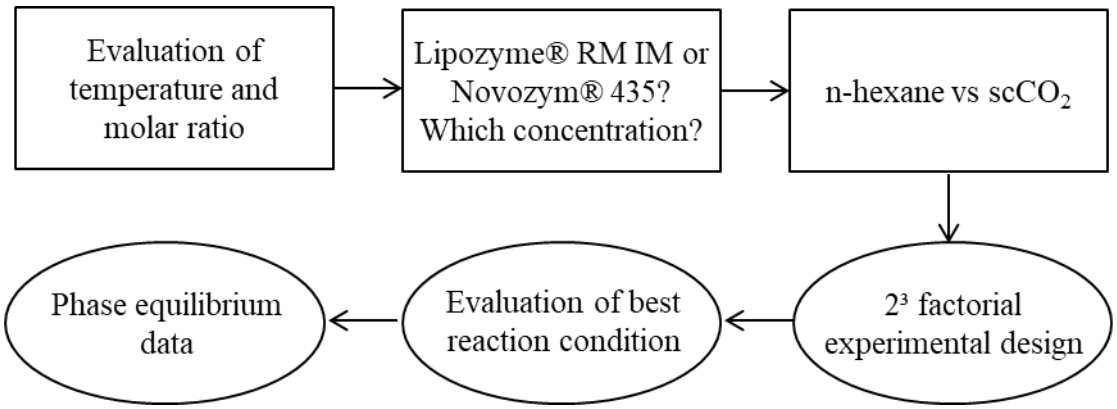

FIGURE 10 – BLOCK DIAGRAM INDICATING THE EXPERIMENTAL STRATEGY AND ITS SEQUENCE
NOTE: SQUARES AND ELLIPSES REPRESENT EXPERIMENTS CONDUCTED IN A BSTR AND IN A VVR, RESPECTIVELY

4 MATERIAL AND METHODS

In this section, a description of the materials and the methods utilized in this work is presented. In the next topics, the materials and methods utilized to determine the enzyme esterification activity, to perform the esterification reactions, the gas chromatography and experiments in the Variable Volume Reactor are presented.

4.1 ENZYME ESTERIFICATION ACTIVITY

Assays of the enzyme's esterification activity were the first step to be done. The enzymes evaluated were two commercial lipases: Lipozyme® RM IM and Novozym® 435. Lipozyme® RM IM is a lipase from the filamentous fungi *Mucor miehei* immobilized in macroporous ion exchange resin. Novozym® 435, on the other hand, is the type B lipase from the yeast *Candida antarctica* immobilized in acrylic resin.

In order to measure the esterification activity of the enzymes by an adapted Lowry-Tinsley method (LOWRY-TINSLEY, 1976), an oleic acid standard-curve was elaborated. The goal of the oleic acid standard-curve is to determine the content of free fatty acids (FFA) in the esterification reaction between ethanol and oleic acid.

To elaborate the oleic acid standard-curve the following materials were used:

- Cupric acetate (analytical standard, Nuclear, Brazil);
- Pyridine **(**analytical standard, Panreac, Spain);
- Oleic acid (analytical standard, Exodo Científica, Brazil);
- Ethanol (analytical standard, 99.8%, Neon, Brazil);
- Hexane (analytical standard, 99.0%, Anidrol, Brazil).

First, the 5.00% (wt./vol.) Lowry Reagent was prepared by the dissolution of cupric acetate in deionized water. Pyridine was added to adjust the solution pH to 6.0-6.2. Thus, proper amounts of oleic acid were dissolute in n-hexane to obtain 5.0 mL- acid oleic solutions in different concentrations (5.0 mM, 20.0 mM, 35.0 mM, 50.0 mM, 65.0 mM, and 80.0 mM) in furtherance of constructing the standard-curve. In sequence, 2.5 mL of the Lowry Reagent, 11.5 mL of toluene and 1.0 mL of the oleic acid solution corresponding to the concentration of the point of interest of the standard-curve were added in Falcon tubes. Each point was

made in triplicate. After that, each tube was vortexed (Kasvi Multifunctional Vortex, model K40-1010) for 1.0 min and centrifuged at 4,000 RPM for 10 min, until phase separation. Next, 3.0 mL of the organic layer was removed from each tube and inserted in the glass spectrophotometry cuvettes. The absorbance was read at 715 nm (Global Analyzer UV-Vis Spectrophotometry, software MetaSpec Pro version: 2.2) and the *oleic acid x absorbance* was plotted, attaining the standard-curve.

The following materials were used to determine the esterification activity of the lipases (Lipozyme® immobilized from *Mucor miehei*, Sigma-Aldrich, Denmark; or Novozym® 435) proposed by (MADALOZZO, 2011):

- Lowry-reagent (prepared as described in the section above);
- Toluene (analytical standard, Neon, Brazil);
- Hexane (analytical standard, Anidrol, Brazil);
- Oleic acid (analytical standard, Exodo Científica, Brazil);
- Ethanol (analytical standard, 99.8%, Neon, Brazil);
- Lipase (Lipozyme® immobilized from *Mucor miehei*, Sigma-Aldrich, Denmark or Novozym® 435)

First, a sufficient amount of ethanol and oleic acid were weighted to reach a concentration of 210 mM and 70 mM, respectively, in the reaction mean. Therefore, the alcohol to acid molar proportion was 3:1. Then, approximately 50.0 mL of hexane was added to an empty jacked 50.0 mL glass vessel of reaction to obtain a final reaction volume of 50.0 mL. After that, only ethanol was inserted in the vessel of the reaction containing hexane and the mixture was mixed well. Then, 0.5 mL of the mixture was withdrawn and put in a Falcon tube already containing 1.25 mL of Lowry-reagent and 5.75 mL of toluene (blank point). Once the blank point is done, 18.3 mg of the enzyme was added to the reaction mixture and another 0.5 mL was removed from the reaction mean and inserted in another Falcon tube already containing 1.25 mL of Lowry-reagent and 5.75 mL of toluene (control point). Subsequently, oleic acid was included in the reaction mean and the reaction was started. The esterification reaction was performed at 55 °C for 30 min and was magnetically stirred (Edutec, model EEQ-9008, Brazil). Immediately after its start, an aliquot of 0.5 mL of the reaction mean was extracted and put in another Falcon tube already containing 1.25 mL of Lowry-reagent and 5.75 mL of toluene (start point). Aliquots of 0.5 mL were withdrawn from

the reaction mixture and inserted in Falcon tubes containing 1.25 mL of Lowry-reagent and 5.75 mL of toluene every 5 min. All points were made in triplicate. Once all the aliquots were removed, each Falcon tube was vortexed (Kasvi Multifunctional Vortex, model K40-1010, Brazil) for 1.0 min and, thereafter, centrifuged for 10 min, following the method proposed by Lowry-Tinsley (1976). Afterward, 0.75 mL of the organic layer was collected and inserted in glass spectrophotometry cuvettes and its absorbance was read at 715 nm (Global Analyzer UV-Vis Spectrophotometry, software MetaSpec Pro version: 2.2). The free fatty acid (FFA) content (mM) was determined by the oleic acid standard-curve previously described and the extent of conversion of the reactants calculated as the equation below (12):

$$Conversion(\%) = \frac{FFA_0 - FFA_n}{FFA_0} \tag{12}$$

where, FFA_0 and FFA_n represent the concentration of FFA at the start (point zero) (mM) and concentration of FFA at a specific time of reaction (point *n*) (mM), respectively.

The enzyme esterification specific activity was determined by the following equation (13):

$$Enzyme\ Activity = \frac{(FFA - FFA_0) \times V}{(t - t_0) \times m_{enzyme}} \tag{13}$$

where, FFA_0 is the concentration of FFA at the start (point zero) (mM), *FFA* is the concentration of FFA at end of the reaction (mM), t_0 and *t* represent the initial and final time of reaction (min), respectively, *V* is the sample volume (mL) and m_{enzyme} represents the mass of enzyme utilized (g). The *Enzyme Activity* of esterification is expressed as specific activity ($U.g^{-1}$).

4.2 ESTERIFICATION REACTIONS

4.2.1 Esterification reactions – Glass Vessels

The esterification reaction conditions were pre-tested in glass vessels first to evaluate the esterification behavior. The reagents used in the pre-test esterification reactions are listed below:

- Geraniol (Food Grade, 99.3%, Sigma-Aldrich, USA);
- Acetic acid (analytical standard, Vetec, Brazil);
- Hexane (analytical standard, Anidrol, Brazil);
- Lipase (Lipozyme® immobilized from *Mucor miehei*, Sigma-Aldrich, Denmark).

The pre-tests esterification reactions occurred in a jacked 50.0 mL glass vessel with a reaction medium of 40.0 mL. The mixing speed was set to 600 RPM by a magnetic stirrer (Edutec, model EEQ-9008, Brazil), which ensured homogenous agitation and mass transfer to the enzyme. Three different reaction temperatures were tested: 30 °C, 45 °C, and 60 ºC. The temperature was reached and controlled using a water bath (Spencer Scientific, USA). The esterification reactions were carried out for 480 min.

The reaction mixture was prepared gravimetrically using an analytical balance (BEL Engineering, Brazil). At first, the initial concentration of the reactants was 100 mM (1:1 alcohol:acid molar proportion) and the enzyme amount was set to 1.0% (wt./wt. of reactants). Later, enzyme concentration was set to 10.0% (wt./wt. of reactants) and further to 20.0% (wt./wt. of reactants). The order of addition of the reactants to the vessel of reaction was the following: alcohol; organic acid; hexane; and, at last, enzyme.

Aliquots were withdrawn in triplicate during the progress of reaction at specific times to evaluate the progress of reaction: at the beginning of the reaction, before enzyme addition (zero), after 15 min, 30 min, 60 min, 120 min, 240 min, 360 min, and after 480 min of reaction.

The acid content of the aliquots was measured by titration, using a digital bottle-top burette (Brand, Titrette®, Germany) to determine the extension of conversion.

4.2.2 Esterification reactions – Batch Reactor

A photograph and a schematic diagram of the closed vessel mini bench top reactor (50 mL Parr® reactor, model 4561, Parr®, USA) equipped with a reactor controller (model 4848, Parr®, USA) are depicted below (FIGURE 11 and FIGURE 12).

FIGURE 11 – PHOTOGRAPH OF THE CLOSED VESSEL MINI BENCH TOP REACTOR (MODEL 4561, PARR®, USA) EQUIPPED WITH A REACTOR CONTROLLER (MODEL 4848, PARR®, USA).

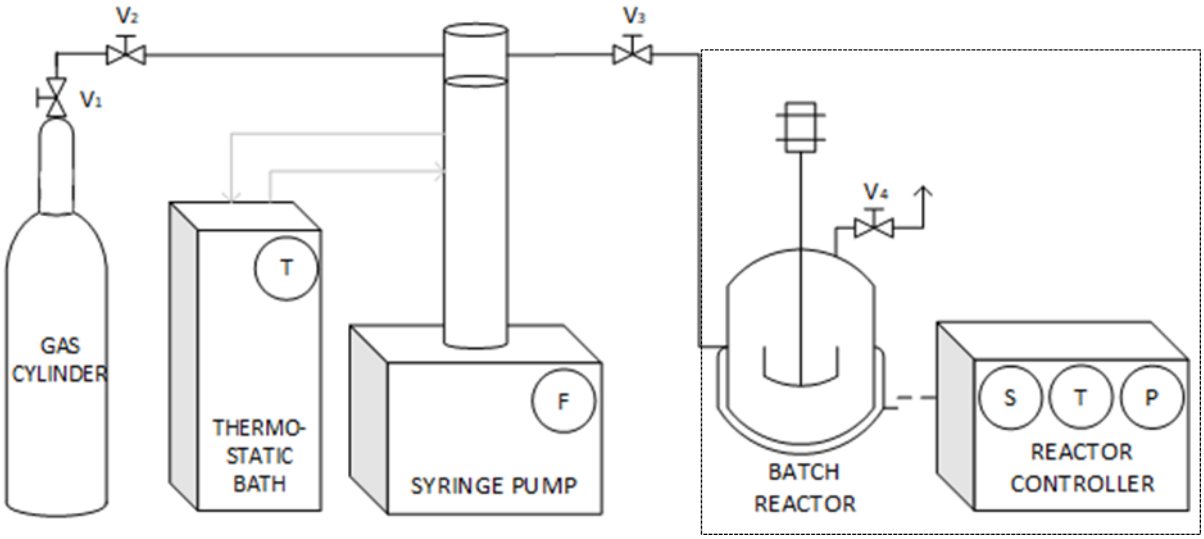

FIGURE 12 – SCHEMATIC DIAGRAM OF THE CLOSED VESSEL MINI BENCH TOP REACTOR (MODEL 4561, PARR®, USA) EQUIPPED WITH A REACTOR CONTROLLER (MODEL 4848, PARR®, USA). WHEN ORGANIC SOLVENT WAS EMPLOYED, ONLY THE HIGHLIGHTED PART BY THE DASHED BLACK LINE WAS USED. GAS CYLINDER, THERMOSTATIC BATH AND SYRINGE PUMP WERE ONLY UTILIZED WHEN CO_2 WAS EMPLOYED AS SOLVENT

The reagents used in the esterification reactions are listed below:

- Geraniol (Food Grade, 99.3%, Sigma-Aldrich, USA);
- Acetic acid (analytical standard, Vetec, Brazil);

- Hexane (analytical standard, Anidrol, Brazil);
- Carbon dioxide (99.9%, White Martins, Brazil);
- Lipase (Lipozyme® lipase immobilized from *Mucor miehei*, Sigma-Aldrich, Denmark; or Novozym® 435).

As pointed out above, part of esterification reactions performed in this study occurred in a 50.0 mL closed vessel mini bench top reactor (model 4561, Parr®, USA) equipped with a reactor controller (model 4848, Parr®, USA) with a reaction medium of 35.0 mL. The mixing speed was set to 600 RPM by the reactor controller (model 4848, Parr®, USA), which ensured homogenous agitation and mass transfer to the enzyme without damaging it. Three different reaction temperatures were tested: 40 °C, 55 °C, and 70 ºC. The temperature was set and controlled by the reactor controller (model 4848, Parr®, USA). The esterification reactions were carried out for 480 min when Lipozyme® was used, and for 240 min when Novozym® 435 was employed.

