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FRANCISCO SIMÃO NETO

USE OF BIOMASS WASTE FOR THE PRODUCTION OF AN ENZYMATIC BIOCATALYST AND APPLICATION IN A REACTION OF INDUSTRIAL INTEREST

FORTALEZA
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Dissertação apresentada à Coordenação do Programa de Pós-graduação em Engenharia Química da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Engenharia Química. Área de concentração: Processos Químicos e Bioquímicos.

Orientadora: Profª. Drª. Maria Alexsandra de Sousa Rios
Coorientador: Prof. Dr. José Cleiton Sousa dos Santos

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Aprovada em 03/03/2023.

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A Deus.

Aos meus pais, Tarcisia e Leonidas.

Aos meus irmãos, Tiago e Emanuel.

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“Pode-se encontrar a felicidade mesmo nas horas mais sombrias, se a pessoa se lembrar de acender a luz.” (Harry Potter 3, 2004).

RESUMO

A prática de reutilização de materiais descartados da indústria é importante para preservar os recursos naturais e reduzir a geração de resíduos. Nesse cenário os materiais lignocelulósicos estão se tornando cada vez mais relevantes como solução ambientalmente sustentável. Uma maneira de reaproveitá-los é através da imobilização enzimática, que aumenta a eficiência e segurança dos processos de produção, permitindo uma melhor estabilidade, reutilização e separação das enzimas. Nesse contexto, uma análise bibliométrica foi realizada. Para a análise bibliométrica, a base de dados foi adquirida do site WebOfScience e foi composta por 1.211 manuscritos, classificados como artigos, artigos de conferência e artigos de revisão. Esses artigos são realionados a imobilização enzimática em materiais lignocelulósicos e foram publicados no período de 2000 a 2022. A partir da análise realizada, observou-se a cooperação entre China, EUA e Índia. A China detém 28,07% das publicações nessa área. A instituição brasileira mais presente no assunto foi a Universidade de São Paulo (USP). Foi constatado que os campos de busca constantes eram o uso de biomassa microbiana como suporte enzimático e a imobilização enzimática. Considerandos os resultados observados, os próximos estudos devem focar em aplicações concretas desses novos suportes. Para alcançar esses objetivos, elabourou-se um estudo da produção do éster de aroma butirato de geranila, utilizando a lipase eversa® transform 2.0 numa reação de esterificação, realizada com planejamento estatístico utilizando o método Taguchi, variando os parâmetros de temperatura de 30 a 50°C, razão molar entre ácido e álcool de 1:1 a 1:9, porcentagem de biocatalisador 5% a 15% e tempo de reação de 2 a 6 horas. O tempo de reação foi o fator mais influente sob o processo com percentual de contribuição igual a 74,4%. Os níveis ideias para a reação obtidos após o tratamento estatístico foram: L3 (6 horas) para o tempo, L3 (50°C) para a temperatura, L2 (1:5 ácido/álcool) para a razão molar e L3 (15%) para a porcentagem de biocatalisador, nessas condições, a conversão teórica foi de 95,9%. Após a realização da reação proposta, notou-se uma conversão de 85,2±0,1%. Finalmente, um estudo de docking e dinâmica molecular foi conduzido para avaliar a estabilidade dos complexos formados entre o ligante e a Eversa. Com a imobilização da lipase eversa® transform 2.0 na bagana da carnaúba (*Copernicia prunifera*), resultou num aumento do rendimento de sintese do butirato de geranila para 91,8±0,7%.

Palavras-chave: bibliometria avançada. método taguchi. docking molecular. imobilização enzimática.

ABSTRACT

The practice of reusing discarded materials from the industry is important to preserve natural resources and reduce waste generation. In this scenario, lignocellulosic materials are becoming increasingly relevant as an environmentally sustainable solution. One way to reuse them is through enzymatic immobilization, which increases the efficiency and safety of production processes, allowing for better stability, reuse and separation of enzymes. In this context, a bibliometric analysis was performed. For the bibliometric analysis, the database was acquired from the WebOfScience website and consisted of 1,211 manuscripts, classified as articles, conference articles and review articles. These articles are related to enzymatic immobilization in lignocellulosic materials and were published in the period from 2000 to 2022. From the analysis carried out, cooperation between China, USA and India was observed. China holds 28.07% of publications in this area. The Brazilian institution most involved in the subject was the University of São Paulo (USP). It was found that the constant search fields were the use of microbial biomass as enzymatic support and enzymatic immobilization. Considering the observed results, the next studies should focus on concrete applications of these new supports. To achieve these objectives, a study was carried out on the production of the aroma ester of geranyl butyrate, using the lipase eversa® transform 2.0 in an esterification reaction, carried out with statistical planning using the Taguchi method, varying the temperature parameters from 30 to 50 °C, molar ratio between acid and alcohol from 1:1 to 1:9, percentage of biocatalyst from 5% to 15% and reaction time from 2 to 6 hours. The reaction time was the most influential factor in the process with a contribution percentage equal to 74.4%. The ideal levels for the reaction obtained after the statistical treatment were: L3 (6 hours) for the time, L3 (50°C) for the temperature, L2 (1:5 acid/alcohol) for the molar ratio and L3 (15%) for the percentage of biocatalyst, under these conditions, the theoretical conversion was 95.9%. After carrying out the proposed reaction, a conversion of 85.2±0.1% was observed. Finally, a docking and molecular dynamics study was conducted to evaluate the stability of the complexes formed between the ligand and Eversa. With the immobilization of the eversa® transform 2.0 lipase on the carnauba berry (*Copernicia prunifera*), it resulted in an increase in the synthesis yield of geranyl butyrate to 91.8±0.7%.

Keyword: advanced bibliometrics. taguchi method. molecular docking. enzymatic immobilization.

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CHAPTER 1 – INTRODUCTION

1.1 Introduction

The reuse of discarded materials from the industry is an important practice for the preservation of natural resources and the minimization of waste generation (HOSSAIN et al., 2020). When materials are repurposed, they don't have to be produced again from virgin raw materials, which reduces the demand for natural resources and helps preserve the environment (BOUKAYOUHT; BAZZI; EL HANKARI, 2023; NGENO et al., 2022). In addition, the reuse of materials can be an economical alternative for the industry, as it can reduce the costs associated with the acquisition of new raw materials and waste management (LU et al., 2020).

There are several examples of materials that can be reused in industry, such as plastics, glass, metals, and paper (AHMAD et al., 2021; ANDIÇ-ÇAKIR et al., 2021). For example, recycled plastic can be used to produce new products such as toys, bags, and packaging (WU et al., 2023). Recycled glass can be used in the manufacture of new bottles and flasks, while recycled metals can be transformed into new components for industry (RUAN et al., 2022). Recycled paper can be used to make new papers, such as printer paper and writing paper (RUAN et al., 2021). The reuse of discarded materials from industry is an effective way to promote a more sustainable economy (SINGH et al., 2021).

The reuse of lignocellulosic biomass has gained increasing prominence as an environmentally sustainable solution (LIN et al., 2022; SHRIVASTAVA; SHARMA, 2022). Lignocellulose is a source of renewable raw material that is found in plants such as wood, rice husks, and wheat straw, among others (SINGH et al., 2022). This type of material is widely used in the paper, cellulose, and bioenergy industry, but it can also be used as a source of materials for the production of other products, such as bioplastics and composites (LI et al., 2022). The reuse of these materials is a viable alternative for the disposal of agricultural and forestry waste, in addition to reducing the need to produce new materials and the emission of greenhouse gases (THAPA et al., 2020). Valuing reuse is an important step toward creating a circular economy, where resources are used efficiently (NAHAK et al., 2022).

Lignocellulosic biomass has a porous structure and a large specific surface, which makes them ideal for enzyme immobilization (PALMQVIST; HAHN-HÄGERDAL, 2000; RODRÍGUEZ-RESTREPO; ORREGO, 2020). Furthermore, they are biodegradable and inexpensive, making them a viable alternative to synthetic enzyme supports (BASSAN et al., 2016). Immobilization in lignocellulosic biomass allows for better stability, reuse, and separation of enzymes during production processes, increasing process efficiency and safety (JIA et al., 2017). In summary, this application is a growing trend in biotechnological processes,

offering a sustainable and cost-effective alternative for the production of chemicals and pharmaceuticals (BANDGAR; JAIN; PANWAR, 2022; NAHAK et al., 2022). To achieve this goal, it is important to use an experimental design. It allows for optimizing the immobilization conditions to obtain a stable and highly active enzyme (DEIVAYANAI et al., 2022; RODRÍGUEZ-RESTREPO; ORREGO, 2020).

Experimental planning includes choosing the appropriate support, determining the best relationship between the enzyme and the support, as well as adjusting the concentration of reagents used in the immobilization process (RENJITH; DEVASENA; ABEENS, 2023). Furthermore, it is important to evaluate the influence of variables such as temperature and reaction time on enzyme immobilization (CHEN et al., 2022; VENKATARAGHAVAN; THIRUCHELVI; SHARMILA, 2020). All these steps are carried out to maximize the enzymatic activity and the stability of the immobilization (MAAMOUN; ELJAMAL; ELJAMAL, 2023). The use of experimental design is therefore essential for the successful immobilization of Eversa® lipase and for optimizing its use in industry. Following, the experimental stages, theoretical analyzes are carried out (MOULA ALI; BAVISETTY, 2020; RAVURI; SHIVAKUMAR, 2020).

The theoretical study of docking and molecular dynamics is a computational approach widely used in the pharmaceutical and chemical industry to predict the interaction between molecules and identify candidate molecules to be used as active compounds (CULLETTA; ALMERICO; TUTONE, 2020; SÁNCHEZ-CRUZ, 2023). Molecular docking allows virtual modeling of the binding between a target molecule and a small molecule, also known as a ligand (AVIZ-AMADOR; CONTRERAS-PUENTES; MERCADO-CAMARGO, 2021; GUO et al., 2023). Molecular dynamics is a simulation technique that allows the analysis of how molecules move and interact in a solution (BARREALES et al., 2021; BRELA et al., 2022). These approaches are important because they provide valuable information about the affinity between molecules and the stability of the union, allowing a better understanding of molecular interactions and the identification of molecules with high potential for use in industry (GONZÁLEZ-TORTUERO; GARRIDO; RODRÍGUEZ, 2023; LI et al., 2020; XU et al., 2021).

Thus, this work presents independent studies on enzymatic immobilization in lignocellulosic biomass and the synthesis of aroma esters. In chapter two, a bibliographic review is presented that involves the central themes discussed, to contextualize and define relevant concepts for the understanding of the work.

In chapter three, a comprehensive bibliometric analysis on enzymatic immobilization in lignocellulosic materials from 2000 to 2022 was carried out, including an in-depth examination of all articles published in the specified period systematically. This analysis will make the countries relevant to the theme in question, the main organizations involved in the research, the journals that stand out in publishing results on the subject, the main authors active in the theme, and the analysis of collaborations between these areas: countries, organizations, authors and journals. This analysis can also predict the future trend of this field of research, even using several years of progress data.

Following, chapter four presents a study on the application of free enzyme in the synthesis of Geranyl Butyrate, an aromatic ester, added to a theoretical study of this reaction. The experimental stage will be guided by the experimental design of the Taguchi method, which is based on the function of bigger is better. For the theoretical stage, analysis of enzyme homology modeling, molecular docking, and molecular dynamics will be applied. This chapter ends with a correlation between the two steps taken.

In chapter five, enzymatic immobilization is approached, where a study of a lignocellulosic material applied as a support for lipase Eversa® is carried out. After immobilization, based on the Taguchi experimental design, this new biocatalyst was applied in the synthesis of industrial aroma and fragrance, for comparison purposes with biocatalysis with the free enzyme. Finally, chapter six provides an overview with final considerations.