The reaction mixture was prepared gravimetrically using an analytical balance (BEL Engineering, Brazil). At first, the initial concentration of the reactants was 100 mM with alcohol to acid molar proportion of 1:1. Later, keeping the acid concentration at 100 mM, alcohol to acid molar proportion was set to 1.25:1.0 and 1.5:1.0. Enzyme concentration was set to 20.0% (wt./wt. of reactants).

When organic solvent was used, the order of addition of the reactants to the reaction vessel was the following: alcohol; organic acid; hexane; and, at last, enzyme. Meanwhile, when CO_2 was the solvent, the order was: alcohol; organic acid and lipase. Therefore, with the vessel of reaction closed and sealed, the proper amount of carbon dioxide was added using a syringe pump (ISCO, model 260D, USA, 16.63 nL uncertainty).

When n-hexane was used as solvent, aliquots were withdrawn in triplicate during the progress of reaction at specific times to evaluate the reaction kinetics. When Lipozyme® was used, aliquots were withdrawn at the beginning of the reaction, before enzyme addition (zero), after 120 min, 240 min, 360 min, and after 480 min of reaction. When Novozym® 435 was used, aliquots were withdrawn at the beginning of the reaction, before enzyme addition (zero); after 30 min, 60 min, 120 min, and after 240 min of reaction.

Using the organic solvent, the acid content of the aliquots was measured by titration, using a digital bottle-top burette (Brand, Titrette®, Germany) to determine the extension of conversion. When the solvent was CO_2, conversion in terms of geraniol was measured by gas

chromatography (Shimadzu CG-2010 Plus with auto-injector AOC-20i coupled with flame ionization detector (FID)), following the methodology described in the next section. This is possible as no side reactions were detected by gas chromatography analysis.

4.2.3 Titration to determine the extent of conversion

The titration of the aliquots to determine their acid content, and, therefore, measure the extent of the conversion of the reactants followed the methodology proposed by Cirillo et al. (2018).

In order to titrate the aliquots, the following materials were used:

- Ethanol (analytical standard, 99.8%, Neon, Brazil);
- An alcoholic solution of phenolphthalein 1.0% (wt./vol.) (Ethanol, analytical standard, 99.8%, Neon, Brazil and Phenolphthalein, analytical standard, Vetec, Brazil);
- Aqueous solution of NaOH 0.01 M.

The titration occurred in screw-cap glass cups and was magnetically stirred in order to guaranty the homogeneity and the mass transfer in the system.

First, 10.0 mL of ethanol (Analytical Standard (99.8%), Neon, Brazil) was added to the screw-cap glass cup and its mass was measured. Then, ca. 200 mg aliquots were withdrawn from the reaction and added to the screw-cap glass cup and its mass measured again to obtain the exact mass of the aliquot added to the solvent. Titration was made in triplicate. Next, two drops of the alcoholic solution of phenolphthalein 1.0% (w/v) (Ethanol (Analytical Standard (99.8%), Neon, Brazil; Phenolphthalein (Analytical Standard, Vetec, Brazil) were included in the mixture. After that, the titration could begin using a digital bottle-top burette (Brand, Titrette®, Germany).

The volume used during the titration was annotated in furtherance of calculating the acid content of the aliquot (14) and, thereafter, the extension of conversion of the reactants (15).

$$Acid\ Content\ (\%) = \frac{V_{NaOH} \times MW_{acid}}{m_{aliquot} \times 10} \times \frac{1}{100} \quad (14)$$

where, V_{NaOH} is the volume of NaOH solution 0.01 M used along with the titration (mL), MW_{acid} represents the molecular weight of the acid being titrated (g.mol^{-1}) and $m_{aliquot}$ is the mass of the aliquot withdrawn from the reaction (g);

$$Conversion\ (\%) = \frac{Acid\ Content_0 - Acid\ Content}{Acid\ Content_0} \times 100 \quad (15)$$

where, *Acid Content$_0$* and *Acid Content* represent the acid content at the beginning and at a specific time of reaction of the esterification reaction (%).

4.3 GAS CHROMATOGRAPHY ANALYSIS

Gas chromatograph was performed using a Shimadzu (Japan) CG-2010 Plus with auto-injector AOC-20i clopped with flame ion detector (FID). The column used was the Restek (USA) SH-Rtx-Wax Crossbond© Carbowax© polyethylene glycol (length: 30 m; internal diameter: 0.32 mm; layer thickness: 0.25 μm). The injection volume was 1.0 μL (split ratio of 1:20). Injector temperature and detector temperature was set to 230 °C and 240 °C, respectively. The initial temperature was set to 130 °C. After that, temperature was increased to 185 °C at a 10 °C.min^{-1} rate. When the temperature reached 185 °C and it was held for 1 min. Pure helium was used as the mobile phase at 1.75 mL.min^{-1}. Methyl laurate was used as internal standard. Total run time was 6.5 min.

4.3.1 Geraniol standard-curve

The following reagents were used while preparing the geraniol standard-curve, which was analyzed in the gas chromatograph (Shimadzu CG-2010 Plus with auto-injector AOC-20i clopped with flame ion detector (FID)):

- Geraniol (Food Grade (99.3%), Sigma-Aldrich, USA);
- n-Heptane (HPLC (>99.0%), Sigma-Aldrich, France).

First, a solution containing 0.05 mL of geraniol and 4.95 mL of n-heptane was gravimetrically prepared in a 5.0 mL volumetric flask (9.1356 mg.mL^{-1}). Afterward, this

solution was transferred to an amber flask and aliquots of different volumes were withdrawn from this amber flask and inserted in 2.0 mL amber vials to prepare diluted solutions of different concentrations, which would be used further to assemble the standard-curve points. Sufficient volume of n-heptane was later added to each vial to attain a final volume of 1.5 mL (TABLE 3).

TABLE 3 – GERANIOL DILUTED SOLUTIONS PREPARATION

Point	Aliquot from the geraniol solution (mL)	Volume of n-heptane (mL)	Total volume (mL)	Concentration ($mg.mL^{-1}$)	Concentration (ppm)
1	0.020	1.480	1.500	0.1218	122
2	0.050	1.450	1.500	0.2853	285
3	0.075	1.425	1.500	0.4280	428
4	0.100	1.400	1.500	0.5706	571
5	0.200	1.300	1.500	1.1413	1141
6	0.300	1.200	1.500	1.7119	1712
7	0.400	1.100	1.500	2.4362	2436
8	0.500	1.000	1.500	3.0452	3045
9	0.600	0.900	1.500	3.6542	3654
10	0.700	0.800	1.500	3.9945	3995

For these analyses, 0.05 mL was withdrawn from each vial and inserted in a new amber vial, in which was added 0.950 mL of n-heptane to reach a final volume of 1.000 mL. The volumes utilized and the concentration of each point can be seen on the table below (TABLE 4):

TABLE 4 – GERANIOL STANDARD CURVE PREPARATION

Point	Aliquot from the geraniol solution (mL)	Volume of n-heptane (mL)	Total volume (mL)	Concentration ($mg.mL^{-1}$)	Concentration (ppm)
1	0.050	0.950	1.000	0.0061	6
2	0.050	0.950	1.000	0.0143	14
3	0.050	0.950	1.000	0.0214	21
4	0.050	0.950	1.000	0.0285	29
5	0.050	0.950	1.000	0.0571	57
6	0.050	0.950	1.000	0.0856	86
7	0.050	0.950	1.000	0.1218	122
8	0.050	0.950	1.000	0.1523	152
9	0.050	0.950	1.000	0.1827	183
10	0.050	0.950	1.000	0.1997	200

Therefore, the range of detection is from 0.0061 $mg.mL^{-1}$ to 0.1997 $mg.mL^{-1}$.

4.3.2 Geranyl acetate standard-curve

The following reagents were used while preparing the geranyl acetate standard-curve, which was analyzed in the gas chromatograph (Shimadzu CG-2010 Plus with auto-injector AOC-20i clopped with flame ion detector (FID)):

- Geranyl acetate (Analytical Standard (99.2%), Sigma-Aldrich, USA);
- n-Heptane (HPLC (>99%), Sigma-Aldrich, France).

The procedure is equal to the described in the previous section (Geraniol standard-curve), except for the concentration of geranyl acetate initial solution (9.5430 $mg.mL^{-1}$).

The volumes utilized in the preparation of the geranyl acetate diluted solutions can be seen on the table below (TABLE 5):

TABLE 5 – GERANYL ACETATE DILUTED SOLUTIONS PREPARATION

Point	**Aliquot from geranyl acetate solution (mL)**	**Volume of n-heptane (mL)**	**Total volume (mL)**	**Concentration ($mg.mL^{-1}$)**	**Concentration (ppm)**
1	0.020	1.480	1.500	0.1286	129
2	0.050	1.450	1.500	0.3214	321
3	0.075	1.425	1.500	0.4821	482
4	0.100	1.400	1.500	0.6428	643
5	0.200	1.300	1.500	1.2856	1286
6	0.300	1.200	1.500	1.9284	1928
7	0.400	1.100	1.500	2.5713	2571
8	0.500	1.000	1.500	3.1810	3181
9	0.600	0.900	1.500	3.8569	3857
10	0.700	0.800	1.500	4.4997	4500

From each vial, 0.050 mL was withdrawn and inserted in a new amber vial, in which was added 0.950 mL of n-heptane to reach a final volume of 1.000 mL. The volumes utilized and the concentration of each point can be seen on the table below (TABLE 6):

TABLE 6 – GERANYL ACETATE STANDARD-CURVE PREPARATION

Point	Aliquot from the geraniol solution (mL)	Volume of n-heptane (mL)	Total volume (mL)	Concentration ($mg.mL^{-1}$)	Concentration (ppm)
1	0.050	0.950	1.000	0.0064	6
2	0.050	0.950	1.000	0.0161	16
3	0.050	0.950	1.000	0.0241	24
4	0.050	0.950	1.000	0.0321	32
5	0.050	0.950	1.000	0.0643	64
6	0.050	0.950	1.000	0.0964	96
7	0.050	0.950	1.000	0.1286	129
8	0.050	0.950	1.000	0.1591	159
9	0.050	0.950	1.000	0.1928	193
10	0.050	0.950	1.000	0.2250	225

Therefore, the range of detection is from 0.0064 $mg.mL^{-1}$ to 0.2250 $mg.mL^{-1}$.

4.3.3 Sample preparation

The following materials were used while preparing the samples to analyze them in the gas chromatograph (Shimadzu CG-2010 Plus with auto-injector AOC-20i clopped with flame ion detector (FID)):

- Samples from reactions;
- n-Heptane (HPLC, >99%, Sigma-Aldrich, France);
- Internal standard solution.

To minimize the impact of solvent loss, an internal standard was used in the sample analysis. Among the possibilities of internal standards, methyl laurate (Sigma-Aldrich) was selected due to its retention time is between the geranyl acetate and geraniol retention times. The solution of internal standard was prepared as the geraniol solution described in the previous section, reaching a concentration of 8.4774 $mg.mL^{-1}$. By using the equation provided by the standard-curve, the analysis result and considering the dilution correction, it was possible to determine the concentration of each sample, and, therefore, establish the extent of conversion of the reagents/yield of reaction.

Two different methods for the sample preparation were used, depending on whether an organic solvent was used in the reaction or not.

When n-hexane was used, samples were prepared by taking 0.250 mL of sample diluted with 1.000 mL of n-heptane in a 15 mL screw-capped glass vial. Afterward, 0.200 mL

of internal standard solution was added to this vial. After properly mixing to ensure homogeneity, 0.200 mL of this solution was withdrawn and inserted in another 15 mL screw-capped glass vial, in 4.800 mL of n-heptane was added. At last, 1.0 mL of the solution containing internal standard was collected and put in 2.0 mL amber vials in order to analyze in the gas chromatograph.

When no solvent was used, samples were prepared by taking 0.250 mL of sample diluted with 9.750 mL of n-heptane in a 15 mL screw-capped glass vial. After properly mixing to ensure homogeneity, 0.050 mL of this solution was withdrawn and inserted in another 15 mL screw-capped glass vial, in which was added later 0.035 mL of internal standard solution and 9.915 mL of n-heptane. At last, 1.0 mL of the solution containing internal standard was collected and put in 2.0 mL amber vials in order to analyze in the gas chromatograph.

4.4 EXPERIMENTS IN THE VARIABLE VOLUME REACTOR (VVR)

The general basis of the experimental apparatus and procedure utilized when the Variable Volume Reactor (VVR) was used, both in esterification and phase equilibrium experiments, have been described extensively (ARAÚJO et al., 2012; GIACOMIN JUNIOR et al., 2019; PINTO et al., 2012; VEIGA et al., 2017) and it is described below (FIGURE 13 and FIGURE 14):

FIGURE 13 – PHOTOGRAPH OF THE EXPERIMENTAL APPARATUS UTILIZED IN THE ESTERIFICATION REACTIONS CONDUCTED IN THE VARIABLE VOLUME REACTOR (VVR) AND IN THE PHASE EQUILIBRIUM EXPERIMENTS.

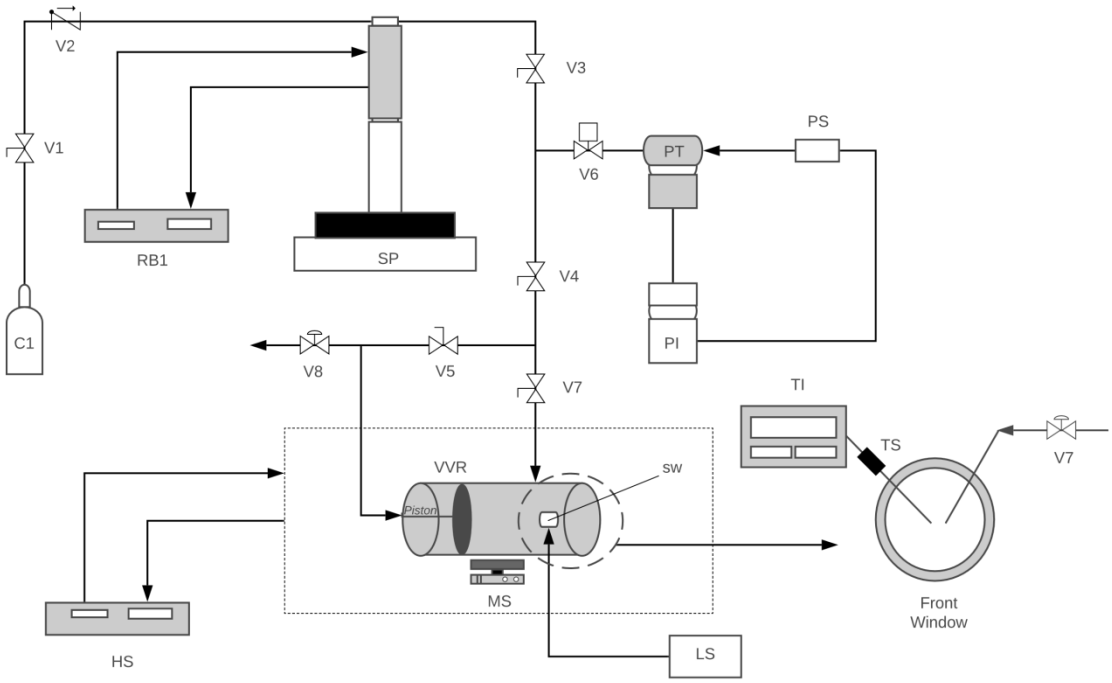

FIGURE 14 – EXPERIMENTAL APPARATUS SCHEMATIC DIAGRAM. VARIABLE VOLUME REACTOR IS HIGHLIGHTED BY THE DASHED BLACK LINE.

where:

C1 – Solvent Cylinder. It is used for storage of the solvent utilized in the experiments (CO_2);

HS – Heating Source. It is an electrical heating jacket for temperature control.

MS – Magnetic Stirrer (IKA®, model C-MAG HS4, Germany). The function of the stirrer system is to fasten the equilibrium phase achieve, ensure the homogeneity and efficient mass and heat transport in the system. A magnetic bar inside the VVR is driven by the magnetic stirrer positioned below the heating source;

LS – Light Source.

Piston - there are two nitrile rubber N90 rings in the piston, which allow its movement in the interior of the VVR and ensure the sealing of the system;

PI – Pressure Indicator. It is a universal indicator (Novus, model N1500, Brazil) for pressure data acquisition;

PS – Power Source. It is used to keep a constant tension and feed a constant current to PT and PI;

PT – Pressure Transducer (Smar, model LD 301, Brazil, uncertainty of ± 0.03 MPa). The transducer is coupled to the line coming from the SP to evaluate the real pressure of the system;

RB1 – recirculating bath. Used to keep the temperature in the syringe pump cylinder constant;

SP – Syringe Pump (ISCO, model 260D, USA, 16.63 nL uncertainty). For injecting CO_2 into the cell and for manipulating pressure in the equilibrium unit;

SW – side window. There are two sapphire windows in the VVR, one in the frontal portion of the reactor (Front Window – diameter: 25.4 mm; thickness: 9.52 mm) and another (SW – diameter: 15.87 mm; thickness: 4.76 mm) in its side;

TI – Temperature Indicator. It is equipped to a temperature sensor (thermocouple) J-type (Ecil, Brazil) and a temperature indicator (COELMATIC, model H242000, Brazil). It is used to measure the real temperature of the mixture inside the VVR. The sensor is put inside the VVR in a way that the cold junction is positioned in the center (considering the radial dimension) of the VVR;

V1 – Sphere Valve. When opened allows solvent flux from C1 to SP;

V2 – Single-way valve. This valve function is to allow flux only in one way. It is put between C1 and SP (after V1) to avoid elevate pressures in the head of the solvent cylinder during the experiments;

V3 – Sphere Valve. Its function is to isolate the system from the syringe pump;

V4 – Needle Valve. It is used to allow solvent flux to the back of the piston, aiming the pressurization of the VVR;

V5 – Needle Valve. It is used to discharge the solvent;

V6 – Valve to control the pressure transducer;

V7 – Needle Feed Valve. It allows a controlled opening of the valve, thus permitting control of the solvent flow fed to the VVR;

V8 – Discharge Valve. It is used to discharge the system after the conclusion of the experiments;

VVR – Variable Volume Reactor. It consists of a 316 stainless steel cylinder with the following dimensions: maximum volume: 27.0 mL; internal diameter: 17.2 mm; length: 176 mm.

Succinctly, the experiments were performed in high-pressure variable volume view cell containing a movable piston, which allows pressure control inside de cell. The apparatus also includes a syringe pump (ISCO, model 260D, USA, 16.63 nL uncertainty) for injecting CO_2 into the cell and for manipulating pressure in the equilibrium unit and an electrical heating jacket for the temperature control.

Coupled to the cell, there are a pressure transducer (Smar, model LD 301, Brazil, uncertainty of ± 0.03 MPa), a universal indicator (Novus, model N1500, Brazil) for pressure data acquisition, and a thermocouple (J-type, Ecil, Brazil) to measure and register the temperature inside the cell. Agitation is provided by a magnetic stirrer (IKA®, model C-MAG HS4, Germany). The visual observations were achieved through two sapphire windows, one on the side and another frontal to the cell.

The VVR was utilized to verify the extension of conversion of geraniol and acetic acid in geranyl acetate when both temperature and pressure are kept constant. Moreover, it was used to clarify the phase equilibrium data in the geraniol + CO_2 binary system and in the geraniol + acetic acid + CO_2 ternary system.

The materials used in the VVR were the following:

- Geraniol (Food Grade, 99.2%, Sigma-Aldrich, USA);
- Acetic acid (analytical standard, 99.8%, Neon, Brazil);
- Ethyl acetate (analytical standard, 99.0%, Honeywell, Germany);
- Carbon dioxide (99.9%, White Martins, Brazil);
- Novozym® 435.

4.4.1 Esterification reactions using supercritical carbon dioxide

The experimental procedure in the Variable Volume Reactor was similar to the described by Veiga et al. (2017) and Giacomin Junior et al. (2019): first, the VVR was flushed with CO_2 (288.15 K and 6.5 MPa) to remove any residual air. Then, it was first loaded with the respective amount of enzyme and then with reactants, previously gravimetrically measured (RADWAG, model AS220/C/2, USA, ±0.0001 g uncertainty). Further, using the syringe pump, with the proper amount of CO_2 (288.15 K and 10.0 MPa), in order to obtain the desired mass ratio between CO_2 and reactants. In sequence, the system was pressurized to 6.0 MPa and then to the desired reaction pressure condition at a 0.5 $MPa.min^{-1}$ pressure rate and heated to the desired reaction temperature. When the aimed pressure and temperature were attained, the reaction was conducted according to corresponding time. Depressurization occurs at the same pressurization rate. Agitation was kept constant at approximately 600 RPM. Reactant molar ratio was kept 1.0:1.0.

4.4.2 Phase equilibrium experiments

For each system composition, the procedure was the same as described by Veiga et al. (2017) and Giacomin Junior et al. (2019): flush of the VVR with CO_2 (288.15 K and 6.5 MPa) to remove any residual air; load of the cell with the respective amount of liquid solute (geraniol, geraniol + acetic acid or geraniol + ethyl acetate), previously gravimetrically prepared in a precision balance (RADWAG, model AS220/C/2, USA, ±0.0001 g uncertainty) according to the desired molar fraction of CO_2 (x_1) in each experiment, and further with the CO_2 (288.15 K and 10.0 MPa); and set the pressure to reach homogenous phase condition (single-phase).

At a fixed temperature (30 °C, 40 °C, 50 °C, 60 °C and 70 °C), the phase transition pressure (the phase saturation condition) was measured by the slow depressurization of the system (using the syringe pump) and read at the incipient new phase formation.

The molar ratio between geraniol and acetic acid was 1.0:1.0 in all experiments. The molar fraction of CO_2 was set accordingly to the system studied.

The uncertainties related to the mole fraction, temperature, and pressure measurements were estimated using the method of uncertainties (type B) as suggested by Taylor and Kuyatt (1994) and are reported in tables with the experimental data.

4.5 THERMODYNAMIC MODELING OF PHASE EQUILIBRIUM AT HIGH-PRESSURE

The thermodynamic modeling used in this work followed a previously reported (GIACOMIN JUNIOR et al., 2019; VEIGA et al., 2017) procedure using the Peng-Robinson (PR) equation of state (PENG and ROBINSON, 1976) with the conventional quadratic van der Waals mixing rule (vdW2), as shown in Eqs (16) and (17).

The estimation of binary system parameters (k_{ij} and l_{ij}) was carried out by minimizing the least-squares objective function of experimental and calculated pressure values using the Nelder-Mead Simplex method from the Matlab optimization toolbox. The calculation of saturation points followed the procedure present by Michelsen (1982) Thermodynamic parameters for pure components are disposed in TABLE 7.

$$a = \sum_{i=1}^{n}\sum_{j=1}^{n} x_i x_j \sqrt{a_i a_j}\left(1 - k_{ij}\right) \tag{16}$$

$$b = \sum_{i=1}^{n}\sum_{j=1}^{n} x_i x_j \frac{1}{2}\left(b_i + b_j\right)\left(1 - l_{ij}\right) \tag{17}$$

TABLE 7 – CHARACTERISTIC THERMODYNAMIC PARAMETERS FOR PURE COMPOUNDS EMPLOYED IN THIS WORK

Compound	M_w/(g mol^{-1})	T_c/K	p_c/MPa	Ω	Ref.
CO_2	44.01	304.21	7.383	0.22362	(ROWLEY et al., 2006)
Ethyl Acetate	88.106	523.3	3.88	0.36641	(ROWLEY et al., 2006)
Acetic acid	60.053	591.95	5.786	0.46652	(ROWLEY et al., 2006)
Geraniol	154.25	684.0	2.6404	0.689131	Aspen [a]

T_c, critical temperature; p_c, critical pressure; ω, acentric factor; M_w, molar mass. [a]Aspen Plus databank v. 8.4.

5 RESULTS AND DISCUSSION

In this section, the experimental results of the synthesis of geranyl acetate and the phase equilibrium measurements data are presented. First, the experimental results of the esterification conducted in a BSTR using Lipozyme® RM IM as biocatalyst and n-hexane as solvent are presented. Next, experimental results when the biocatalyst was changed to Novozym® 435, but solvent and reactor remained the same, are given. Further, the experimental results when CO_2 was employed as an alternative solvent are shown. At last, the phase equilibrium measurements data and their modeling are disposed.

Following the adapted Lowry-Tinsley method (LOWRY-TINSLEY, 1976), Lipozyme® RM IM showed an activity of 403.1 $U.g^{-1}$ and Novozym® 435 an activity of 210.0 $U.g^{-1}$ ($R^2 = 0.9933$)

5.1 GERANYL ACETATE KINETICS IN N-HEXANE USING LIPOZYME® RM IM AS BIOCATALYST

As mentioned in the Material and Methods section, pre-tests of the esterification reaction were conducted in glass vessels. In these experiments, it was observed that when only 1.0% (wt./wt.) of Lipozyme® RM IM was used, the conversion was very low independently of the reaction condition. Thus, enzyme concentration was set to 10.0% (wt./wt.). With higher concentration of biocatalyst, the enzyme was sheared due to the friction between the agitator, the enzyme and the vessel's walls, losing activity. Therefore, after 240 min of reaction, more enzyme was added to the reaction to achieve an enzyme concentration of 20.0% (wt./wt.). However, in consequence of the operator's lack of experience, the titration was conducted using a 0.1 M aqueous solution of NaOH proceeding in an inaccuracy in the results. Hence, as in the BSTR the enzyme remained preserved, further esterification reactions using n-hexane and Lipozyme® RM IM were conducted only in the BSTR.

As mentioned in the Material and Methods section, BSTR experiments with n-hexane were performed using three different temperatures (40 °C, 55 °C and 70 °C) using 20% (wt./wt.) of enzyme and geraniol to acetic acid molar ratio of 1.0:1.0. Furthermore, at the central temperature, 55 °C, two other molar ratios were tested (1.25:1.0 and 1.5:1.0). The results obtained for this set of experiments were compiled according to the temperature in FIGURE 15(a) and to the molar ratio in FIGURE 15 (b).

All results presented for BSTR experiments are shown as symbols, representing average values, and error bars that are equal to average ± 2.05, which represent the expanded uncertainties with 95% confidence level. This value was calculated by multiplying the average experimental standard deviation obtained by 4.30 (t for a probability of 0.05 and, since each sample was analyzed in triplicates, the degree of freedom is equal to 2) and dividing by the square root of the number of samples (HECKERT et al., 2002). Furthermore, this set of experiments reports conversions obtained for geraniol, but were calculated according to Eq. (4) which is related to the conversion of acetic acid. This is possible since no side reactions were observed, as confirmed by GC analysis.