1.2 Objectives

The main objective of this communication was to immobilize Eversa® lipase (*Aspergillus oryzae*) in carnauba straw (*Copernicia prunifera*) by applying the new biocatalyst in an industrial flavor synthesis reaction.

1.2.1 Specific objectives

- Analyze the world context of research involving enzymatic immobilization in lignocellulosic biomass, based on an advanced bibliographical analysis;
- Apply free Eversa in esterification reactions to obtain the aroma ester of geranyl butyrate;
- Perform a theoretical and computational analysis of the reception reaction of geranyl butyrate;
- Immobilize Eversa in the carnauba berry activated with glutaraldehyde;
- Optimize lipase immobilization;
- Apply the new biocatalyst in the esterification reactions.

CHAPTER 2

CHAPTER 2 – BIBLIOGRAPHIC REVIEW

2.1 Bibliographic review

2.1.1 Enzymes

Enzymes, for the most part, are defined as proteins focused on accelerating chemical reactions (ZHANG et al., 2021). Their advantages are related to its application as extremely efficient biocatalysts, capable of acting in low concentrations, conditions of variable temperatures and pH. Because they are proteins, they have several characteristics of biomolecules, such as maintaining concentration and activity during a reaction. (LI; WU; SU, 2020; TANG et al., 2019).

These biomolecules are highly specific, where each enzyme has its structural organization that only allows the binding of its substrate (BILAL et al., 2018). Enzymes are indispensable in carrying out cellular biochemical processes, such as the degradation of nutritional molecules, maintenance and conversion of chemical energy, in addition to the synthesis of biomacromolecules using basic precursor molecules (BHATTACHARJEE; MORYA; GHOROI, 2020; CHUNG et al., 2018).

In the International Union of Biochemistry and Molecular Biology (IUBMB), information such as enzyme classification, structure, peptide sequence, catalytic reaction type, substrate, product, and kinetic parameters is provided. Table 2.1 presents some of this information.

Table 2.1 — Classification of enzymes according to IUBMB.

Class	Name	catalyzed reaction
1	oxidoreductases	Electron transfer (hybrid ions or H atoms).
2	Transferases	Functional group transfer reactions (aldehyde, acyl, glycosyl, phosphate groups).
3	hydrolases	Hydrolysis reactions (transfer of functional groups to water molecules). act about Connections ester, glycosidic, peptide, and C -N.
4	Liases	Cleavage of C-C, CO, CN, or other bonds by elimination, breaking double bonds or rings or adding groups to double bonds.
5	Isomerases	Transfer of groups within the same molecule, producing isomeric forms.
6	Ligases	Formation of C-C, CS, CO, and CN bonds by condensation reactions coupled with the hydrolysis of ATP or other cofactors.

Source: Adapted from Pacheco and Mendes (2021).

2.1.1.1 Enzymes as a biocatalyst

In the context of environmental sustainability, the application of biocatalysts adds advantages to processes (MONTEIRO et al., 2020). Although this is not an advantage restricted

to biocatalysis alone, a point to be highlighted is that its application complies with Principle 9 of Green Chemistry, which deals with the use of a catalyst to increase selectivity, reducing waste generation, time reaction and energy demand (CHAPMAN; ISMAIL; DINU, 2018).

Faced with a variety of possible catalysts, what justifies the choice of biocatalysts, rather than other catalysts, is that enzymes are catalysts developed naturally over the years, to make life possible through effective and stable (BARBOSA et al., 2014; CHAPMAN; ISMAIL; DINU, 2018)biosynthetic pathways. However, these biomolecules have evolved to catalyze certain reactions with naturally specific substrates for certain physiological reaction media, specificities distinct from those commonly required in the large-scale production of commercially valuable organic compounds (XU et al., 2018).

Thus, what defines whether an enzyme will be applied in industrial processes, it must remain active at high concentrations of substrate and product, emphasizing that these values are on average about one hundred times higher than normal conditions (CAVALCANTE et al., 2021). Furthermore, it is known that enzymes use water as a solvent, however, several organic compounds used as substrates exhibit the behavior of hydrophobic elements. In these cases, small amounts of organic solvents must be added to help dissolve the substrate, so these organic solvents must be tolerated by the enzyme (CHI et al., 2021).

Enzymes used industrially must withstand high salinity concentrations, heterogeneities of temperature, pH, substrate and product concentrations in the reactor, and many other operational factors due to limitations of extensive mass and heat transfer in mixtures (CHAPMAN; ISMAIL; DINU, 2018; MONTEIRO et al., 2020). Consequently, methods must be developed to produce more resistant enzymes, and only then will the use of biocatalysts on an industrial scale continue to be developed (BARBOSA et al., 2014).

Enzymatic engineering involves the remodeling of a protein, where an amino acid residue is usually changed by other similar residues to enhance the properties of the studied proteins (CAVALCANTE et al., 2021; CHI et al., 2021). Finally, scientific advances in enzymatic engineering make it possible to modify and even synthesize new biocatalysts within the standards used in industrial production chains.

2.1.1.2 Lipase

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are relatively easy-to-obtain enzymes that act in the catalysis of the hydrolysis of oils and fats (FERREIRA MOTA et al., 2022). In addition to not requiring cofactors, it is widely used due to its versatility in carrying out the catalysis, availability on the market, and low cost compared to other enzymes (BOLINA;

GOMES; MENDES, 2021). This family of enzymes is useful in the catalysis of chemical hydrolysis reactions, but in addition can also be well employed in the esterification and transesterification reactions of water-insoluble esters (SARNO; IULIANO, 2019). In all biocatalysis, the use of lipases presents several gains over other synthetic transformations, since they act in mild reaction conditions and the high level of selectivity demonstrated by this class of catalysts, thus enzymes are most used in biocatalysis (ZHANG et al., 2020).

Lipase has attracted a lot of interest for its application as an industrial biocatalyst, being used in the field of dairy products, performing the hydrolysis of milk fat, in the field of cleaning materials, in the synthesis of detergents, in the field of textile industries, in the manufacture and leather and paper treatment, food industry and other chemical industries, as well as wastewater treatment (CONTESINI et al., 2020; GUO; SUN; LIU, 2020; MOKHTAR et al., 2020). In the present work, lipase from *Aspergillus oryzae* (Eversa®) was used in its free form and immobilized on a carnauba straw support, to develop enzymatic catalysts for the esterification reaction in the development of the aroma ester, geranyl butyrate.

2.1.1.2.1 Lipase from Aspergillus oryzae (Eversa®)

Faced with the need to develop new lipases, numerous studies have been encouraged to go deeper into this class of enzymes, aiming at different specific uses (FRAGA et al., 2019). In this case and after this context, Novozymes began the development of a new enzyme, which would become lipase Eversa® (ALVES et al., 2022). It was only in 2014 that it was possible to commercialize these new catalysts, which would have the purpose of synthesizing biofuels derived from waste oils or animal fat (CARVALHO et al., 2021). From then on, these enzymes gained prominence in biodiesel synthesis reactions (SUN; GUO; CHEN, 2021).

In all works where this enzyme was applied, its free form was used following the recommendations given by the supplier (CAVALCANTE et al., 2022). Given this, it is possible to verify the lack of well-developed and complete characterization of the enzyme in terms of stability, and activity, among other specificities (GUIMARÃES et al., 2021). In this context, the work by Guimarães et al. (2021), stands out from the others because the immobilized Eversa® enzyme was used, however, no characterization studies were carried out to define the properties of this enzyme being immobilized and, in addition, applied in the synthesis of biodiesel (GUIMARÃES et al., 2021).

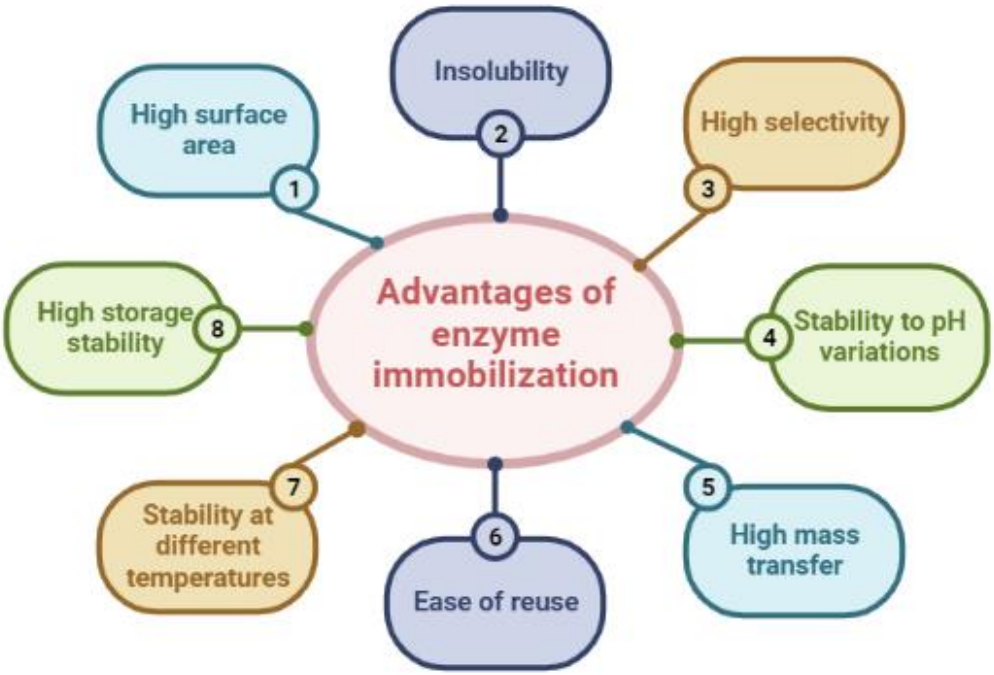
Like other enzymes, Eversa® can also be applied in free form (CAVALCANTE et al., 2022). However, it is worth noting that if this enzyme is subjected to the immobilization

process, many of its properties may be enhanced (CHOI et al., 2022). Improvements in enzyme stability, activity, selectivity or specificity, resistance to inhibitors or chemicals, and even purity, can be a result of successful and proper immobilization of this enzyme (LEE et al., 2019). The constant application of different hydrophobic supports in the synthesis of biodiesel confirms the importance of immobilization for enzymatic engineering (ALVES et al., 2022).

2.1.2 Enzymatic immobilization

Enzymes are biocatalysts that can be applied in different situations (PINHEIRO et al., 2019). This versatility makes the enzymes need to go through modifications that guarantee them the necessary resistance for each application (SECUNDO, 2013). Thus, enzymatic immobilization presents itself as a possibility for optimizing these biocatalysts, allowing their use in the most diverse areas of research (ADLERCREUTZ, 2013). In Figure 2.1, it is possible to observe the advantages of this immobilization process, which may be responsible for several improvements in its stability in the reaction medium, increased resistance to different temperatures, pH, and organic solvents, in addition to increasing its storage capacity and even allowing its reuse.

Figure 2.1 — Advantages associated with enzymatic immobilization.