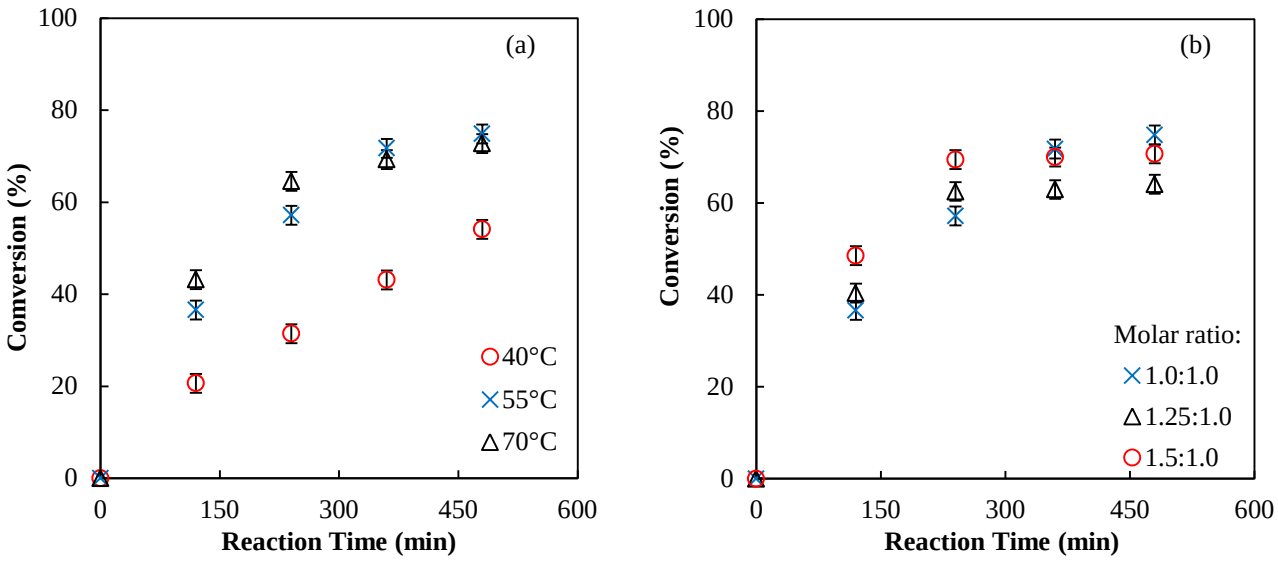

FIGURE 15 – EXPERIMENTAL RESULTS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID, N-HEXANE AND 20% OF LIPOZYME® RM IM (WT. OF BIOCATALYST/WT. OF REACTANTS) IN THE BSTR: (a) RESULTS OBTAINED FOR GERANIOL TO ACETIC ACID MOLAR RATIO OF 1.0:1.0 AT DIFFERENT TEMPERATURES; AND (b) RESULTS OBTAINED AT 55 °C FOR DIFFERENT MOLAR RATIOS.

As it can be seen in FIGURE 15(a), using equimolar proportions of geraniol to acetic acid, higher conversions were achieved at 70 °C, although, when the temperature was 55 °C, the conversion was commensurate, while the lower conversions were obtained when the temperature was set to 40 °C. For both cases, the molar conversions were directly related to the thermal energy provided to the system, demonstrating that the reaction was favored as

more thermal energy was disposed. When the intermediate temperature (55 °C) was set, conversions were closer to those obtained at 70 °C, rather than to those obtained at 40 °C.

Furthermore, FIGURE 15(b) shows that for reactions conducted at 55 °C, conversions obtained using a molar proportion of 1.5:1.0 were similar to those obtained for the equimolar proportion reaction. On the other hand, when only a slight excess was wielded (molar proportion of 1.25:1.0), the conversion was lower. This peculiar behavior should be further studied in future works by scrutinizing the chemical equilibrium of the reaction through robust thermodynamic models.

The effect of the molar ratio was also checked at 70 °C, as presented in FIGURE 16. As noticed from the results obtained at 55 °C and 70 °C, reactions performed with an excess of geraniol presented higher initial reaction rates since the conversions were higher for reaction times up to 240 min. For further reaction times, lower conversions were observed at alcohol to acyl donor molar ratio of 1.5:1.0, indicating a possibility that the slight excess of geraniol may have been sufficient to cause an inhibition of Lipozyme® RM IM, diminishing its activity. This distinct behavior should be also further studied in future works by scrutinizing the chemical equilibrium of the reaction through robust thermodynamic models.

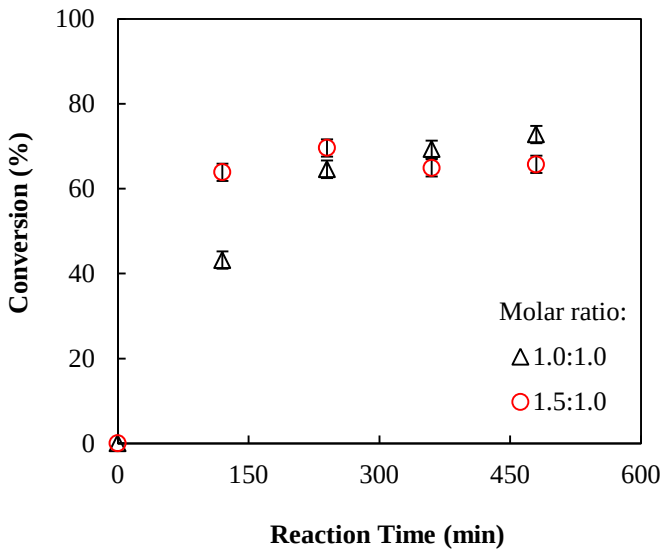

FIGURE 16 – EXPERIMENTAL RESULTS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID, N-HEXANE AND 20% OF LIPOZYME® RM IM (WT./WT.) IN THE BSTR: COMPARISON OF RESULTS OBTAINED AT 70 °C FOR DIFFERENT MOLAR RATIOS.

Regarding the alcohol inhibition, Chulalaksananukul, Condoret and Combes (1992) and Claon and Akoh (1993) also observed geraniol inhibition towards *Mucor miehei* lipase (IM 20) and *Mucor miehei* lipases (IM 20 and IM 60), respectively. Yee and Akoh (1996) verified the same enzyme inhibition using nonspecific lipase from *Pseudomonas* sp immobilized on glass beads.

In terms of conversion, Yee and Akoh (1996) utilized the latter enzyme and acetyl acetate as an acyl donor in n-hexane and obtained, after 24 hours, a conversion of 97% at 50 °C. Chulalaksananukul, Condoret, and Combes (1992) attained an 85% conversion after 3 days of reaction at 40 °C using propyl acetate as an acyl donor in great excess (alcohol to acyl donor molar proportion of 1:20) in n-hexane and Lipozyme® as biocatalyst. In contrast, only in 8 hours, a conversion of 70.7% was reached at 55 °C, 1.5:1.0, using Lipozyme®, but acetic acid as acyl donor, in the experiments described here.

Acetic acid has been reported by many authors as a lipase inhibitor, the reason the previously cited authors chose esters instead of acetic acid to obtain geranyl acetate. Following this acyl donor switch, Rosa et al. (2017), Murcia et al. (2018), and Badgujar and Bhanage (2014) used vinyl acetate as an acyl donor in the transesterification with geraniol in n-hexane as solvent. Rosa et al. (2017) using free *Candida rugosa* lipase, at 45 °C, got, after 480 min of reaction, a conversion of 79%. These results are similar to those obtained in this work, although Rosa et al. (2017) used a non-commercial lipase.

Badgujar and Bhanage (2014) also employed non-commercial lipase from *Pseudomonas cepacia* immobilized on a ternary blend biodegradable polymer film in the synthesis of geranyl acetate. They employed alcohol to acyl donor molar proportion of 1:4 and obtained almost 100% of conversion after 3 hours of reaction at 55 °C (BADGUJAR and BHANAGE, 2014). Murcia et al. (2018), on the other hand, employed Novozym® 435 as biocatalyst and achieved a 98.4% conversion after 160 minutes of reaction.

In contrast to these authors, Chen, Lin, and Chang (2002), Molinari, Villa, and Aragozzini (1998) and Bartling et al. (2001) used acetic acid as acyl donor and achieved considerable conversions – 95% and 94%, respectively. Chen, Lin, and Chang (2002) employed an alcohol to acyl donor molar proportion of 2:1 in n-hexane, but *Candida cylindracea* lipase entrapped in hybrid sol-gel formed with nonwoven fabric and reached, at 35 °C, after 24 hours, a conversion of 95%. Bartling et al. (2001) operated at 30 °C with equimolar proportions of reactants in n-hexane, exploiting Novozym® 435 as enzyme, reached, after 12 hours of reaction, 94% of conversion when a water-control method was not

applied and near 100% of conversion when a water-selective membrane was used. Instead, Molinari, Villa, and Aragozzini (1998) used free cells of *Rhizopus delemar* as biocatalyst and obtained a 54% conversion after 24 hours of reaction using an equimolar proportion of substrates but with semi-continuous addition, at 55 °C, in n-heptane as solvent.

5.2 GERANYL ACETATE SYNTHESIS KINETICS IN N-HEXANE USING NOVOZYM® 435 AS BIOCATALYST

After the previous study, while still using BSTR and n-hexane, the enzyme was switched and the synthesis of geranyl acetate was conducted with Novozym® 435 as biocatalyst at 55 °C with an equimolar proportion of geraniol and acetic acid to evaluate the biocatalyst influence. When comparing the synthesis of geranyl acetate using Lipozyme® RM IM and Novozym® 435, clearly the latter is more attractive as it exhibits higher conversions for lower reaction times, as shown in FIGURE 17. After only 240 min of reaction, the conversion was 82.9% when Novozym® 435 was utilized, while the esterification using Lipozyme® RM IM took 480 min to achieve 74.8% of conversion. These results present a good agreement with the literature data cited previously, especially when no methods to shift the equilibrium were used.

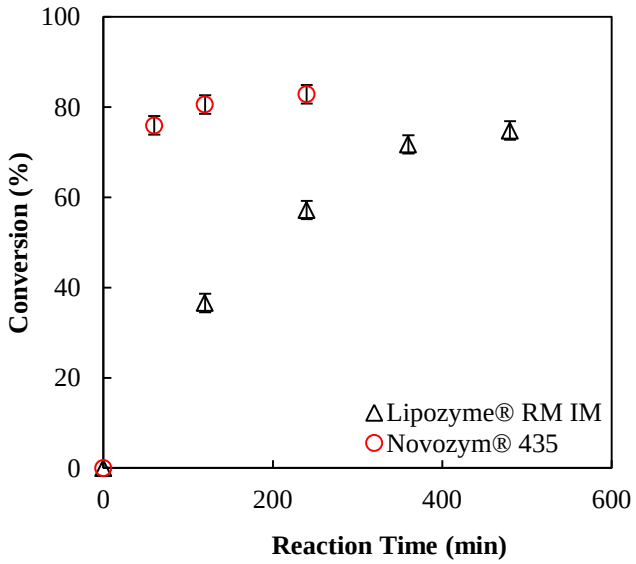

FIGURE 17 – EXPERIMENTAL RESULTS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID, N-HEXANE AND 20% OF BIOCATALYST (WT./WT.) IN THE BSTR: COMPARISON OF RESULTS OBTAINED AT 55 °C AND MOLAR RATIO OF 1.0:1.0, USING DIFFERENT COMMERCIAL LIPASES.

The difference in the conversion using different lipases lies in intrinsic discrepancies between the enzymes. According to Nelson, Foglia and Marmer (1996), lipases from *Mucor miehei* are more efficient hydrolyzing esters, while lipases from *Candida antarctica* are better for transesterification. Besides that, in the case of Lipozyme® RM IM, the flapping lid of this lipase protrudes into the binding pocket nearby, producing steric hindrance in the binding site, which is called interfacial inactivation (SALVI, KAMBLE and YADAV, 2017). On the other hand, the flapping lid is inexistent in Novozym® 435 and, therefore, less steric hindrance happens, which leads to higher conversions (SALVI, KAMBLE and YADAV, 2017). In addition, Novozym® 435 bears a chemical environment around the lipase active site, resulting in the correct interaction of the substrate with the enzyme, also a small quantity of water remains in the matrix pores of this biocatalyst, which contributes to maintaining this lipase structural stability (SALVI, KAMBLE and YADAV, 2017).

Finally, since Novozym® 435 presented a higher activity, the concentration of enzyme towards the mass of reactants was reduced from 20% (wt./wt.) to 10% (wt./wt.). The results are presented in FIGURE 18 and demonstrated, as expected, that when 20% of enzyme (wt./wt.) was used, a higher conversion was achieved in less reaction time (75.9% after 60 min). However, after 120 min of reaction, both conditions resulted in similar conversions, in such a way that when 10% of Novozym® 435 (wt./wt.) was used, the conversion reached 83.7% after 240 min of reaction, while using 20% (wt./wt.) the conversion for the same reaction time was 82.9% because enzyme saturation is reached at 10% as well. Therefore, aiming for a more economically attractive synthesis and considering that when there is a high concentration of biocatalyst an enzymatic inhibition may occur, further esterification reactions could be conducted using 10% of enzyme (wt./wt.).

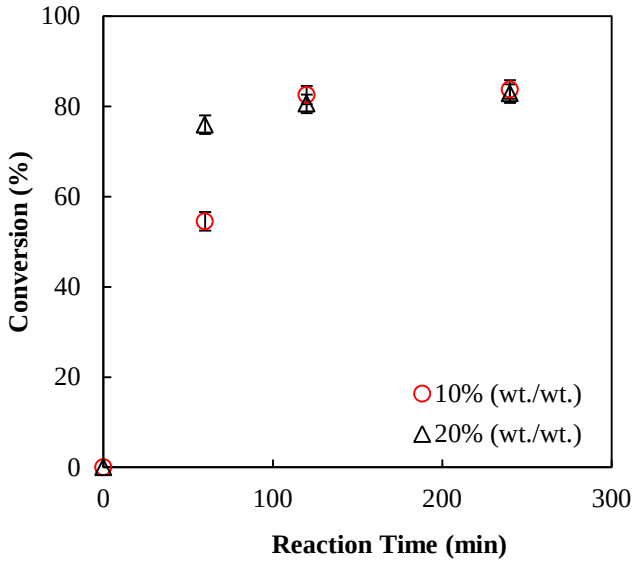

FIGURE 18 – EXPERIMENTAL RESULTS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID AND N-HEXANE IN THE BSTR: COMPARISON OF RESULTS OBTAINED AT 55 °C AND MOLAR RATIO OF 1.0:1.0 FOR DIFFERENT CONCENTRATIONS OF NOVOZYM® 435.