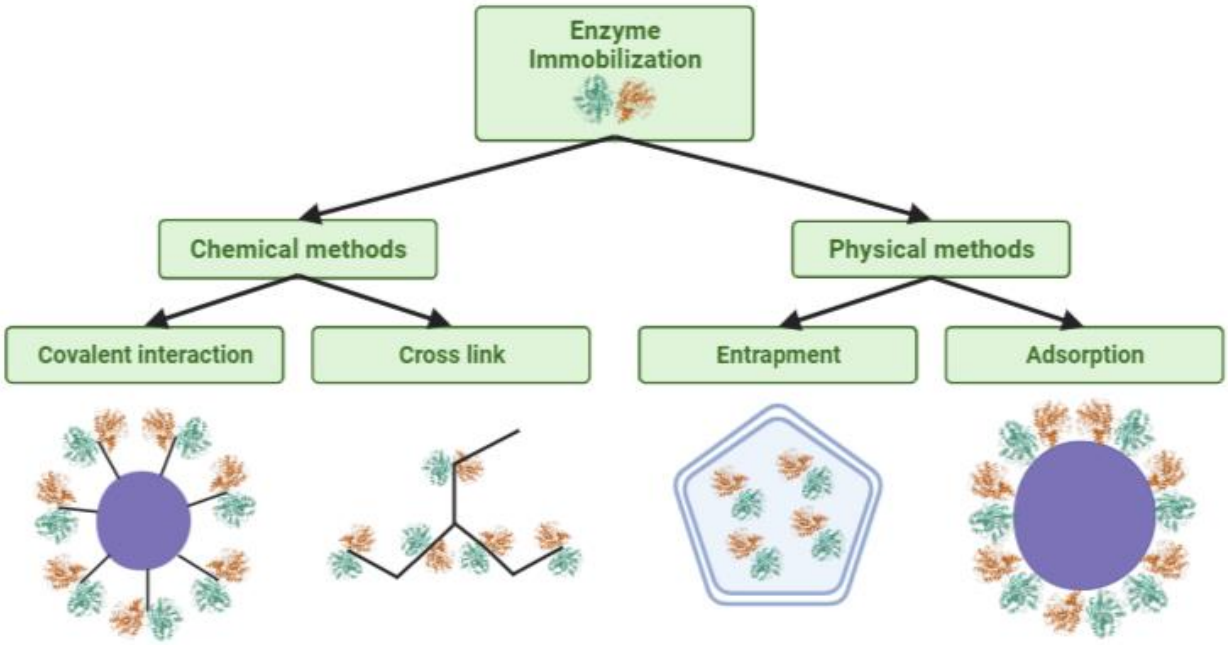


Source: Author (2023).

The enzymatic immobilization process can occur by different methods, whether physical or chemical (HOMAEI et al., 2013). Physical methods are divided into entrapment, where the enzyme is immobilized within an inert support, and the physical adsorption method

by direct binding to the support. Chemical methods are covalent coupling and cross-linking, which is the mutual link between enzymes or proteins that are not soluble (FARHAN et al., 2021). All methods have their associated advantages and disadvantages, so it is up to the researcher to analyze the characteristics of their application to define the most appropriate immobilization method (CABRERA et al., 2018). Figure 2.2 exemplifies the biocatalysts obtained in each method.

Figure 2.2 — Main methods of enzymatic immobilization.



Source: Author (2023).

The method of immobilization by entrapment is within the classification of a physical method in which the enzyme is trapped inside the chosen support, in addition to that, it should be noted that in the case of a physical method, this immobilization becomes irreversible (ALMUTAIRI et al., 2022; CAO et al., 2021). Immobilization by entrapment has its methodology based on the polymer chain of the support, which after activation starts to react with the enzymes, resulting in the formation of a polymeric matrix that will become a trapping structure for these biocatalysts (CAO et al., 2021; ELAGLI et al., 2014). Like other immobilization methods, entrapment allows greater resistance to pH variation, and control of polarity and affinity, in addition to other properties of this material to increase the catalytic power of these biocatalysts (DE OLIVEIRA et al., 2018; JALKH; GHAZALY; EL-RASSY, 2020). Regarding the materials used in immobilization by entrapment, it is possible to highlight collagen, gelatin, polyurethane, and alginate (XING et al., 2022).

Enzymatic immobilization by physical adsorption was one of the pioneering

methods in this line of research (ALAMSYAH et al., 2017). This is an easy-to-perform stabilization method that can be reversible, occurring from hydrophobic interactions, hydrogen bonds, and van der Waals forces (GAO et al., 2018; KHARRAT et al., 2011). The advantages of enzymatic immobilization by adsorption are the simplicity of the technique, which allows stabilization under mild conditions, the potential for maintaining the catalytic activity of the enzyme due to the absence of major structural changes in the biomolecules, and the support that favors reuse (CARVALHO et al., 2020; KHADEMIAN et al., 2020). However, the disadvantage is the stochastic nature of the enzymatic reaction and possible desorption due to changes in temperature, pH, and ionic strength (MOKHTAR et al., 2020; SUGAHARA; VARÉA, 2014). Thus, alternative ways of overcoming these disadvantages have resulted in the development of several in recent years, such as the chemical modification of support materials and the use of crosslinking agents (MACHADO et al., 2019; ZHOU et al., 2019).

Covalent binding is one of the most widely used enzymatic stabilization methods in proteomic research and is based on the occurrence of covalent interactions involving the functional groups on the surface of the supports and amino acid residues of the enzyme (BONAZZA et al., 2018; RUEDA et al., 2015). In this immobilization method, spacer reagents are often used, whose purpose is to allow greater contact of the enzyme with the reaction medium, where the real need for this will depend on the volume of functional groups added to the support (BONAZZA et al., 2018; KASHEFI; BORGHEI; MAHMOODI, 2019). The advantage of this method is the greater resistance to variation in pH, temperature, and organic solvents stand out (DE OLIVEIRA et al., 2020).

The cross-linking technique is an irreversible immobilization method and does not require the use of support (ABDUL WAHAB et al., 2019; HONG et al., 2021). . In this method, the binding is mutual between the enzymes, but bindings with inactive proteins can also occur, thus forming complex three-dimensional structures (GUAJARDO; AHUMADA; DOMÍNGUEZ DE MARÍA, 2021). The occurrence of cross-linking occurs through the formation of an intermolecular bond involving the enzyme and the bifunctional or multifunctional reagent, making them insoluble in the reaction medium (NOURI; KHODAIYAN, 2020). Binding with specific amino acid groups of the enzymes is only possible due to the presence of at least two interacting ends in the molecules that are the cross-linking agents (JEGAN ROY; EMILIA ABRAHAM, 2004). Binding with specific amino acid groups of the enzymes is only possible due to the presence of at least two interacting ends in the molecules that are the cross-linking agents (SHELDON, 2011). This technique is laborious and

requires a lot of time, in addition to the potential loss of half of the enzymatic activity, reduced mechanical stability, and low reproducibility, especially when working with a large number of enzymes (JIA et al., 2017; SCHOEVAART et al., 2004).

2.1.3 Supports

The properties of immobilized enzymes are subject to the specific properties of the support and the enzyme, as well as the conditions of use of the biomolecule used (BETANCOR et al., 2006). The relationship between the two provides important kinetic properties for their practical applications (MULINARI; OLIVEIRA; HOTZA, 2020). Therefore, the conformational structure and the specific activity of the enzyme must be preserved when the immobilization is performed without the enzyme being defunctionalized when it comes into contact with the surface of the support (RODRIGUES et al., 2019).

Thus, carefully selected support can significantly enhance the performance of the system's operation (RUEDA et al., 2016). In terms of morphology, solid supports can be divided into two categories: porous and non-porous (CHEN et al., 2020). In terms of chemical composition, solid supports can be divided into organic and inorganic materials (LOU et al., 2021).

2.1.3.1 Agroindustrial waste

Agricultural and industrial wastes are generally the by-products resulting from manufacturing processes and industrial treatment of agricultural or animal products (SKORONSKI et al., 2016). Straw, logs, bark, vegetable pulp, legumes, and many others are examples of by-products of industrial processes that do not have added value, as these residues are often not used directly (MOTAUNG; LINGANISO, 2018). However, sugars, proteins, fibers, and minerals are compounds extremely present in the composition of these by-products, thus providing alternative sources of carbohydrates and nitrogen, to the detriment of synthesizing sources of these nutrients applied in bioprocesses (DE CASTRO COÊLHO et al., 2021).

Agribusiness and agricultural residues are alternatives for supplementing ruminant animals in critical periods when the availability of dry food decreases due to the lower availability of forage (FUENTES et al., 2021). Residues such as corn straw, bean pods, husks and straw from cereals such as rice, carnauba straw, and wheat straw have reduced nutritional value, despite their high availability (MARTÍNEZ et al., 2023). These by-products still have a high lignin content, reduced soluble carbohydrate content, and a percentage of crude protein

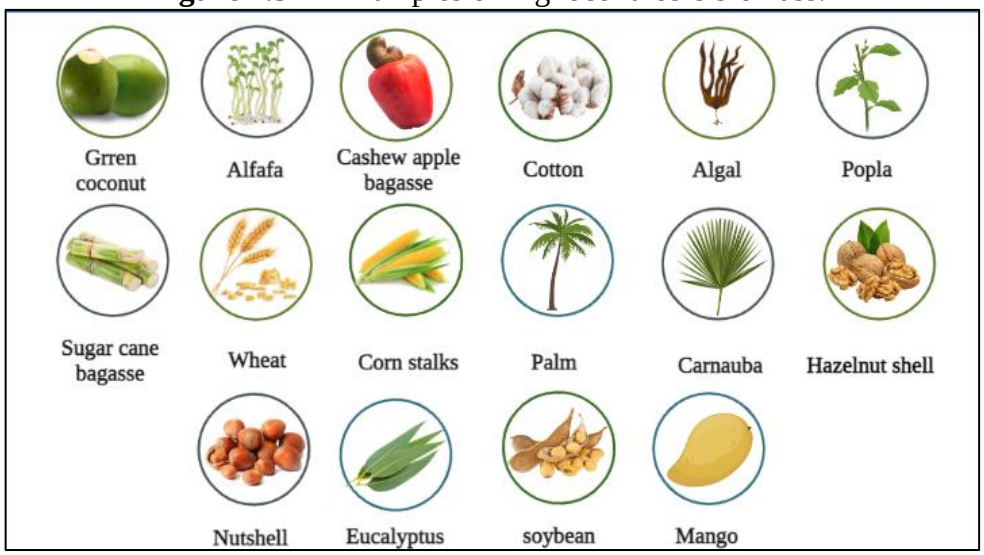
below the necessary (CARLOSAMA ADRIANA et al., 2021).

Currently, the search for effective utilization of various agricultural and industrial wastes has increased (GRESES; TOMÁS-PEJÓ; GÓNZALEZ-FERNÁNDEZ, 2020). Numerous bioprocesses have been designed to make it possible to use these materials as substrates in the synthesis of multiple molecules with high-added value, such as microbial proteins, organic acids, ethanol, and enzymes (FUENTES et al., 2021). Using agroindustrial by-products as a substrate for bioprocesses not only makes economic sense but also helps to solve the environmental problems arising from the accumulation of these materials in the environment (DE CASTRO COÊLHO et al., 2021).

2.1.3.2 Lignocellulosic biomass

It is estimated that 1.5 trillion tons of lignocellulosic biomass are produced annually, providing a renewable source of raw materials for the synthesis of bioproducts such as ethanol and biodiesel (THAPA et al., 2020). This vast collection of biomasses is mainly due to the management of crops (RODRÍGUEZ-RESTREPO; ORREGO, 2020). In this context, it is important to highlight the large amount of solid biomass that remains unused after harvesting seeds and grains, as well as residues from industrial production (BASSAN et al., 2016). Figure 2.3 presents a series of lignocellulosic biomasses applied in the stabilization of enzymes.

Figure 2.3 — Examples of Lignocellulosic biomass.



Source: Author (2023).

Brazil is responsible for growing a wide variety of grains and crops, resulting in high amounts of agroindustrial waste that could be converted into ethanol. Worldwide, Brazil has the production of 95 million tons of soy, placing it in second place in the production of this legume, in addition, it is also responsible for the production of 12 million tons of rice, placing

it among the ten countries that produce more of this cereal (CONAB, 2015).

The composition, molecular structure, and physical-chemical properties of lignocellulosic biomass give them remarkable resistance to hydrolysis reactions (LI et al., 2022). Such a characteristic (resilience to hydrolysis) made it difficult to produce knowledge about economic techniques used to convert lignocellulosic biomass into fermentable, energetically viable, and environmentally correct sugars (AHMAD FARID; ANDOU, 2022).