5.3 GERANYL ACETATE SYNTHESIS USING SUPERCRITICAL CO_2 AS SOLVENT

In order to substitute the harmful n-hexane as the solvent for the esterification, supercritical carbon dioxide ($scCO_2$) was employed, due to its qualities and properties prior explained. Preliminary experiments were performed using the BSTR, at 55 °C, reactants molar ratio of 1.0:1.0, 10.0 MPa, 240 min of reaction and using both biocatalysts already employed in this work. The esterification reaction between geraniol and acetic acid using 20% of Lipozyme® RM IM (wt./wt.) as biocatalyst and 39.0 g of $scCO_2$ in the Parr® reactor did not show any conversion, while using 20% of Novozym® 435 (wt./wt.) and 38.7 g of $scCO_2$, 12.0% of conversion was obtained. The difference observed in the results provided by the two biocatalysts was explained in the previous section.

Furthermore, an additional experiment was performed at 55 °C, reactants molar ratio of 1.0:1.0 and 10% of Novozym® 435 (wt./wt.) but with the addition of a small fraction of the previous amount of $scCO_2$ (19.6 g), which corresponds to a mass ratio of reactants to CO_2 of 0.6. Due to the experimental apparatus, the pressure inside the BSTR has a direct relationship with the amount of $scCO_2$ fed to the reaction system. Even though the role of CO_2 in esterification reactions is not entirely known, apparently a higher amount of such solvent is

prejudicial to the reactions, due to the dilution of both catalysts and reactants (MELFI et al., 2020).

For the aforementioned reaction, the conversion after 240 min was 60.5%, demonstrating, once again, the negative effect of a great excess of CO_2 on the conversion of acids. This value of conversion is still worse than that obtained when n-hexane was used as solvent, showing similar behavior to that presented by Peres, Gomes da Silva and Barreiros (2003), who used $scCO_2$ and scEthane and noticed that as CO_2 has more affinity with water, rather than higher chain hydrocarbons, the water formed remains in the reaction media.

Therefore, to thoroughly investigate the effects of both the pressure and the amount of CO_2, another experimental apparatus was required. The next set of experiments using $scCO_2$ as solvent was conducted in a Variable Volume Reactor (VVR), following a 2^3 experimental design with triplicate in the central point (TABLE 8), in which three reaction parameters were evaluated: temperature, pressure, and reactants to CO_2 mass ratio. The biocatalyst employed was only Novozym® 435 due to the low conversion observed when Lipozyme® RM IM was used. Agitation and reactants molar ratio were kept constant at 600 RPM and 1.0:1.0, respectively, and time of reaction was 60 min for all runs.

TABLE 8 – EXPERIMENTAL DESIGN OF REACTIONS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID AND $SCCO_2$ IN THE VVR, MOLAR RATIO OF 1.0:1.0 AND REACTION TIME OF 60 MIN, WITH RESPONSES IN TERMS OF CONVERSION OF GERANIOL.

Run	Temperature (°C)	Pressure (MPa)	Mass of Reactants/CO_2	Conversion (%)
P1	45	8.0	0.2	25.8
P2	65	8.0	0.2	43.1
P3	45	16.0	0.2	30.9
P4	65	16.0	0.2	46.9
P5	45	8.0	1.0	19.9
P6	65	8.0	1.0	44.4
P7	45	16.0	1.0	24.1
P8	65	16.0	1.0	45.4
P9	55	12.0	0.6	29.2
P10	55	12.0	0.6	29.4
P11	55	12.0	0.6	31.1

As there is a frontal window in the VVR, the system can be visually inspected to verify the homogeneity and mixture of the system. Thus, during a screening experiment in this reactor, the system was pressurized at 10.0 MPa and, at first, the reaction media was

homogeneous. However, after 60 min of reaction, probably due to water and ester formation, a two-phase (liquid-liquid) system was formed. Therefore, the pressure was increased to 12.0 MPa to ensure homogeneity and efficient mass transport in the system and this new value was used as the pressure of the central point.

When the pressure was 8.0 MPa, there was a vapor-liquid-solid system, which may diminish mass and heat transfer, as the enzyme and reactants are not mixed with CO_2, as can be seen in FIGURE 19(a). This might explain why, when maintaining other parameters constant, the conversion was slightly lower when pressure was 8.0 MPa. At 12.0 MPa and 16.0 MPa, FIGURE 19(b) and FIGURE 19(c), respectively, the reaction system comprises a single liquid phase (composed by reactants and solvent) in contact with the solid enzyme, enhancing mass and heat transfer, what favors the esterification.

(a) (b) (c)

FIGURE 19 – INSIDE VIEW OF THE VVR FOR REACTIONS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL USING ACETIC ACID AND $SCCO_2$: (a) VAPOR-LIQUID-SOLID REACTION SYSTEM AT 8.0 MPa (AGITATION OFF); (b) LIQUID-SOLID PHASE AT 12.0 MPa (AGITATION ON); AND (c) LIQUID-SOLID PHASE AT 16.0 MPa (AGITATION OFF).

For such experiments, the best conversion (46.9%) was achieved when the temperature was 65 °C, the pressure was 16.0 MPa and the mass ratio of reactants to CO_2 was 0.2. A comparison with literature data indicated that better conversions were obtained in the present work compared to studies performed either in VVR, as Peres, Gomes da Silva and Barreiros (2003) obtained less than 25%, or in BSTR, as conversions presented by Couto et al. (2011) were lower than 20% after 60 min. As compared to the work of Peres, Gomes da Silva and Barreiros (2003), the main difference of the experimental procedure is that in this work the pressure was not related to the amount of CO_2 added to the system.

Moreover, the conversion results presented in TABLE 8 were statistically evaluated by analysis of variance (ANOVA), tested using the software Statistica for Windows 6.0

(Statsoft Inc., USA) to measure the main effects of the variables. According to the Pareto Chart, presented in FIGURE 20, only temperature is statistically relevant to the reaction.

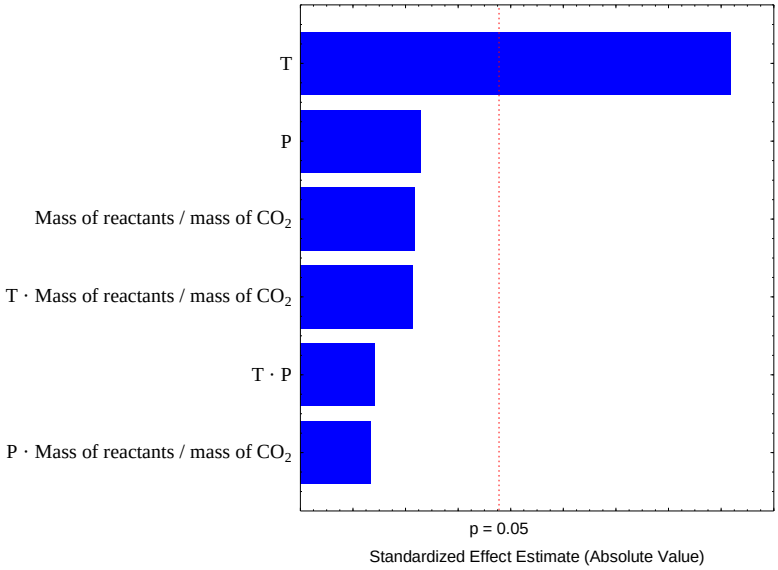

FIGURE 20 – PARETO CHART OF STANDARDIZED EFFECT FOR REACTIONS PERFORMED IN THE VVR FOLLOWING THE EXPERIMENTAL DESIGN, WITH RESPONSES IN TERMS OF CONVERSION OF GERANIOL.

The results reported in TABLE 8 also indicated different behaviors depending on the pressure, since at 45 °C and 8.0 MPa, the lower reactants to CO_2 mass ratio (or higher amount of CO_2) resulted in the higher conversion, while at 45 °C and 16.0 MPa, the behavior was the opposite, as higher reactants to CO_2 mass ratio (or lower amount of CO_2) presented a higher conversion. At 65 °C, the pressure effect was override and the differences obtained for the conversions lied within the experimental error. Therefore, some of the conditions presented in TABLE 8 were further studied for other reaction times, to evaluate the effects of both pressure and CO_2 amount. In this sense, reactions were performed using the conditions of the central point (P9 to P11) and of reactions P4 (in which higher conversions were obtained) and P8 (so as to evaluate the effect of the amount of CO_2).

The results obtained can be seen in FIGURE 21 and indicated that when a higher amount of CO_2 was employed, a higher conversion was obtained only for a reaction time of 30 min. However, after this reaction time, every other conversion values were nearly identical. Furthermore, until 120 min, conversion values were lower for reactions performed at 55 °C, but after 240 min, this reaction presented similar conversions to those obtained at 65 °C. The results presented in FIGURE 21 are shown as symbols, representing average values for the conversion of geraniol, and error bars that are equal to average ± 3.21 which represent

the expanded uncertainties with 95% confidence level. This value was calculated by multiplying the average experimental standard deviation obtained by 4.30 and dividing by the square root of the number of samples (t for a probability of 0.05 and degree of freedom equal to 2, as the analyses were performed in triplicates) (HECKERT et al., 2002).

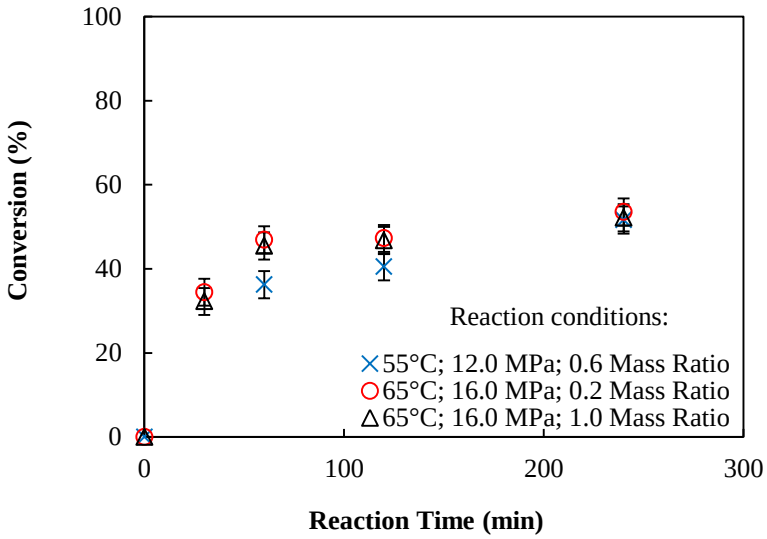

FIGURE 21 – EXPERIMENTAL RESULTS OF GERANYL ACETATE SYNTHESIS FROM THE ESTERIFICATION OF GERANIOL AND ACETIC ACID USING $SCCO_2$ WITH MOLAR RATIO OF 1.0:1.0 AND 10% OF NOVOZYM® 435 (WT./WT.) IN THE VVR.

Yet, this conversion obtained still lower than some described in the literature. So, to investigate the thermodynamic properties of the system and understand better the system behavior, phase equilibrium measurement experiments were conducted.

5.4 PHASE EQUILIBRIUM MEASUREMENT AND MODELING

Knowledge about phase behavior during the synthesis of geranyl acetate is fundamental to comprehend the experimental results. Therefore, as there is a lack of this information in the literature, phase equilibrium measurements and modeling of the {CO_2(1) + geraniol(2)} binary system and the {CO_2(1) + geraniol(2) + ethyl acetate(3)} and {CO_2(1) + geraniol(2) + acetic acid(3)} ternary systems were conducted and presented below. The results are also presented in Tavares et al. (2020).

5.4.1 Phase equilibrium measurement and modeling for the binary system {CO_2(1) + geraniol(2)}

TABLE 9 and TABLE 10 present the experimental phase transition data for the binary system {CO_2(1) + geraniol(2)} at temperatures of 303 K to 343 K. TABLE 9 is organized by isotherms and molar fraction of CO_2 (x_1) and geraniol (x_2). TABLE 10 shows transition points where a vapor-liquid-liquid equilibrium (VLLE) was observed.

TABLE 9 – PHASE EQUILIBRIUM MEASUREMENTS FOR THE BINARY SYSTEM {CO_2(1) + GERANIOL(2)}

x_1	x_2	p/MPa	σ/MPa	Transition type	x_1	x_2	p/MPa	σ/MPa	Transition type
T = 303.2 ± 0.3 K									
0.4119	0.5881	4.05	0.05	VLE(BP)	0.7949	0.2051	15.19	0.02	LLE
0.4990	0.5010	4.91	0.10	VLE(BP)	0.8187	0.1813	16.96	0.05	LLE
0.6111	0.3889	6.07	0.02	VLE(BP)	0.9006	0.0994	20.55	0.20	LLE
0.6991	0.3009	7.57	0.05	LLE	0.9209	0.0791	18.97	0.25	LLE
0.7505	0.2495	10.47	0.06	LLE	0.9325	0.0675	18.21	0.25	LLE
T = 313.1 ± 0.2 K									
0.4119	0.5881	4.69	0.08	VLE(BP)	0.7949	0.2051	14.66	0.02	VLE(BP)
0.4990	0.5010	5.90	0.04	VLE(BP)	0.8187	0.1813	15.98	0.09	VLE(BP)
0.6111	0.3889	7.22	0.03	VLE(BP)	0.9006	0.0994	17.87	0.16	VLE(DP)
0.6991	0.3009	9.21	0.01	VLE(BP)	0.9209	0.0791	17.26	0.06	VLE(DP)
0.7505	0.2495	11.22	0.05	VLE(BP)	0.9325	0.0675	16.85	0.02	VLE(DP)
T = 323.1 ± 0.2 K									
0.4119	0.5881	5.42	0.02	VLE(BP)	0.7949	0.2051	14.93	0.04	VLE(BP)
0.4990	0.5010	6.92	0.02	VLE(BP)	0.8187	0.1813	15.60	0.11	VLE(BP)
0.6111	0.3889	8.70	0.06	VLE(BP)	0.9006	0.0994	17.48	0.02	VLE(DP)
0.6991	0.3009	10.75	0.03	VLE(BP)	0.9209	0.0791	17.32	0.07	VLE(DP)
0.7505	0.2495	12.54	0.07	VLE(BP)	0.9325	0.0675	17.16	0.01	VLE(DP)
T = 333.1 ± 0.3 K									
0.4119	0.5881	6.05	0.08	VLE(BP)	0.7949	0.2051	15.87	0.04	VLE(BP)
0.4990	0.5010	7.81	0.04	VLE(BP)	0.8187	0.1813	16.67	0.06	VLE(BP)
0.6111	0.3889	9.89	0.09	VLE(BP)	0.9006	0.0994	18.29	0.02	VLE(BP)
0.6991	0.3009	12.35	0.02	VLE(BP)	0.9209	0.0791	18.08	0.12	VLE(DP)
0.7505	0.2495	13.90	0.05	VLE(BP)	0.9325	0.0675	17.80	0.07	VLE(DP)
T = 343.0 ± 0.3 K									
0.4119	0.5881	6.69	0.06	VLE(BP)	0.7949	0.2051	17.19	0.08	VLE(BP)
0.4990	0.5010	8.49	0.06	VLE(BP)	0.8187	0.1813	17.59	0.06	VLE(BP)
0.6111	0.3889	11.13	0.03	VLE(BP)	0.9006	0.0994	19.44	0.01	VLE(BP)
0.6991	0.3009	13.94	0.04	VLE(BP)	0.9209	0.0791	19.25	0.04	VLE(DP)
0.7505	0.2495	15.53	0.07	VLE(BP)	0.9325	0.0675	19.09	0.06	VLE(DP)

Standard uncertainties u are $u(T)$ = 0.5 K, $u(p)$ = 0.20 MPa, $u(x)$ = 0.005. σ/MPa represents the standard deviation of triplicate measurements of phase transition for the same condition. VLE(BP) are representing bubble point transitions, VLE(DP) dew point transitions, LLE represents liquid to two-liquid phases transitions (liquid-liquid equilibrium).