Lignocellulose is the most important part of the composition of plants, it is directly linked to its structural composition and it is a renewable material (BAPTISTA; DOMINGUES, 2022). This biomass is formed by three main components, cellulose (up to 50%), hemicellulose (20 to 40%), and lignin (20 to 30%), but it is possible to find extracts and inorganic compounds included in the material under analysis (SHRIVASTAVA; SHARMA, 2022; ZHANG et al., 2023). Each plant has its composition and, in addition, factors such as age, harvest time, and state or stage of development also affect this composition (WANG et al., 2023). The three most common polymers found in structures are cellulose, hemicellulose, and lignin, which are highly entangled and chemically linked by variable bonds between non-covalent and cross-covalent (NAHAK et al., 2022).

Cellulose is defined as a semicrystalline linear polymer formed from D-glucose subunits linked by β-1 glycosidic bonds to form cellobiose dimers (LIN et al., 2022). Here there is the formation of long elemental chains that are held together by hydrogen bonds and van der Waals forces (SINGH et al., 2022). Cellulose is commonly found in crystalline form and compared to amorphous form (BANDGAR; JAIN; PANWAR, 2022).

Hemicellulose is defined as a polysaccharide that has a lower molecular weight than cellulose (DEIVAYANAI et al., 2022). It is composed of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, -O-methyl-glucuronic acid, D-galacturonic acid, and D-glucuronic acid (AHMAD FARID; ANDOU, 2022). Polysaccharides are linked by β-1, and sometimes β-1,3 glycosidic bonds (ZHANG et al., 2023). Hemicellulose differs from cellulose in that it has filaments with short side chains made of numerous and different sugars, while cellulose consists of oligomers that are very susceptible to hydrolysis (BAPTISTA; DOMINGUES, 2022).

Finally, there is lignin, which binds to hemicellulose and cellulose and forms a physical barrier, which is impermeable to plant cell walls (LI et al., 2022). It is in the composition of cell walls to provide structural support, prevents permeability, and ensures resistance to attack by microorganisms, in addition to providing the cell wall with protection against oxidative stress (AHMAD FARID; ANDOU, 2022). It is defined as an amorphous,

water-insoluble, and optically inactive macromolecule composed of phenylpropane molecules coupled from non-hydrolyzable bonds (SHRIVASTAVA; SHARMA, 2022).

2.1.3.2.1 Carnauba (Copernicia prunifera)

The carnauba tree (*Copernicia prunifera*) is native to Brazil, is a palm that varies in height from 7 to 10 meters and reaches a maximum of 15 meters (GONÇALVES et al., 2020; SILVA JÚNIOR et al., 2014). The plant has a straight cylindrical stem, 15 to 25 cm in diameter. It commonly occurs near rivers, with a preference for clayey (heavy) and silty (river) soils, being able to withstand prolonged flooding during the rainy season, in addition to being resistant to high levels of salinity (GONÇALVES et al., 2020). It is also very resistant to heat, being able to withstand up to 3,000 hours of sunlight per year (SILVA JÚNIOR et al., 2014). It is worth mentioning that the age of the palm tree, the soil in which it grew, the climate, and the distance from the sea are points that can cause variation in the production of straw and consequently by-products (SUSMITA DEVI et al., 2022).

Carnauba is native to Northeastern Brazil, without adverse conditions, it grows at a rate of about 30 cm each year, and it reaches vegetative maturity (first flowering) at 12 years old (SILVA et al., 2017). Its production varies between 5 and 60 sheets per year. Even so, there was no progress in research on genetic improvement to make it leafy early, nor in the possibility of joint cultivation with crops and pastures, or even replanting for the recovery of land with high salinity through irrigation operations (SILVA et al., 2020).

The carnauba leaves are arranged in an entire spherical shape, the crown is slightly bluish green due to the wax that covers the leaves, the fan formed is up to 1.5 m long, the surface is wrinkled, the tips are divided into lengths, hairs nearly erect and stiff (GOMES et al., 2009). The leaves are attached to the stem by hard petioles that can reach 2 m in length, partially covered, especially at the edges, by massive thorns with a curved shape (SILVA et al., 2020).

Finally, straw, a by-product of carnauba, is important in the Northeast due to its versatility (GONÇALVES et al., 2020). The straw preparation stage, in some cases, is manual, as it is useless for handicrafts after crushing them (GOMES et al., 2009). Another use of straw is as a food source for ruminants, even though it does not have good energy values, thus it acts as a complement to the diet of these animals (SILVA JÚNIOR et al., 2014). It can also be applied as an agricultural fertilizer, as it prepares the soil for food crops such as beans, corn, and fruit, which must be processed and placed in the soil (SILVA et al., 2020).

2.1.4 Biocatalyst application

Flavorings are substances that are very present in the processing of the food industry, being used as food additives (API et al., 2022; COSTA et al., 2022). According to Ordinance No. 540, of October 27, 1997, a food additive is any ingredient added to food production, which contributes to the nutrients of the final product (BRASIL, 1997). These additives are used to improve the olfactory characteristics of the product so that it is more attractive to the consumer, but they are also used due to the possibility of improving the technological characteristics and increasing the conservation of the product, guaranteeing longer storage time, without prejudice to their quality.

Flavorings are classified as either natural or synthetic (AMORNRUK et al., 2022; UPSTILL; ROWLAND; JORDAN, 2022). Where natural aromas are defined as those synthesized from physical, microbiological, or enzymatic methods, while synthetic ones are those obtained by chemical processes (CHEN et al., 2022; YU et al., 2023). Blended, interacted, or cured and smoked aromas are considered natural or synthetic, depending on the nature of the raw material or the manufacturing process. Flavorings can be sold in solid (powder, granules, or tablets), liquid (solution or emulsion), or paste form. (GUCKENBIEHL et al., 2022; WU et al., 2023).

Fragrances are volatile organic chemicals, because they are composed of short-chain molecules and, therefore, have low molecular weight (COSTA et al., 2022). These compounds are a sensory component of several products and can generate new flavors, as well as enhance, replace, or mask existing aromas (AMORNRUK et al., 2022).

Esters are widely used as flavoring agents and are prized for the fruity aroma they impart. They are added to fruit-flavored products (drinks, jams, and candies) and also in dairy products such as yogurts (UPSTILL; ROWLAND; JORDAN, 2022). These compounds, derived from the first synthetic fragrances a century ago, can also be produced by microbial and enzymatic pathways, the latter being the route of application of the biocatalysts produced in this work (YU et al., 2023).

2.1.5 Theoretical study

One stage of the theoretical study concerns molecular docking, which is a computational modeling technique used in the area of chemistry and biology to predict the interaction between molecules (IQBAL et al., 2023; NOUR et al., 2022). This technique consists of simulating the interaction between a target molecule and a ligand molecule, to determine the most favorable position and orientation for a bond to occur between them (AVIZ-AMADOR; CONTRERAS-PUENTES; MERCADO-CAMARGO, 2021; GUO et al., 2023).

Molecular docking is used in several areas of science, such as the development of new drugs, the study of enzymes and proteins, and the analysis of interactions between molecules in a biological system (SINGH et al., 2022a).

docking process consists of several steps, which include the preparation of molecules, the definition of the binding site of the target molecule, the generation of conformations of the binding molecule, the evaluation of interactions between molecules, and the selection of the most suitable conformations (GOVINDARASU et al., 2021; SARKAR; SRIVASTAVA; AILANI, 2022). The final objective is to find the conformation that presents the best affinity between the molecules, that is, the one that presents the highest binding free energy (SÁNCHEZ-CRUZ, 2023). This technique is utilized for the development of new drugs, as it allows the identification of candidate molecules for inhibitors of proteins and enzymes involved in diseases (CHEDADI et al., 2021).

Molecular dynamics is a technique used to simulate the behavior of molecules and biological systems in a computational environment (LI et al., 2020; XU et al., 2021). This technique makes it possible to study how molecules move, interact and change conformation in response to different environmental conditions, such as temperature, pressure, and solute concentration (YANG et al., 2022). Molecular dynamics is applied in many areas of science, including chemistry, biology, physics, and engineering, and is particularly useful for understanding the dynamics of proteins and other biological macromolecules (GONZÁLEZ-TORTUERO; GARRIDO; RODRÍGUEZ, 2023).

Molecular dynamics methodology involves simulating the movement of molecules in a simulated environment, using mathematical equations that describe the forces acting on each atom (BRELA et al., 2022; PAHLANI; SCHWARTZENTRUBER; JAMES, 2023). These equations are numerically solved by computers, allowing the trajectories of molecules over time to be evaluated (BARREALES et al., 2021). The molecular dynamics technique is particularly important for understanding how molecules interact with other molecules and for predicting how different environmental conditions affect the behavior of molecules, thus allowing the identification of new therapies or interventions in different fields of science (CULLETTA; ALMERICO; TUTONE, 2020; SHARIATI et al., 2021).

CHAPTER 3

CHAPTER 3 – USE OF LIGNOCELLULOSIC BIOMASS AS SUPPORTS FOR ENZYMATIC IMMOBILIZATION: AN ADVANCED BIBLIOMETRIC ANALYSIS

3.1 Abstract

Lignocellulosic biomasses are used in several applications, such as energy production, materials, and biofuels. These applications result in increased consumption and waste generation of these materials. However, alternative uses are being developed to solve the problem of waste generated in the industry. Thus, research is carried out to ensure the use of these biomasses as enzymatic support. These surveys can be accompanied using the advanced bibliometric analysis tool that can help determine the biomasses used and other perspectives on the subject. With this, the present work aims to carry out an advanced bibliometric analysis approaching the main studies related to the use of lignocellulosic biomass as an enzymatic support. This study will be carried out by highlighting the main countries/regions that carry out productions, research areas that involve the theme, and future trends in these areas. It was observed that there is a cooperation between China, USA, and India, where China holds 28.07% of publications in this area, being the country with the greatest impact in the area. Finally, it is possible to define that the use of these new supports is a trend in the field of biotechnology.

Keywords: Enzymatic immobilization. Lignocellulosic biomass. Bibliometric analysis.

3.2 Introduction

Faced with the development of the industry, new materials needed to be developed or studied to analyze the possibility of use or reuse (PAPADAKI et al., 2018; UNUGUL et al., 2020). In this context, the use of waste from the industry itself came to be considered, since, as the industry increases its production, more waste is generated (ADELEKE et al., 2021; JAISWAL et al., 2020). These residues are intended for different reapplications, or what is not ideal, they are disposed of incorrectly causing negative effects on the environment (ARUN et al., 2020; DARWESH; MATTER; EIDA, 2019; DO et al., 2019). Thus, the proper destination of this waste came into focus and studies are carried out to improve this area (CHU et al., 2021; GARCÍA-GALÁN et al., 2020).

With the advancement of research focused on reuse, it was concluded that agroindustrial waste had the potential for reuse (PALMQVIST; HAHN-HÄGERDAL, 2000; RODRÍGUEZ-RESTREPO; ORREGO, 2020). Within this class of residues, there are residues of lignocellulosic biomass, which have great potential for use in the most diverse lines of research (BASSAN et al., 2016; JIA et al., 2017; THAPA et al., 2020). These materials are represented by different residues, such as sugarcane bagasse (BHATTACHARJEE; MORYA; GHOROI, 2020), used in the production of ethanol, cashew bagasse, carnauba straw, among others (CHAGAS et al., 2022; DE SOUZA et al., 2016; SUSMITA DEVI et al., 2022). In studies of potential applications for these materials, the possibility of using them as a support for enzymes was seen.

Enzymes are macromolecules that have the potential to catalyze the most diverse reactions (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; LI et al., 2012; LIVAGE; CORADIN, 2018). These macromolecules are obtained from different microorganisms in different ways, thus resulting in the classification as a biocatalyst (CHAPMAN; ISMAIL; DINU, 2018; ZHU; WU; HUA, 2019). These biocatalysts have a high degree of selectivity and purity, thus reducing the generation of by-products in the reactions where they are applied (MOHAMAD et al., 2015; RUFER, 2021; SECUNDO, 2013). However, these biocatalysts may suffer interference from the reaction medium, as they are susceptible to denaturation by organic solvents and damage to their catalytic site (MEGHWANSHI et al., 2020; PIERRE, 2004; SCHMID; VERGER, 1998). In addition to these points, another disadvantage is the impossibility of reusing these enzymes (DROUT; ROBISON; FARHA, 2019; LIU; CHEN; SHI, 2018; MARIZ et al., 2021).

Because they are materials that require very specific technology and still little

industrial demand, these bioproducts have a high market value (FARHAN et al., 2021; HOMAEI et al., 2013; PINHEIRO et al., 2019; ROCHA et al., 2021). Adding the economic factor and the technical factor of not enabling reuse, there is the main negative point in the use of these catalysts (ADLERCREUTZ, 2013; CABRERA et al., 2018; MONTEIRO et al., 2020). Soon, a new area of research emerged, where studies are carried out to enable enzymatic immobilization. This immobilization results in the potentization of the catalytic capacities of these enzymes to improve stability and resistance to denaturation in an organic medium and enable the separation of the reaction medium, thus allowing the reuse of this biocatalyst (BEZERRA et al., 2020; CUI et al., 2018; DATTA; CHRISTENA; RAJARAM, 2013; DURAIARASAN et al., 2016; MONTEIRO et al., 2021, 2022; VERMA; BARROW; PURI, 2013). In addition, in the studies, new materials are analyzed to be used as a support and this resulted in the possibility of applying lignocellulosic biomass (BINHAYEEDING et al., 2020; CARVALHO et al., 2020; MONTEIRO et al., 2020).

The analyzes carried out will be based on the advanced bibliometrics method. This method consists of a thorough review of all papers published within the period delimited in the methodology (DUTRA et al., 2022; JIANG; GINOCCHIO; ROSENKRANTZ, 2016). Such analyzes will result in the visualization of relevant countries for the subject in question, main organizations involved in the research, journals that stand out in the publication of results in this area, and main authors acting in the subject, in addition to the analysis of cooperation between these spheres: country, organization, authors, and journals (BORNMANN; LEYDESDORFF, 2013; MALEKLI et al., 2022; SALES et al., 2022). This analysis can also result in forecasting the future for this line of research, even using progression data from previous years (MARVUGLIA et al., 2020; ROSENKRANTZ; JIANG, 2016).

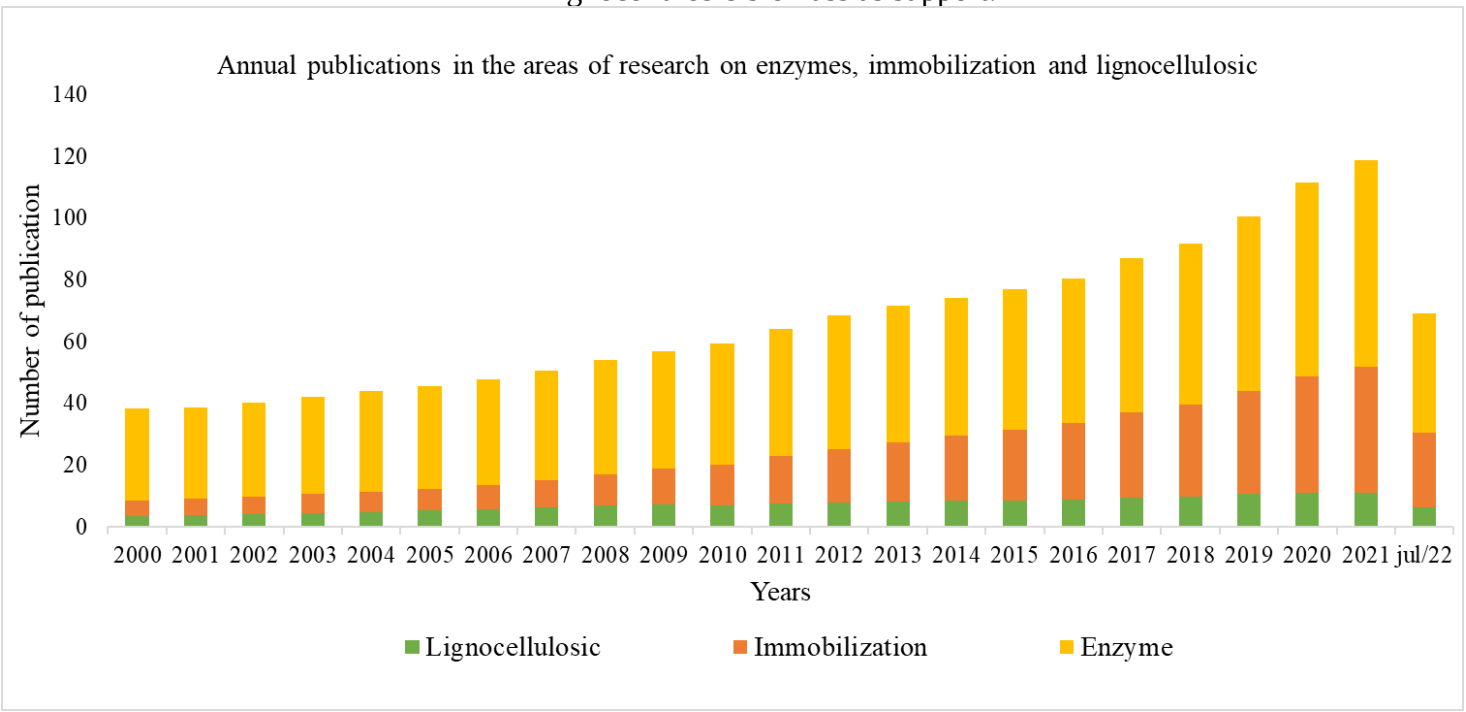
Thus, an advanced bibliometric analysis will consider keywords, co-citation links, and bibliographic coupling networks as the main units of analysis to address the follow-ing research questions (RQs):

- RQ1. What are the advances in scientific production on lignocellulosic biomass and enzymatic immobilization?
- RQ2. Research hotspots (keywords) that stand out in studies of lignocellulosic biomass and enzymatic immobilization?
- RQ3. Which authors, organizations, and countries stand out in studies on lignocellulosic biomass and enzymatic immobilization?
- RQ4. What are the main emerging research subfields of lignocellulosic biomass

and enzyme immobilization in recent literature?

As shown in Figure 3.1, there has been an increase in publications involving enzymatic immobilization. Such research is mainly motivated by environmental issues, which have recently been gaining prominence in scientific discussions. Thus, the year 2021 can be highlighted with the number of publications related to enzymatic immobilization in lignocellulosic materials, proportionally higher compared to previous years, with about 118 academic productions. Then there is the year 2020, with 112 academic productions, and then the year 2019, with about 112 academic productions. The productions for the year 2022 are less expressive since only the data from January to July were considered the time of construction of the database, however, even in this reduced period, about 70 articles had already been published. that there has been a growing interest in the scientific community in relation to this area of research.

Figure 3.1 — Annual production of research on enzymatic immobilization using lignocellulosic biomass as support.



Source: Author (2023).

Still, in relation to Figure 3.1, it is possible to verify that, separately, the themes of immobilization and enzymes were more addressed in the year 2021, with 41 and 66 articles being published this year, respectively. On the other hand, the topic of biomass and lignocellulosic materials was addressed more frequently in 2020, with a total of 11 articles.

Thus, the present work aims to carry out an advanced bibliometric analysis of the applications of lignocellulosic biomass as a support for enzymatic immobilization. It is justified

by the need for new materials for enzymatic support and the development of new applications of these materials, thus reducing the environmental impacts involved in the inappropriate disposal of these residues.

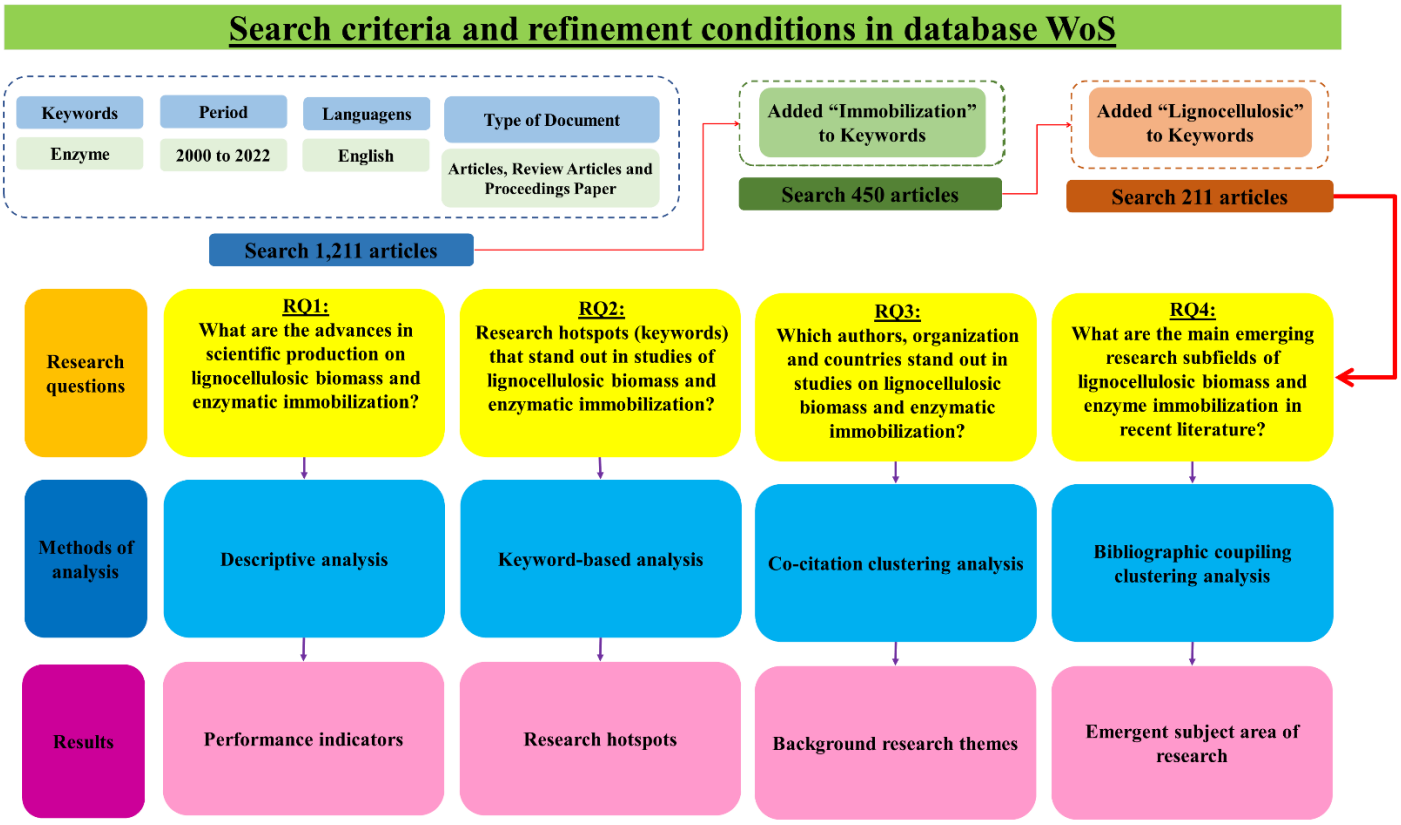
3.3 Methodology

3.3.1. Data source

The data used in the work were acquired from the database of the website *Web of Science - Core Collection.* For data selection, classification parameters referring to advanced bibliometric analysis were applied. In accessing the platform, the login provided by the Agency for the Improvement of Higher Education Personnel (CAPES) in the Capes Periódicos system was used.