TABLE 10 – LL → VLL PHASE TRANSITIONS FOR THE BINARY SYSTEM {CO_2(1) + GERANIOL(2)}

T/K	*p*/MPa	*σ*/MPa
x_1 = 0.9006		
303.1	6.45	0.30
305.1	7.18	0.25
306.9	7.39	0.30
309.1	7.74	0.28
x_1 = 0.9325		
302.1	6.88	0.31
304.6	7.24	0.20
306.3	7.50	0.25
307.8	7.79	0.28
308.6	7.89	0.30
309.7	8.05	0.30

Standard uncertainties *u* are *u(T)* = 0.5 K, *u(p)* = 0.20 MPa, *u(x)* = 0.005. σ/MPa represents the standard deviation of triplicate measurements of phase transition for the same condition. LL represents two-liquid phases coexistence (liquid-liquid equilibrium) to vapor-liquid-liquid three-phases coexistence (VLL).

For the binary system {CO_2 (1) + geraniol (2)}, at all temperatures examined, the dew points were only detected when the molar fraction of CO_2 was above 0.81. For this binary system, liquid-liquid equilibrium (LLE) was detected at 303.2 K and when x_1 ranged from 0.69 to 0.93. Liquid-liquid to vapor-liquid-liquid phase transition was observed when the molar fraction of CO_2 was 0.90 and 0.93 for the 303.1 K to 309.7 K temperature range (TABLE 10).

For the ternary system {CO_2 (1) + geraniol (2) + ethyl acetate (3)} bubble point transitions (V → VL) were experimentally observed at temperatures up 313 K. Heterogeneous phase transitions were detected at higher temperatures (above 313 K) and high CO_2 molar fraction, i.e., for this ternary system, only when the temperature was 323.2 K or higher and the molar fraction of CO_2 was 0.89, LLE to VLLE or vapor (one-phase) to LLE transition occurred. On the other hand, for the ternary system {CO_2(1) + geraniol(2) + acetic acid(3)} bubble points, regarding V → VL transitions, were experimentally observed at all temperatures evaluated. Comparing the binary system {CO_2(1) + geraniol(2)} with its associated ternary systems adding either ethyl acetate or acetic acid, it is observed that the acetic acid acts as a better cosolvent decreasing the liquid-liquid immiscibility between CO_2 and geraniol. It is due to the hydrogen bonds between a carboxylic acid group with the alcohol and with the electron pairs available on CO_2, and due to the dispersion energy between the molecules involved in these ternary systems. Therefore, from the results presented in TABLE 9, 10, 12 and 13, it can be observed that different reaction approaches, i.e., whether geraniol esterification or transesterification reactions are designed to work in a supercritical CO_2

process, different conditions of CO_2 to reactants ratio, temperature and pressure must be set in order to get homogeneous phase conditions for the reaction. Using acetic acid as a reactant should demand lower conditions of pressure when compared to transesterification reaction using ethyl acetate to perform the geraniol esterification within a homogeneous region. Furthermore, the phase behavior of these reactant systems must be taken into account to design and optimize, mainly, enzyme-catalyzed geraniol (trans)esterification reactions using supercritical CO_2 as a solvent.

After all phase transition measurements for the systems presented in this study, samples of the liquid solute phase were withdrawn after releasing the CO_2 from the view cell, and these samples were analyzed by GC, aiming to detect any ester formation (geranyl acetate) or side components, as presented in section 4.3. Thus, for all samples, geraniol esterification or transesterification reactions or any side components were not detected by gas chromatography for both {CO_2 (1) + geraniol (2) + ethyl acetate (3)} and {CO_2 (1) + geraniol (2) + acetic acid (3)} ternary systems and for the {CO_2 (1) + geraniol (2)} binary system while and after phase equilibrium data acquisition. All results have demonstrated that the system was inert during the period of phase equilibrium measurements.

Thermodynamic modeling using the PR model was performed by fitting a unique set of binary interaction parameters for all isotherms (global fitting) for the binary system {CO_2(1) + geraniol(2)}, and in addition, temperature-dependent binary interaction parameters were obtained as well. TABLE 11 summarizes all the binary interaction parameters used or obtained, where k_{ij} represents the binary energy parameter and l_{ij} the repulsive energy parameter. CO_2 + ethyl acetate binary parameters in the {CO_2(1) + geraniol(2) + ethyl acetate(3)} ternary system are from Kloc et al. (2019). The root-mean-square-deviation (*rmsd*) was calculated using Eq. (18), where $P_{cal,i}$ and $P_{exp,i}$ represent, respectively, the saturation pressure calculated and obtained experimentally in the "i" measurement and *NOBS* the number of experimental observations.

$$rmsd = \sqrt{\sum_{i=1}^{NOBS} \left(P_{cal,i} - P_{exp,i}\right)^2} \tag{18}$$

TABLE 11 – FITTED INTERACTION PARAMETERS OF THE PENG-ROBINSON USED IN THIS WORK

System	*T* / K	k_{12}	l_{12}	*rmsd*/MPa
{CO_2(1)+ geraniol(2)}	303.15 – 343.15 K	0.0748	-0.0246	1.48
		$0.1807 - 0.3335x10^{-3}T$	$-0.5186x10^{-3} - 0.4704x10^{-3}T$	0.32
{CO_2(1) + ethyl acetate(2)}[a]	313 – 353 K	-0.0171	-0.0334	0.10
{CO_2(1) + acetic acid(2)}[b]	313.2 – 353.2 K	-0.0103	-0.0424	0.12

[a] Interaction parameters fitted using experimental data presented by Kloc et al. (2019). Interaction parameters fitted using VLE data presented by Bamberger, Sieder and Maurer (2000).

When the binary interaction parameters were fixed for the binary system {CO_2(1) + geraniol(2)}, the model did not accurately predict the phase behavior of this system. However, when these parameters are temperature-dependent, the PR model with vdW2 mixing rule was capable of fitting well the complex phase behavior experimentally observed, including the prediction of VLE, LLE, and VLLE with a unique set of binary interaction parameters (k_{12} and l_{12}). These increases in the PR equation of state accuracy, taking into account the binary interaction parameters with temperature can be observed as the root-mean-square-deviation reduces from 1.48 to 0.32. As geraniol is a polar molecule, its intermolecular interactions with the system involve hydrogen bonds, quadrupolar-polar interactions, which are affected by temperature.

FIGURE 22 depicts the pressure-composition diagram for the binary system {CO_2(1) + geraniol(2)} at 303.2 K. In this phase diagram, the experimental data is compared to the calculated using the PR model, where the continuous line represents the saturation curve. The area between the dashed line and the continuous line corresponds to the observed liquid-liquid equilibrium. As can be inferred by FIGURE 22, the model proposed is in accordance with the experimental data. It is worth mentioning that the system aforementioned possess an upper critical endpoint (UCEP) located next to 309 K, as exemplified in FIGURE 23(a) and suggested by experimental data in TABLE 10. From the experimental results presented in TABLE 9 and 10, and prediction using the PR equation of state, it can be observed that CO_2 + geraniol presents a type-III diagram according to the classification of Scott and van Konynenburg (1980). This type-III diagram behavior is confirmed by FIGURE 22, which, when magnified to the region near 0.995 molar fraction (x_1), shows a three-phase characteristic as visually depicted in FIGURE 23(b). This system behavior was confirmed by experiments.

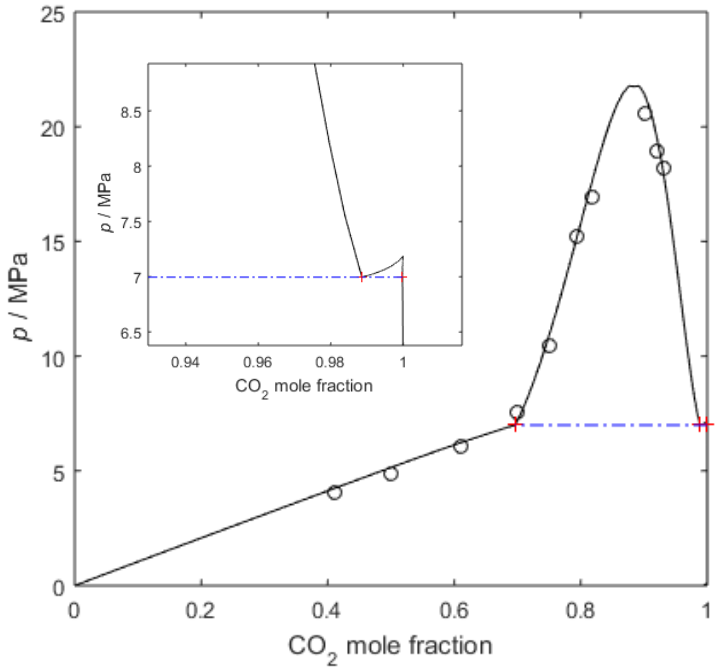

FIGURE 22 – PRESSURE-COMPOSITION DIAGRAM OF {CO_2(1) + GERANIOL(2)} BINARY SYSTEM AT 303.17 K (○, THIS WORK) SHOWING A ZOOM IN THE VLE AT HIGH MOLE FRACTION OF CO_2 (ABOVE 0.93). RED CROSSES AND BLUE DOTTED LINE ARE PRESENTING THE VLLE CALCULATED WITH THE PR EQUATION OF STATE
SOURCE: TAVARES et al. (2020).

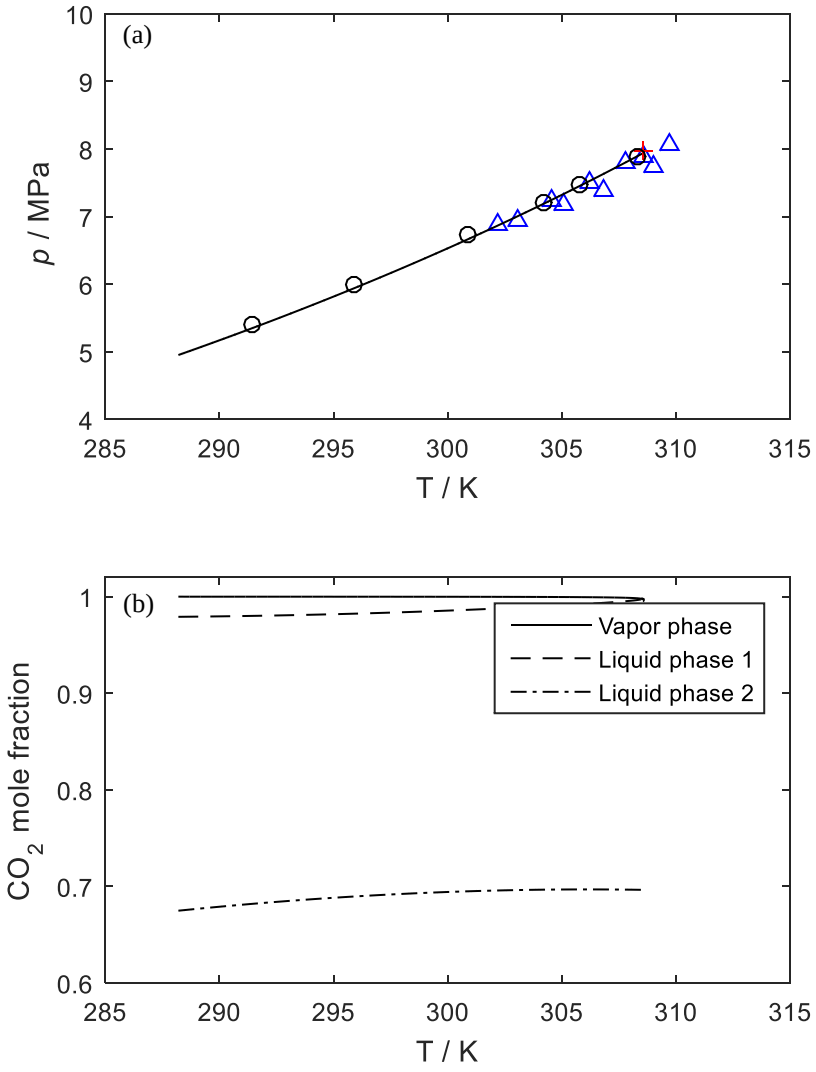

FIGURE 23 – THREE-PHASE DIAGRAM OF {CO_2 (1) + GERANIOL(2)}. (a) COMPARISON BETWEEN VLLE EXPERIMENTAL DATA (△, THIS WORK; ○, TUFEU et al. (1993)) AND THREE-PHASES CALCULATED LINE AND (b) THREE-PHASES COMPOSITIONS CALCULATED USING THE PR EQUATION OF STATE. THE RED CROSS REPRESENTS THE CALCULATED UCEP.
SOURCE: TAVARES et al. (2020).

For temperatures equal or higher of 313 K, liquid-liquid equilibrium (LLE) was not observed, only vapor-liquid equilibrium (VLE). FIGURE 24(a-d) summarizes the phase equilibrium-curves, highlighting the behavior differences when the temperature is above the UCEP condition, in a pressure-composition diagram comparing the experimental data and calculated values using the PR equation of state.

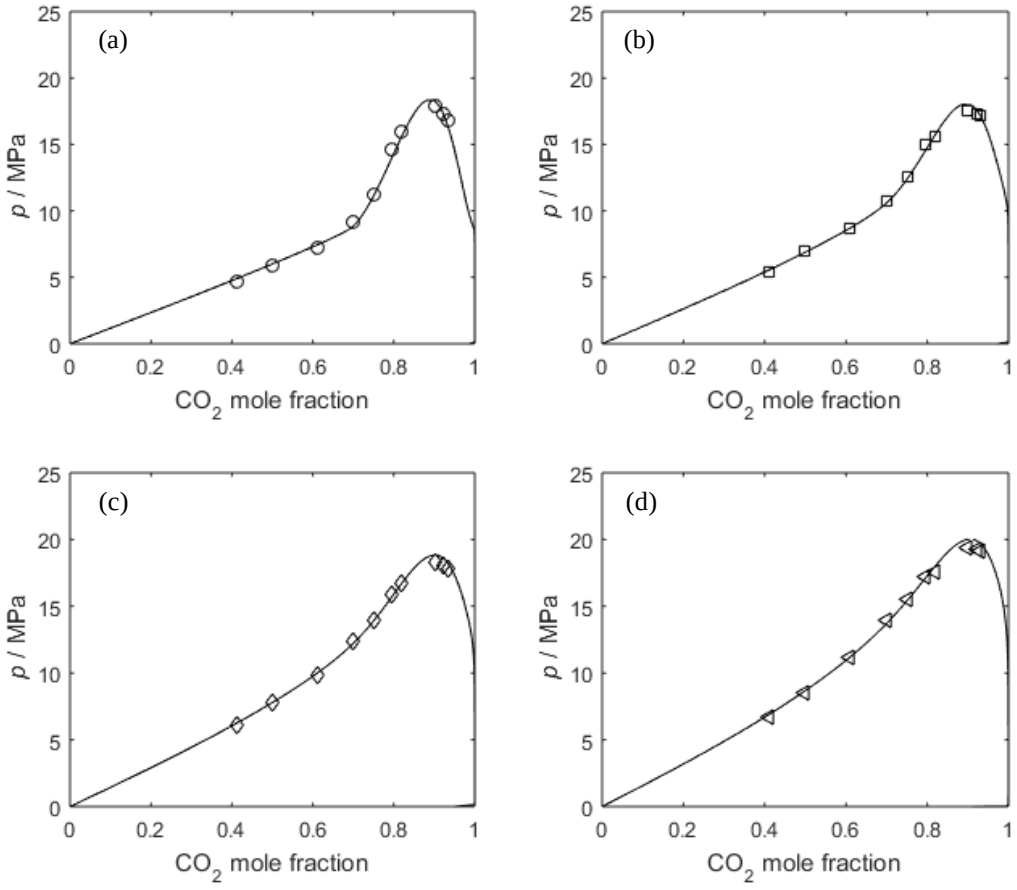

FIGURE 24 – PRESSURE-COMPOSITION DIAGRAM OF {CO_2(1) + GERANIOL} AT (a) 313 K, (b) 323 K, (c) 333 K AND (d) 343 K. COMPARISON BETWEEN EXPERIMENTAL DATA (SYMBOLS) AND PR MODEL (LINES) USING TEMPERATURE-DEPENDENT BINARY INTERACTION PARAMETERS (TABLE 11)
SOURCE: TAVARES et al. (2020).

Tufeu, Subra and Plateaux (1993) and Lamba et al. (2016) have also studied the CO_2 and geraniol binary system. Lamba et al. (2016) presented solubility data of geraniol in supercritical carbon dioxide at temperatures from 308 to 333 K and pressures from 10 to 18 MPa, where the highest solubility (y_2 = 0.0252 mol.mol^{-1}) was found at 308 K and 18 MPa. Those authors reported that the solubility increased as the temperature decreased and the pressured increased. Tufeu, Subra and Plateaux (1993), on the other hand, presented experimental data of critical and three-phase line for the system CO_2 + geraniol, and as it can be seen in FIGURE 23(a), the three-phase data presented in this work are in agreement with those presented by Tufeu, Subra and Plateaux (1993).

5.4.2 Phase equilibrium measurements for the ternary systems {CO2(1) + geraniol(2) + ethyl acetate(3)} and {CO_2(1) + geraniol(2) + acetic acid(3)}

TABLE 12 and TABLE 13 present the phase transitions measurements for the ternary systems {CO_2(1) + geraniol(2) + ethyl acetate(3)} and {CO_2(1) + geraniol(2) + acetic acid(3)}, respectively, at different fixed geraniol to acetic acid (or ethyl acetate) molar ratios.

TABLE 12 – PHASE EQUILIBRIUM MEASUREMENTS FOR THE TERNARY SYSTEM {CO_2(1) + GERANIOL(2) + ETHYL ACETATE(3)}

x_1	x_2	x_3	p/MPa	σ/MPa	Transition type
T = 303.1 ± 0.2 K					
0.6011	0.1996	0.1993	4.28	0.06	VLE(BP)
0.6996	0.1503	0.1501	4.95	0.04	VLE(BP)
0.8002	0.1000	0.0998	5.77	0.07	VLE(BP)
0.8993	0.0504	0.0503	6.39	0.04	VLE(BP)
T = 313.2 ± 0.3 K					
0.6011	0.1996	0.1993	5.32	0.00	VLE(BP)
0.6996	0.1503	0.1501	6.03	0.01	VLE(BP)
0.8002	0.1000	0.0998	6.88	0.02	VLE(BP)
0.8993	0.0504	0.0503	7.75	0.03	VLE(BP)
T = 323.2 ± 0.3 K					
0.6011	0.1996	0.1993	6.12	0.03	VLE(BP)
0.6996	0.1503	0.1501	7.03	0.05	VLE(BP)
0.8002	0.1000	0.0998	8.24	0.06	VLE(BP)
0.8993	0.0504	0.0503	8.86	0.09	LLE → VLLE
0.8993	0.0504	0.0503	9.90	0.05	V → LLE
T = 333.1 ± 0.4 K					
0.6011	0.1996	0.1993	7.00	0.08	VLE(BP)
0.6996	0.1503	0.1501	8.13	0.04	VLE(BP)
0.8002	0.1000	0.0998	9.71	0.06	VLE(BP)
0.8993	0.0504	0.0503	12.00	0.03	V → LLE
T = 343.1 ± 0.3 K					
0.6011	0.1996	0.1993	8.34	0.10	VLE(BP)
0.6996	0.1503	0.1501	9.38	0.01	VLE(BP)
0.8002	0.1000	0.0998	11.47	0.06	VLE(BP)
0.8993	0.0504	0.0503	14.18	0.07	V → LLE

Standard uncertainties u are $u(T)$ = 0.5 K, $u(p)$ = 0.20 MPa, $u(x)$ = 0.005. σ/MPa represents the standard deviation of triplicate measurements of phase transition for the same condition. VLE(BP) are representing bubble point transitions, VLE(DP) dew point transitions, LLE represents two-liquid phases coexistence (liquid-liquid equilibrium), and VLLE represents three-phases coexistence (VLL).

TABLE 13 – PHASE EQUILIBRIUM MEASUREMENTS FOR THE TERNARY SYSTEM {CO_2(1) + GERANIOL(2) + ACETIC ACID(3)}

x_1	x_2	x_3	p/MPa	σ/MPa	Transition type
T = 303.1 ± 0.2 K					
0.5526	0.2238	0.2236	4.99	0.02	VLE(BP)
0.5985	0.2008	0.2007	5.54	0.00	VLE(BP)
0.7003	0.1499	0.1498	5.88	0.01	VLE(BP)
0.8000	0.1000	0.1000	5.91	0.01	VLE(BP)
T = 313.1 ± 0.3 K					
0.5526	0.2238	0.2236	6.12	0.03	VLE(BP)
0.5985	0.2008	0.2007	6.81	0.00	VLE(BP)
0.7003	0.1499	0.1498	7.30	0.05	VLE(BP)
0.8000	0.1000	0.1000	7.35	0.09	VLE(BP)
T = 323.1 ± 0.3 K					
0.5526	0.2238	0.2236	7.01	0.02	VLE(BP)
0.5985	0.2008	0.2007	7.94	0.02	VLE(BP)
0.7003	0.1499	0.1498	8.78	0.06	VLE(BP)
0.8000	0.1000	0.1000	9.62	0.09	VLE(BP)
T = 333.1 ± 0.3 K					
0.5526	0.2238	0.2236	8.35	0.02	VLE(BP)
0.5985	0.2008	0.2007	9.30	0.03	VLE(BP)
0.7003	0.1499	0.1498	10.63	0.08	VLE(BP)
0.8000	0.1000	0.1000	11.92	0.08	VLE(BP)
T = 343.0 ± 0.2 K					
0.5526	0.2238	0.2236	9.60	0.07	VLE(BP)
0.5985	0.2008	0.2007	10.62	0.07	VLE(BP)
0.7003	0.1499	0.1498	12.51	0.07	VLE(BP)
0.8000	0.1000	0.1000	14.01	0.08	VLE(BP)

Standard uncertainties u are $u(T)$ = 0.5 K, $u(p)$ = 0.20 MPa, $u(x)$ = 0.005. σ/MPa represents the standard deviation of triplicate measurements of phase transition for the same condition. VLE(BP) are representing bubble point transitions, VLE(DP) dew point transitions, LLE represents two-liquid phases coexistence (liquid-liquid equilibrium), and VLLE represents three-phases coexistence (VLL).

The ternary systems, {CO_2(1) + geraniol(2) + ethyl acetate(3)} and {CO_2(1) + geraniol (2) + acetic acid(3)}, are represented by pressure-temperature diagrams in FIGURE 25 and FIGURE 26, respectively, where the experimental data are compared to predicted values using the PR equation of state with temperature-dependent binary interaction parameters (TABLE 11). In FIGURE 25, the phase fraction and CO_2 composition of three-phase equilibrium for the system {CO_2(1) + geraniol(2) + ethyl acetate(3)} is presented. For both ternary systems, the binary interaction parameters between geraniol – ethyl acetate and geraniol – acetic acid were set to zero (k_{23} = l_{23} = 0). It can be seen that the thermodynamic model proposed was capable of predict well the phase behavior of both ternary systems, considering only binary interaction parameters for CO_2 and solutes.

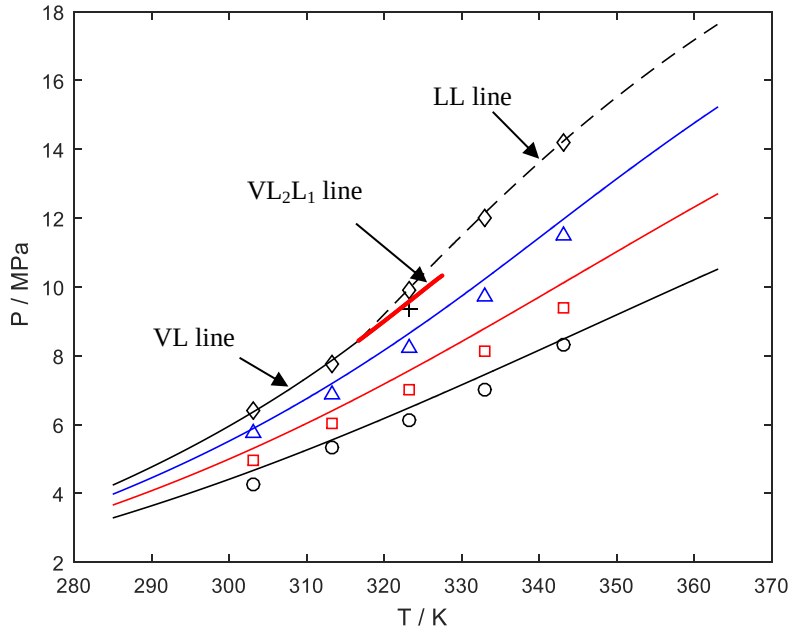

FIGURE 25 – PRESSURE-TEMPERATURE DIAGRAM OF {CO_2(1) + GERANIOL (2) + ETHYL ACETATE (3)} FOR CO_2 MOLAR FRACTION OF 0.60 (○), 0.69 (□), 0.80 (△), AND 0.89 (◇). COMPARISON BETWEEN EXPERIMENTAL DATA AND PR MODEL USING TEMPERATURE-DEPENDENT BINARY INTERACTION PARAMETERS FOR {CO_2(1) + GERANIOL(2)} AS PRESENTED IN TABLE 11
SOURCE: TAVARES et al. (2020).