The scheme in Figure 3.2 shows how the acquisition of data that make up the main database was carried out. This base will serve as support for all the analyzes that will be carried out during the work, so the term “Enzymes” was inserted in the search line and the filter was delimited as “Title”. Next, a new line was inserted with the term “2000-2022” and the filter “Year Published”. The number of articles that were returned was 1,211. These were the articles that fell within the boundaries of the research, however, to improve the analysis, a refinement was necessary to guarantee the fidelity of the data, thus discarding the academic works that were outside the objective of this bibliometric analysis.

Figure 3.2 — Structure that represents the search and analysis criteria.



Source: Author (2023).

The delimitations were carried out as follows: Types of Documents, where the limitation was restricted to Articles, Conference Articles, and Review Articles. Language, where only the English language works would be taken into account. Finally, the data set used was limited to 1,211 articles that will be applied in the advanced bibliometric analysis method. Following, the main database resulted in the generation of two new smaller databases that would be used for the analysis of two other themes of great relevance to the central theme of the research.

Regarding the first derivation, this resulted in the analysis of the raw materials used in enzymatic immobilization. Thus, a new line was added to delimit a new term, here the filter used remained as "All Fields", since the term was defined as "biomass" OR "lignocellulosic" and the other filters and terms remained to continue from the 1,211 articles already defined. After this new delimitation, the presence of 255 articles related to the researched terms was verified, that is, the use of biomass and lignocellulosic biomass in works involving enzymes, in the period between 2000 and 2022.

Finally, the second derivation applied concerns the treatment carried out on the enzymes, to guarantee greater resistance and thus guarantee greater possibilities of applications

in different conditions. This treatment is immobilization, for this reason, the new line used the term “Immobilization” with the filter “All Fields”. Thus, a return of 450 articles related to the researched topic was obtained, once again, within the 1,211 articles in the database.

3.3.2. Data analysis

Three software were applied in carrying out the analyzes of this work, namely: CiteSpace (version 5.8.R6 Philadelphia, Pennsylvania, USA), VOSviewer (version 1.6.17 Leiden, South Holland, Holland), and ArcGIS (version 10.5 Redlands, California, USA). In order of application, in VOSviewer the visualization of the data was carried out in a way that would allow a better understanding and analysis of the data (CATANI et al., 2022). Subsequently, ArcGIS 10.5 was applied to present data distributions on geographic maps. Finally, CiteSpace was used to project the future of research in areas of interest, based on the keywords and clusters generated (CHEN; ZHANG; LI, 2022).

3.4 Results and discussion

3.4.1. Bibliometric analysis

3.4.1.1. Publication result: overall results

We got 1,211 records from the Web of Science, published between 2000 and 2022. One publication from May 2020 had the highest visibility and relevance to the present study. This paper is entitled “Immobilization of enzymes and cells in lignocellulosic biomass” by Rodriguez-Restrepo, YA (2020) regarding using lignocellulosic biomass for enzyme immobilization, aiming at their application in fermentation, remediation of contaminated water and soil, solvent synthesis, and fine chemistry. This paper presents an alternative application of these materials, as their high production and improper disposal can cause environmental damage (RODRÍGUEZ-RESTREPO; ORREGO, 2020).

Lignocellulosic biomass is mostly biomass residues that have been used in different applications, such as energy production, either thermal or electrical energy, materials, and biofuels, such as sugarcane bagasse after bioethanol production (LIU et al., 2021). The numerous applications of these biomasses increase their consumption and consequently the production of waste of these materials, so alternative uses are developed to circumvent this problem. Currently, the application of these biomasses as enzyme support has gained considerable research prominence, as green coconut fibers (BEZERRA et al., 2015), corn cob powder (BASSAN et al., 2016), and wood sawdust are already used (BAROUNI et al., 2016), for covalent bond immobilization of Candida antarctica lipase, *Candida rugosa* lipase, among

others (RODRÍGUEZ-RESTREPO; ORREGO, 2020).

Lignocellulosic biomasses, such as agricultural residues, have been used in different that produce these materials. Such changes will favorably influence the economics and results of by-product production, besides defining the ideal parameters for the growth of its use, thereby causing a decrease in the incorrect disposal of the by-products. The topics addressed by the articles analyzed in the database are used to highlight moments of great importance for environmental concerns and then show how to circumvent such setbacks to ensure a more sustainable lignocellulosic biomass market.

3.4.1.2. Distribution of scientific journals

After analyzing the database collected, it was possible to highlight the eligible works that are part of the theme of lignocellulosic biomass as support for enzyme immobilization in the distribution of scientific journals. Thus, we found 391 journals, with an average of approximately 3.2 publications per journal. This result underlines the importance of this topic for several research areas. However, there is still a need to clarify this research line because of its excessive importance for scientific and industrial society. Another interesting point worth highlighting concerns the diversity of research groups working in this area, which reflects the wide variety of journals that address the topic. In addition, the different groups ensure the experimentation of many methodologies and individuals in each group. Such plurality guarantees a better explanation of the theme and enriches the academic community with new ideas and projects.

Table 3.1 summarizes the list of the ten journals with the highest influence on the theme of this paper, ranked by the number of publications. It is important to note that these journals hold more than a quarter of all publications analyzed here, which are not so expressive and reinforce the diversity of research in the area.

The journal Bioresource Technology stands out by leading the list presented in Table 3.1. This journal has an impact factor of 11,889 and 51 articles on this research topic, which represents 4.21% of the total papers collected. Further, it got 2,913 citations over the years. Bioresource Technology obtained an average of 57.12 citations per article. The journal that ranks second on the list—Applied Biochemistry and Biotechnology—which has an impact factor of 3,094, has 50 articles published on the topic, which represents 4.13% of the documents analyzed in this paper, and has 747 citations.

The list presented in Table 3.1 is mostly composed of European journals. Only Applied Biochemistry and Biotechnology and Enzyme and Microbial Technology are

American journals. Although none of them is the journal with the highest impact factor, they are among the top ten selected. These scientific journals rank second and third, with the highest number of publications. This shows the excessive density of journals in the same region concerning the topics studied here, as about 70% of the major journals are European.

Table 3.1 — The ten main scientific journals published in the area of use of lignocellulosic biomass as support for enzymatic immobilization.

Rank	Journal	Country	Impact factor	Number of Publications	Number of citations	Average citations	Percentage (%)
1	Bioresource technology	ENG	11.889	51	2,913	57.12	4.21
2	Applied biochemistry and biotechnology	EUA	3.094	50	747	14.94	4.13
3	Enzyme and microbial technology	EUA	3.705	37	1,196	32.32	3.06
4	Biomass & bioenergy	ENG	5.774	24	701	29.21	1.98
5	Chemosphere	ENG	8.943	23	368	16.00	1.90
6	Process biochemistry	ENG	4.885	23	1,025	44.57	1.90
7	Science of the total environment	NLD	10.753	21	224	10.67	1.73
8	Waste and biomass valorization	NLD	3.449	21	699	33.29	1.73
9	Soil biology & biochemistry	ENG	8.546	20	1,620	81.00	1.65
10	International journal of biological macromolecules	NLD	8.025	18	250	13.89	1.49

Impact factor in 2021. USA = United States of America; NLD = Netherlands; ENG = England.

Source: Author (2023).

The journals occupying the first and second positions in the ranking of Table 3.1 (Bioresource Technology and Applied Biochemistry and Biotechnology) hold 30% of the most cited articles related to enzyme immobilization. Thus, one can see that there is a large distribution of studies carried out from a methodology of specific scientific groups. All the 10 most cited journals together account for only 23% of all publications in the database collected, among the 391 journals analyzed. Regarding the number of citations, Bioresource Technology has 2,913 (9.71% of the total citations) and Applied Biochemistry and Biotechnology has 1,774 (5.71% of the citations). This shows the relevance of publications from these journals and the wide dispersion of studies with less prominence in a wide variety of journals.

3.4.1.3. Distribution by countries, organizations, and authors

We analyzed the relationship between articles and their countries and institutions. Among the 81 countries cited in the manuscripts, 10 countries concentrated most of the publications—about 87.81% of the papers. The remaining articles are from the other 71 countries. Table 3.2 shows the leading countries.

Table 3.2 — The 10 most productive countries in the area of use of lignocellulosic biomass as support for enzymatic immobilization.

Rank	Country	Number of	Number of	Average	Total link	Percentage

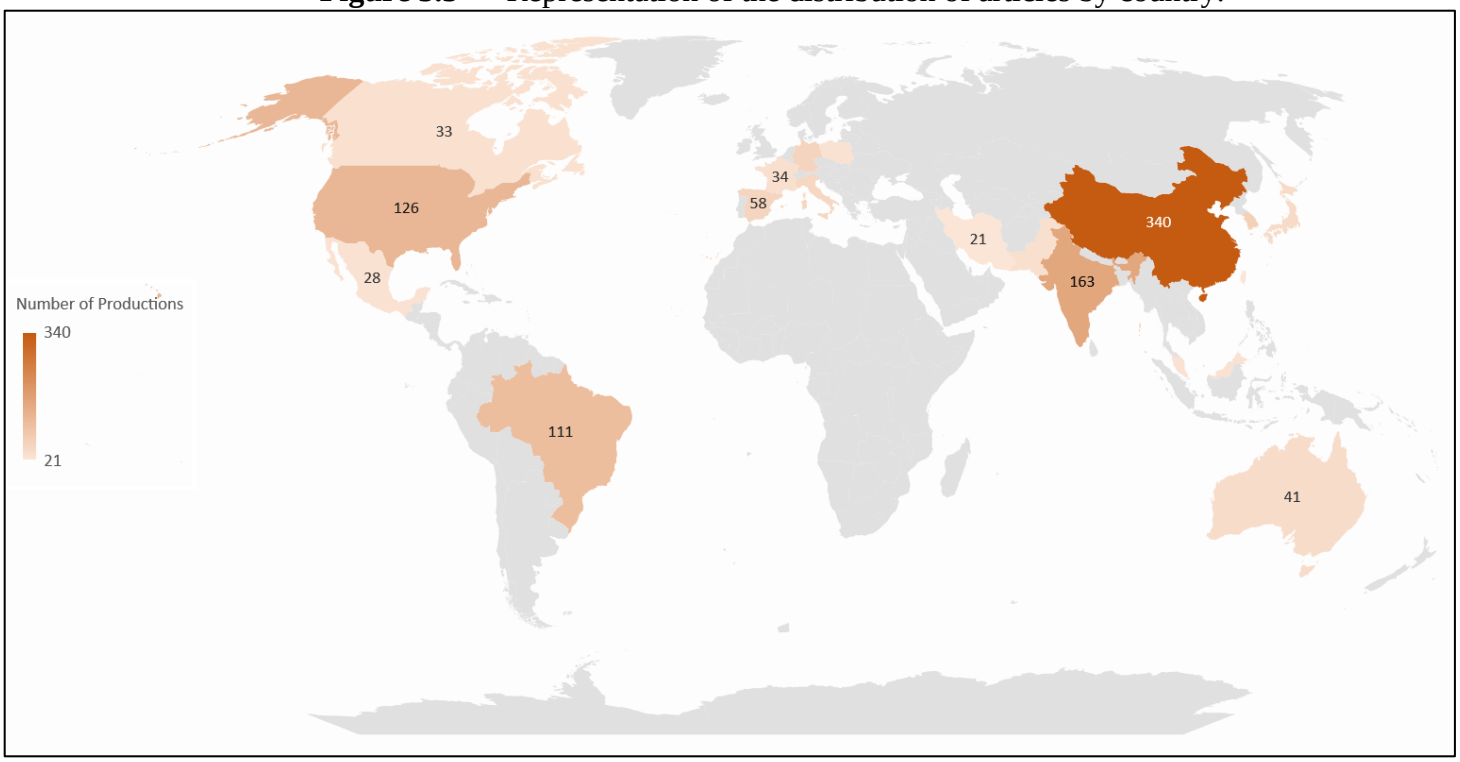
		Publications	Citations	citation	strength	(%)
1	China	340	7419	21.82	57,67	28.07
2	India	163	5399	33.12	37,78	13.46
3	United States of America	126	4126	32.74	23,93	10.4
4	Brazil	111	1598	14.39	28,46	9.16
5	South Korea	68	2462	36.20	14,94	5.61
6	Germany	60	2947	49.11	11,95	4.95
7	Spain	58	1191	20.53	14,24	4.78
8	Italy	54	1184	21.92	9,79	4.45
9	Japan	43	2305	53.60	7,80	3.55
10	Australia	41	1044	25.46	12,72	3.38

Source: Author (2023).