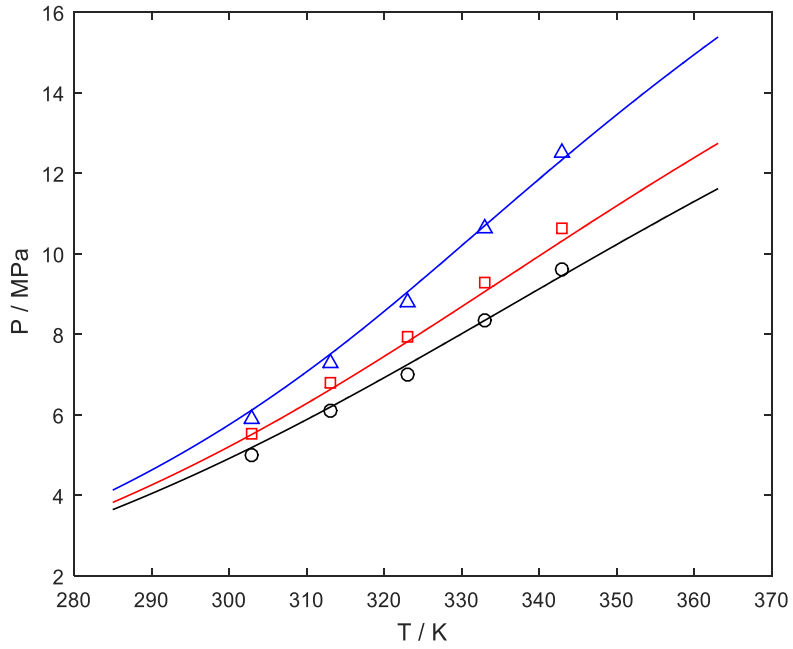

FIGURE 26 – PRESSURE-TEMPERATURE DIAGRAM OF {CO_2(1) + GERANIOL(2) + ACETIC ACID(3)} FOR CO_2 MOLAR FRACTION OF 0.55 (○), 0.59 (□), AND 0.70 (△). COMPARISON BETWEEN EXPERIMENTAL DATA AND PR MODEL USING TEMPERATURE-DEPENDENT BINARY INTERACTION PARAMETERS AS PRESENTED IN TABLE 11
SOURCE: TAVARES et al. (2020).

The ternary system {CO_2(1) + geraniol(2) + ethyl acetate(3)}, similarly to the {CO_2(1) + geraniol(2)} binary system, presents a type-III diagram behavior as shown in FIGURE 27(a) and (b), where both liquid-phases compositions merge to a critical phase. It exhibits an estimated UCEP of 327 K at 0.95 CO_2 molar fraction. FIGURE 27(a) displays the liquid-phase fractions for this ternary system.

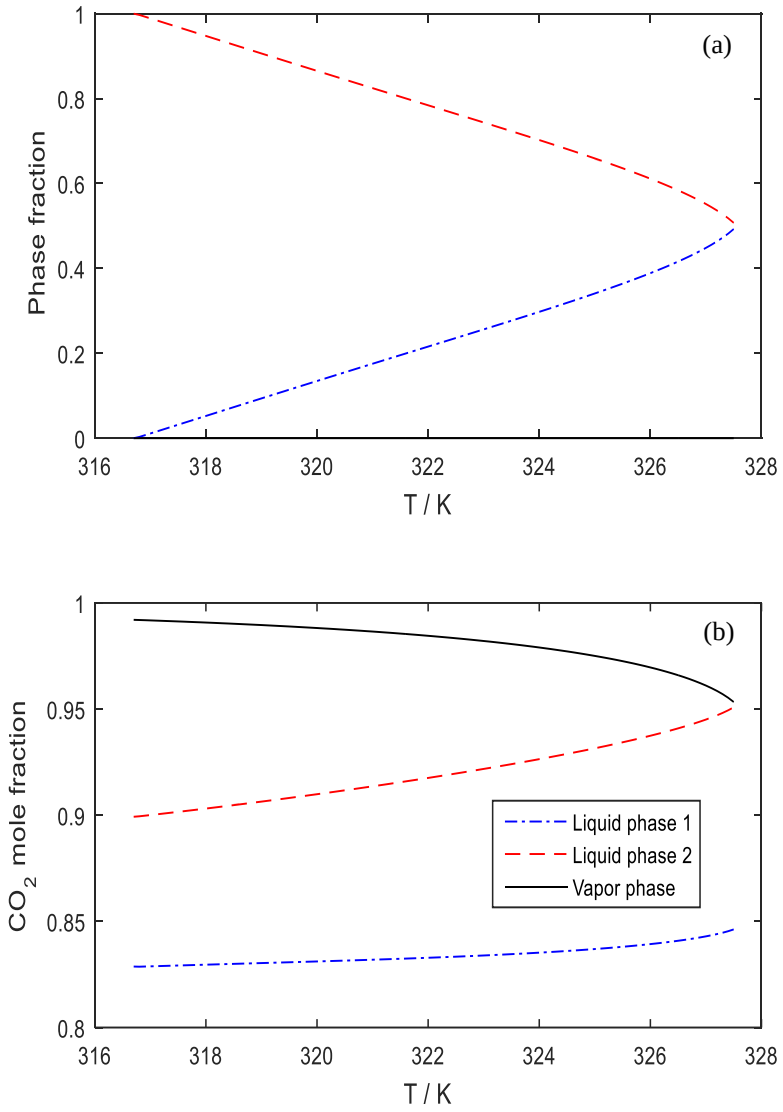

FIGURE 27 – THREE-PHASE FRACTIONS (a) AND (b) CO_2 COMPOSITIONS FOR THE SYSTEM {CO_2(1) + GERANIOL(2) + ETHYL ACETATE(3)}. CALCULATED VALUES USING THE PR EQUATION OF STATE CONSIDERING THE VAPOR PHASE FRACTION AS ZERO ($B^V = 0$)
SOURCE: TAVARES et al. (2020).

At last, the {CO_2(1) + geraniol (2) + acetic acid(3)} ternary system reveals a type-I vapor-liquid equilibrium (VLE) for all points measured (FIGURE 26). At 0.9 CO_2 molar fraction, no phase transition was were observed for the conditions investigated in this work.

Therefore, comparing the two ternary systems it is observed from FIGURE 25 and FIGURE 26 that the presence of ethyl acetate as cosolvent diminished the three-phase region and the immiscibility observed for the CO_2 + geraniol at high-pressures and high CO_2 concentrations, but the acetic acid addition brought the system down to another type of overall phase boundary, without liquid-liquid immiscibility.

5.5 FINAL CONSIDERATIONS

The experimental results obtained in the esterification of geraniol and acetic acid using Lipozyme® RM IM and Novozym® 435, employing n-hexane and scCO_2, and two batch-type reactors, a Batch Stirred Tank Reactor (BSTR) and a Variable Volume Reactor (VVR) were presented in the previous sections. For such reactions, a higher activity of Novozym® 435 was demonstrated, since this biocatalyst took nearly half the time to obtain similar conversions as Lipozyme® RM IM, as it was expected by literature review and intrinsic biocatalysts characteristic previously explained.

A comparison between esterification reactions performed with different solvents indicated that the presence of n-hexane increases the conversion, as compared to the presence of scCO_2. However, in possess of the phase equilibrium data, different reaction conditions using scCO_2, which may ensure single-phase condition, can be performed to reach higher conversions. In addition, mechanisms to remove water from the reaction media, such as the use of molecular sieves, could be implemented to achieve better results.

Concerning the type of reactor, the comparison between reactions performed in a BSTR and in a VVR indicated better results for the former. The ability to set and control the pressure independently of the amount of carbon dioxide added to the reaction system revealed to be crucial. As at higher pressures the homogeneity of the system is reached, if pressure is dependent on the amount of CO_2, a great amount of carbon dioxide must be inserted, which would diminish the esterification, as explained in section 5.3.

Among reaction conditions, the temperature has a major positive effect, while the pressure has a slight positive effect and the influence of the amount of CO_2 depends on both

temperature and pressure, indicating that the phase partition can play an important role in the conversion of esterification reaction employing $scCO_2$ as an alternative solvent.

Phase equilibrium data for the {CO_2(1) + geraniol(2)} binary system and for the {CO_2(1) + geraniol(2) + ethyl acetate(3)} and the {CO_2(1) + geraniol (2) + acetic acid(3)} ternary systems at temperatures ranging from 303.2 to 343.2 K were also presented. The experimental results obtained reinforced that at 16.0 MPa the reaction system is homogeneous, favoring the esterification as better mass and heat transport is provided. These results also indicate that when a great amount of carbon dioxide is used, higher pressure must be set to achieve the single-phase condition. Also, it was evidenced that using acetic acid as a reactant should demand lower conditions of pressure when compared to transesterification reaction using ethyl acetate to perform the geraniol esterification within a homogeneous region.

Also, vapor-liquid equilibria were observed in the binary and ternary systems investigated. Liquid-liquid equilibria were only observed for the binary system at 303.2 K when the molar fraction of CO_2 ranged from 0.6991 to 0.9325. For both ternary systems, only one-phase to two-phase transitions were observed, except when ethyl acetate was used, and the temperature was 323.2 K or higher and the molar fraction of CO_2 was 0.89. In those cases, LLE to VLLE or vapor to LLE transition occurred.

All the systems were adequately modeled with the Peng-Robinson cubic equation of state with the conventional quadratic van der Waals mixing rule (vdW2), indicating that this model is suitable for these systems. For the range of condition evaluated, the thermodynamic model was able to successfully represent the {CO_2(1) + geraniol(2) + ethyl acetate(3)} and the {CO_2(1) + geraniol (2) + acetic acid(3)} ternary systems, when employing a unique set of binary interaction parameters (k_{ij} and l_{ij}), both temperature-independent. However, for the {CO_2(1) + geraniol(2)} binary system, a temperature-dependent approach for the binary interaction parameters was necessary to represent the phase behavior of the system properly due to the intrinsic characteristics of the geraniol molecule, as explained in section 5.4.1.

Thus, considering the promising results obtained in the esterification reactions and the phase equilibrium measurements, it is interesting to consider further scale-up of the enzymatic esterification of geraniol and acetic acid to assess the reaction conversion. Batch esterification reactions may be conducted in a 100 mL Parr® reactor, because of the geometry reactor similarity, aiding the scale-up. Besides, keeping up with Bourkaib et al. (2018), the esterification reaction may be operated as a continuous process in a continuous

tubular reactor, which may enhance productivity and conversion as the product is continuously removed, boosting the esterification. Moreover, as water is produced during the esterification, which favors the hydrolysis of the freshly ester produced, the use of molecular sieves or other water removal methods may be considered to increase esterification in further experiments. Nonetheless, the investigation of the chemical equilibrium of the esterification reactions, through robust thermodynamic models, should be done in order to elicit some distinctive behaviors found and gather additional information about the esterification reactions, such as maximum theoretical conversion and the amount of water produced as a side product.

6 CONCLUSIONS

This work presented the results obtained for reactions of geraniol and acetic acid using Lipozyme® RM IM and Novozym® 435, employing n-hexane and $scCO_2$, and two batch-type reactors, a Batch Stirred Tank Reactor (BSTR) and a Variable Volume Reactor (VVR). For such reactions, a higher activity of Novozym® 435 was demonstrated, since this biocatalyst took nearly half the time to obtain similar conversions as Lipozyme® RM IM. A comparison between reactions performed with different solvents indicated that the presence of n-hexane increases the conversion, as compared to the presence of $scCO_2$. Concerning the type of reactor, the comparison between reactions performed in a BSTR and in a VVR indicated better results for the former.

Among reaction conditions, the temperature has a major positive effect, while the pressure has a slight positive effect and the influence of the amount of CO_2 depends on both temperature and pressure, indicating that the phase partition can play an important role in such reactions. At 65 °C, both pressure and amount of CO_2 show a small influence, considering the ranges used in this work for these parameters. Furthermore, when a great excess of CO_2 is used, this solvent seems to be prejudicial to the reactions.

This work also reported the phase equilibrium data for the binary system {CO_2(1) + geraniol(2)} binary system and for {CO_2(1) + geraniol(2) + ethyl acetate(3)} and {CO_2(1) + geraniol (2) + acetic acid(3)} ternary systems at temperatures ranging from 303.2 to 343.2 K. Vapor-liquid equilibria were observed in the binary and ternary systems investigated. Liquid-liquid equilibria were only observed for the binary system at 303.2 K when the molar fraction of CO_2 ranged from 0.6991 to 0.9325. Dew points were only detected for the binary system as well when the molar fraction of CO_2 was higher than 0.81 at all temperatures tested. Below this molar fraction, for the binary system, at all temperatures, and for all the points scrutinized in the ternary systems, bubble points were detected. For both ternary systems, only one-phase to two-phase transitions were observed, except when ethyl acetate was used, and the temperature was 323.2 K or higher and the molar fraction of CO_2 was 0.89. In those cases, LLE to VLLE or vapor to LLE transition occurred.

All the systems were adequately modeled with the Peng-Robinson cubic equation of state with the conventional quadratic van der Waals mixing rule (vdW2). For the range of condition evaluated, the thermodynamic model was able to successfully represent the {CO_2(1) + geraniol(2) + ethyl acetate(3)} and the {CO_2(1) + geraniol (2) + acetic acid(3)} ternary

systems, when employing a unique set of binary interaction parameters (k_{ij} and l_{ij}), both temperature-independent. However, for the {CO_2(1) + geraniol(2)} binary system, a temperature-dependent approach for the binary interaction parameters was necessary to represent the phase behavior of the system properly. Fundamental to notice that, for all systems scrutinized in the phase equilibrium experiments, no ester or side components were detected by gas chromatography.

Thus, the results presented in this work can contribute to the understanding of the role of CO_2 in such reactions and, therefore, serve as background information for the obtainment of higher conversions using a safer and greener solvent, as compared to the use of organic solvents.

6.1 SUGGESTIONS TO FUTURE WORKS

As suggestion to further studies, it is recommended the study of the water role in the esterification, both in BSTR and in VVR. Besides, more experimental conditions, i.e.: higher temperatures, should be evaluated, both in BSTR and in VVR. Mechanisms of water removal should be tested and a scale-up of the system investigated in the present work may be considered as well.

Extra, the investigation of the chemical equilibrium of the esterification reactions studied in this work, through robust thermodynamic models, should be done in order to elicit some distinctive behaviors found. Also, in possess of the data regarding the chemical equilibrium, additional information about the esterification reactions, such as maximum theoretical conversion and the amount of water produced as a side product, shall be identified and insights to achieve higher conversion, especially when scCO_2 is employed, spurred.

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