There is a greater interest in this topic from some central regions, displayed by the concentration of articles in a smaller portion of the stated countries. China holds 28.07% of the articles published on the topic, thereby securing its position at the top of this list. India holds 13.46% of production—half that seen in the country in the first place. Finally, in third place is the United States of America, with 10.4% of production, a figure close to second place that leaves it even further away from first place.

The following analysis considers the number of citations per country to determine the relevance and impact of these countries. Once again, China holds 7,419 citations, which places it as the country with the highest impact regarding lignocellulosic biomass as a support for enzyme immobilization. India was the second country with the most citations (3,323 citations), followed by the United States of America (2,555). The number of citations is directly linked to the number of manuscripts produced, as these countries are the largest holders of articles (Figure 3). For the construction of this figure, 19 regions that published at least 20 articles were specified.

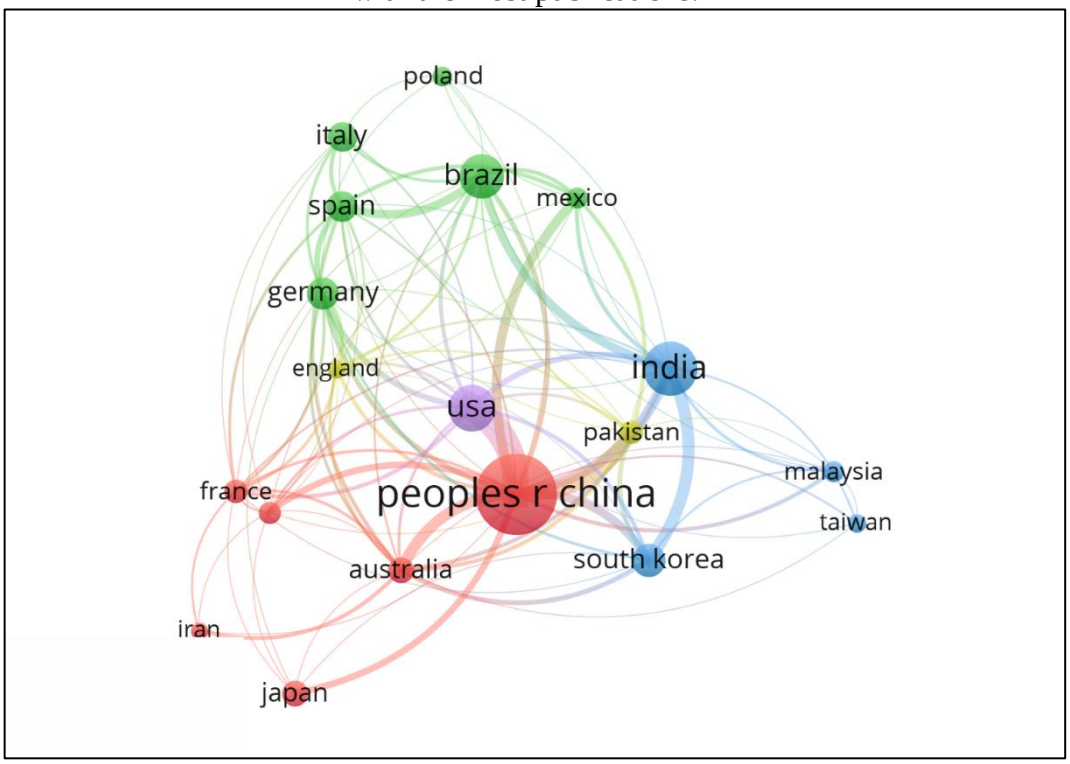
Figure 3.3 — Representation of the distribution of articles by country.



Source: Author (2023).

Considering the filters used in Figure 3.3, Figure 3.4 represents the network of interconnections or the collaboration network between countries. The great interaction between China and the United States is clear, ensuring a good relationship between sharing ideas and working together to build manuscripts, although these countries are on different continents.

Figure 3.4 — Network visualization map related to collaboration among the 19 countries with the most publications.[1]



Source: Author (2023).

The economic factor is also responsible for bringing these countries together since they are world economic powers, and scientific cooperation strengthens both. In addition, there is a concern with environmental problems, a problem that these countries deal with regularly. Thus, cooperation guarantees investments in this area and the possibility of exchanging ideas. Figure 3.4 shows this point, which represents the growing interest in studies regarding lignocellulosic biomass as a support for enzyme immobilization. Finally, the interconnections show a triangle of cooperation between China, India, and South Korea, valid because of their geographical proximity, showing the proximity between their economic and socio-environmental interests.

After analyzing the database, we noticed the involvement of 1,482 organizations from 81 countries. This expressive number testifies to the importance of the present work. However, the greatest volume of manuscripts produced is focused on about 20 countries, which together represent only a quarter of all the countries identified. Emerging and developed economies are related to developing alternative solutions that guarantee a good socio-

[1] The thickness of the lines connecting the two countries indicates the accumulation of co-authorship, and the colors divided into clusters illustrate the groups of countries with a high level of collaboration.

environmental policy, which is in line with the countries highlighted in this analysis: China, India, the USA, and Brazil.

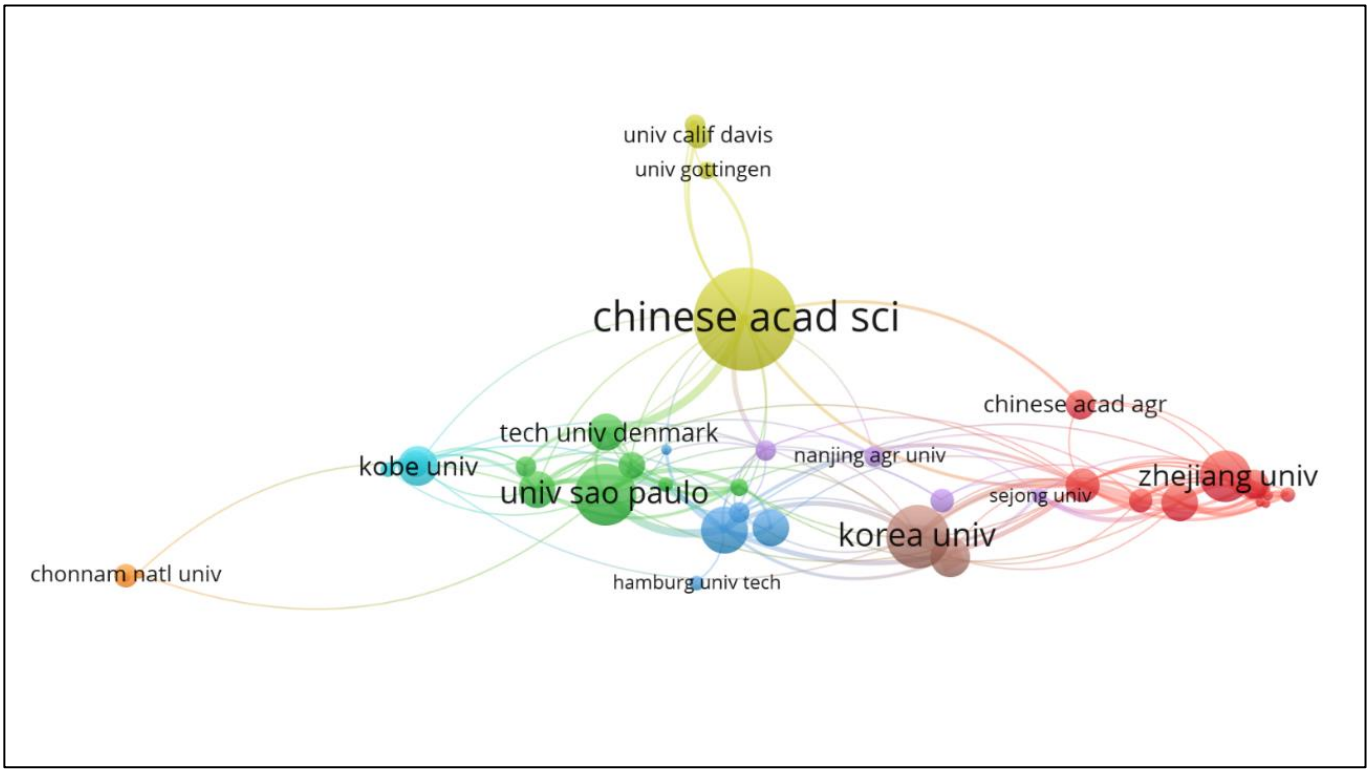
We found that 98% of the organizations cited produced up to 10 publications on this research topic from 2000 to 2022. About 70% produced only one publication. We could verify the contribution of several authors that ensured the research decentralization, even within the same country, thanks to this perspective introduced by the analysis. However, consistency in the production of manuscripts in this area is restricted to only 2% of the organizations.

Two organizations that each have only one manuscript on the topic have a high number of citations. The first is Meisei University (Japan) which owns the manuscript "Ethanol fermentation from biomass resources: current status and prospects" by Lin, Y (2006), with 1,118 citations, which makes it the most cited article in the database. The second is Lund University (Sweden) which has the article "Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification" by Palmqvist, E (2000), with 939 citations, ranking second in the ranking of most cited articles.

The data analyzed show strong links between countries geographically close, which is valid because of cooperation between neighboring organizations. However, some countries have organizations that break out of this geographical barrier and form partnerships with organizations from other continents, such as the partnership between the United States and China. Brazil also stands out in the interconnection of organizations on different continents, having a close relationship with Spain and India. The relationship between Brazil and India is strong in the pharmaceutical market, which may justify academic cooperation between these two countries (MUNJAL et al., 2021).

Figure 3.5 illustrates the interconnections between the organizations. For a proper presentation of the interactions, we delimited a minimum amount of 250 citations accumulated by documents published in the analyzed period, thus identifying 40 institutions in the database that meet the minimum citation quota. We highlighted the most relevant organizations, such as the Chinese Academy of Sciences, the University of São Paulo, and the University of Korea, with the best cooperation results. Finally, among the 47 institutions with at least 250 cumulative citations, only 21 (about 44.7%) had over 10 collaborations with other organizations.

Figure 3.5 — Network visualization map related to collaboration between organizations with at least 250 citations.[2]

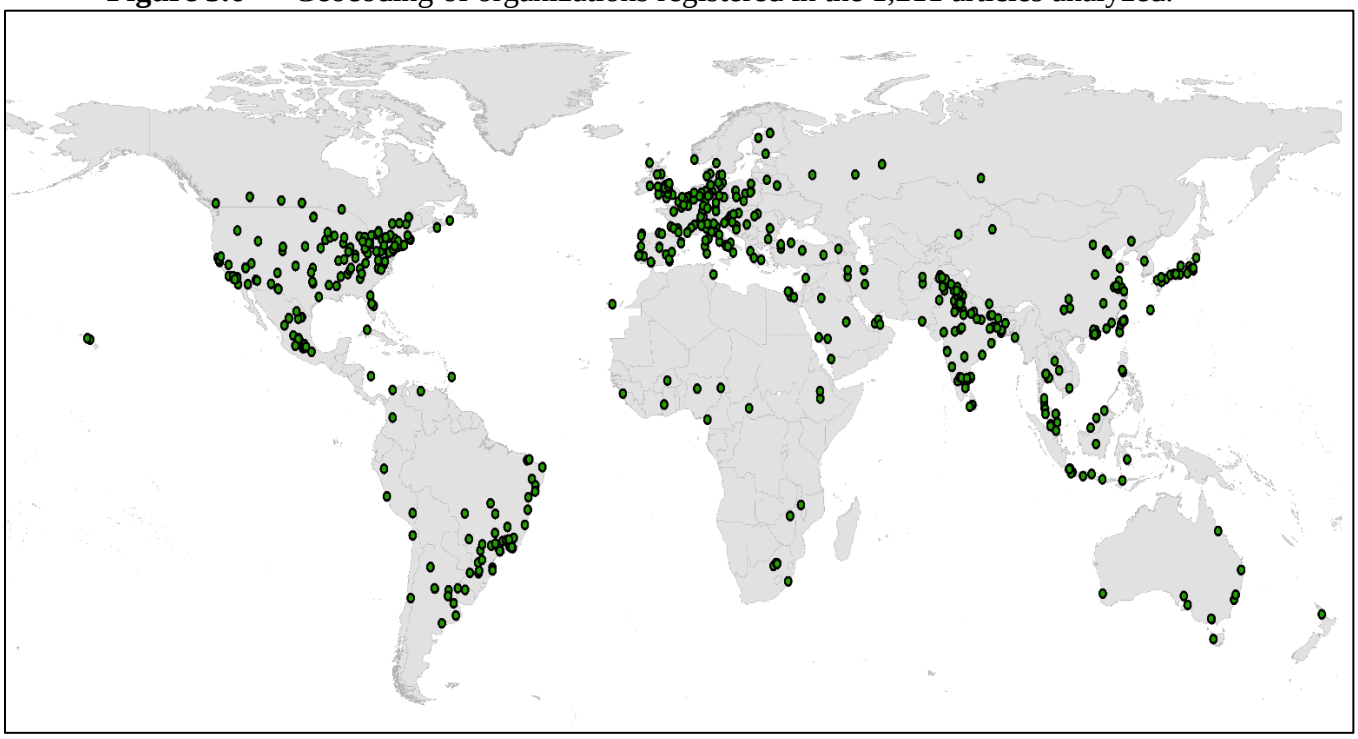


Source: Author (2023).

To complement the network visualization analysis, a geocoding was developed that represents each organization within its country as green dots. This geocoding increases the possibilities of visualization and analysis of the organizational distribution involved in the research focused on the research topic. Figure 3.6 presents an option for analyzing geocoded addresses, showing a higher density of organizations in the regions that comprise North America and Europe, and not far below some regions of South America and Asia.

[2] The thickness of the lines connecting the two organizations is a strong indication of the accumulation of co-authorship, and the colors divided into clusters illustrate groups of institutions with a high level of collaboration.

Figure 3.6 — Geocoding of organizations registered in the 1,211 articles analyzed.



Source: Author (2023).

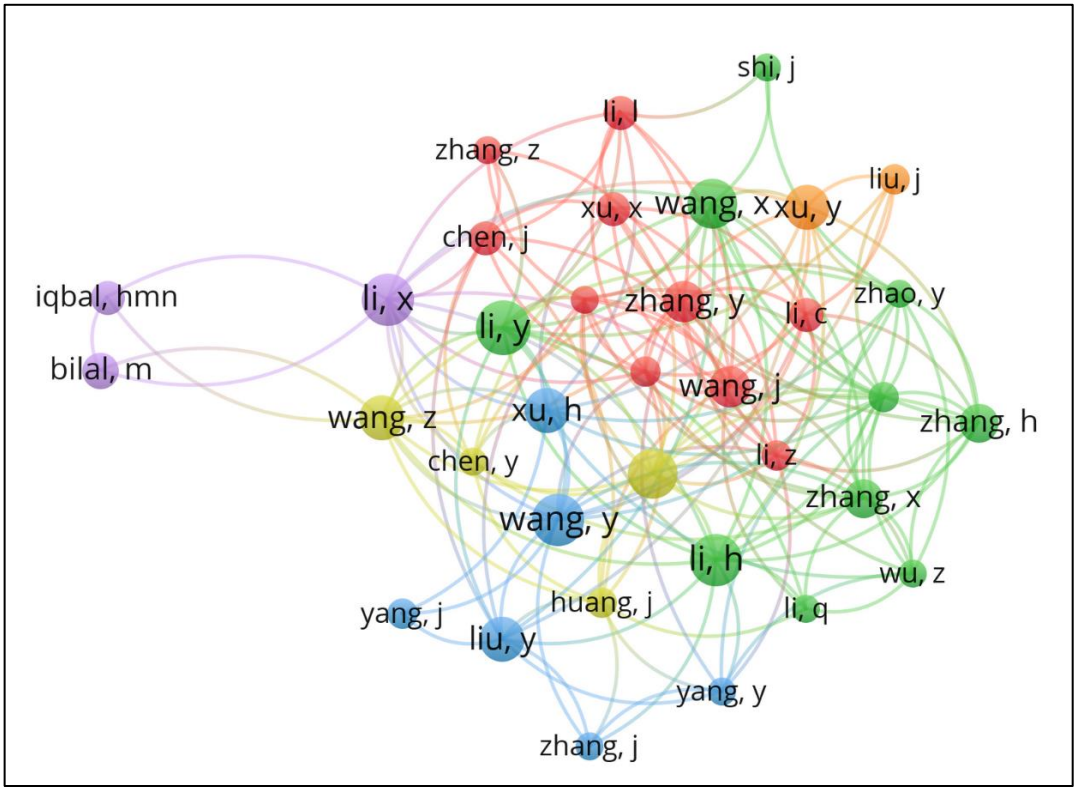
A total of 5,323 different authors were identified. This result shows the great diffusion of research lines carried out in this area, which guarantees a great diversity of researchers and methodologies to apply to the theme. Figure 3.7 presents the relationship between authors, following the delimitation that each author must have at least one published work and have at least 450 citations of their works. This image provides a view of the closest collaboration sets and shows that almost all authors are part of the large collaboration network for co-authorship.

The author Lin, Y authored the most cited paper in the database ("Ethanol ferment from biomass resources: current state and prospects" (LIN; TANAKA, 2006), with 1,018 citations. However, we notice he is not part of an extensive network of collaborations with other papers. On the other hand, Kim, and Seung participated in developing 14 papers (most notably "Enzymatic coproduction of biodiesel and glycerol carbonate from soybean oil and dimethyl carbonate", from 2011), which obtained 69 citations.

We implemented a filter of at least five citations that reduced authors to 3,810, of which only 10 had no co-authorship relationship. These remained outside the primary network, generating ten smaller and independent networks, which represent about 0.26% of the collaborating authors. Thus, it is once again clear the large collaborative network for the production of articles, the local networks between neighboring institutions, and the cooperation

between authors from the same institution, which in the end all collaborations strengthen the studies on this theme.

Figure 3.7 — Network visualization map related to collaboration between authors with at least 450 citations.[3]



Source: Author (2023).

3.4.1.4. The most cited articles

From the analysis of the most cited manuscripts about lignocellulosic biomass as support for enzyme immobilization, 10 articles stood out, which together obtained 5,753 citations. The manuscript "Ethanol fermentation from biomass resources: current state and prospects" (LIN; TANAKA, 2006), which is in the first place, obtained 1,018 citations, thus accounting for 17.7% of the total citations (Table 3.3). This article addresses ethanol fermentation, presenting practical examples and providing information for a general and rather broad overview of the current ethanol fermentation scenario. Above all, it cites the biomass resources, microorganisms, and technology applied in this production. In addition, it presents promising perspectives for ethanol fermentation

[3] The thickness of the lines connecting two authors is a strong indication of the accumulation of co-authorships, and the colors divided into clusters illustrate groups of authors with a high level of collaboration.

Table 3.3 — The most cited works in the area of the use of lignocellulosic biomass as support for enzymatic immobilization.

Rank	Paper	Authors	Year Published	Average Publication	Total Citations
1	Ethanol fermentation from biomass resources: current state and prospects	Lin, Y and Tanaka, S	2006	59.88	1018
2	Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification	Palmqvist, E and Hahn-Hagerdal, B	2000	40.83	939
3	Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: A novel biological approach to nanoparticle synthesis	Mukherjee, P; Ahmad, A; Mandal, D; Senapati, S; Sainkar, SR; Khan, MI; Parishcha, R; Ajaykumar, PV; Alam, M; Kumar, R and Sastry, M	2001	38.41	845
4	White-rot fungi and their enzymes for the treatment of industrial dye effluents	Wesenberg, D; Kyriakides, I and Agathos, SN	2003	38.9	778
5	An overview of enzymatic production of biodiesel	Ranganathan, SV; Narasimhan, SL and Muthukumar, K	2008	29.8	447
6	Fungal dye decolorization: Recent advances and future potential	Kaushik, P and Malik, A	2009	28.71	402
7	Pathways of nitrogen utilization by soil microorganisms - A review	Geisseler, D; Horwath, WR; Joergensen, RG and Ludwig, B	2010	29.46	383
8	Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation	Murthy, HN; Lee, EJ and Paek, KY	2014	36.78	331
9	Enzyme stabilization by nano/microsized hybrid materials	Hwang, ET and Gu, MB	2013	30.6	306
10	The role of additives on anaerobic digestion: A review	Romero-Guiza, MS; Vila, JJ; Mata-Alvarez, J; Chimenos, JM and Astals, S	2016	43.43	304

Source: Author (2023).

The paper "Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification" (PALMQVIST; HAHN-HÄGERDAL, 2000), obtained 939 citations in the analyzed time interval. This manuscript presents a review regarding the effect of different detoxification methods on fermentability and the chemical composition of hydrolysis components. Thus, the inhibition of fermentation is mitigated by the application of a ligninolytic enzyme. lacase, by prior fermentation with the fungus Trichoderma reesei and other pretreatments. Another point analyzed is the yield of the fermentative reactions, identifying possible inhibitors linked to continuous and discontinuous fermentations (PALMQVIST;

HAHN-HÄGERDAL, 2000).

Another prominent paper is entitled "Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach for nanoparticle synthesis" (MUKHERJEE et al., 2001), which is about a pioneering biological method applied to the synthesis of silver nanoparticles with Verticillium. By leaving the fungal-based biomass in contact with aqueous Ag+ ions, intracellular metal ions and the formation of silver nanoparticles of 25 ± 12 nm were reduced. Electron microscopic analysis of fungal cell flakes showed that Ag particles formed under the cell wall, probably because of metal ions from enzymes in the cell wall membrane. Finally, it was concluded that metal ions showed no toxicity to fungal cells and could continue their proliferation after the biological synthesis of Ag nanoparticles (MUKHERJEE et al., 2001).

The article "White rot fungi and their enzymes to treat industrial dye effluents" (WESENBERG, 2003), also stands out as it is a review of the future uses of white rot fungi (WRF) and their embryo-modifying enzymes (LMEs) in treating industrial effluents, especially those contaminated with dyes. The textile industry is by far the most enthusiastic user of synthetic dyes and demands environmentally friendly solutions for colored wastewater. The potential for bleaching and reducing WRF intoxication can be exploited with new knowledge about the physiology of these organisms, their bio-stimulatory properties, and the stability of their enzymes. This knowledge should be translated into robust and reliable waste processing (WESENBERG, 2003).

3.4.1.5. The research areas

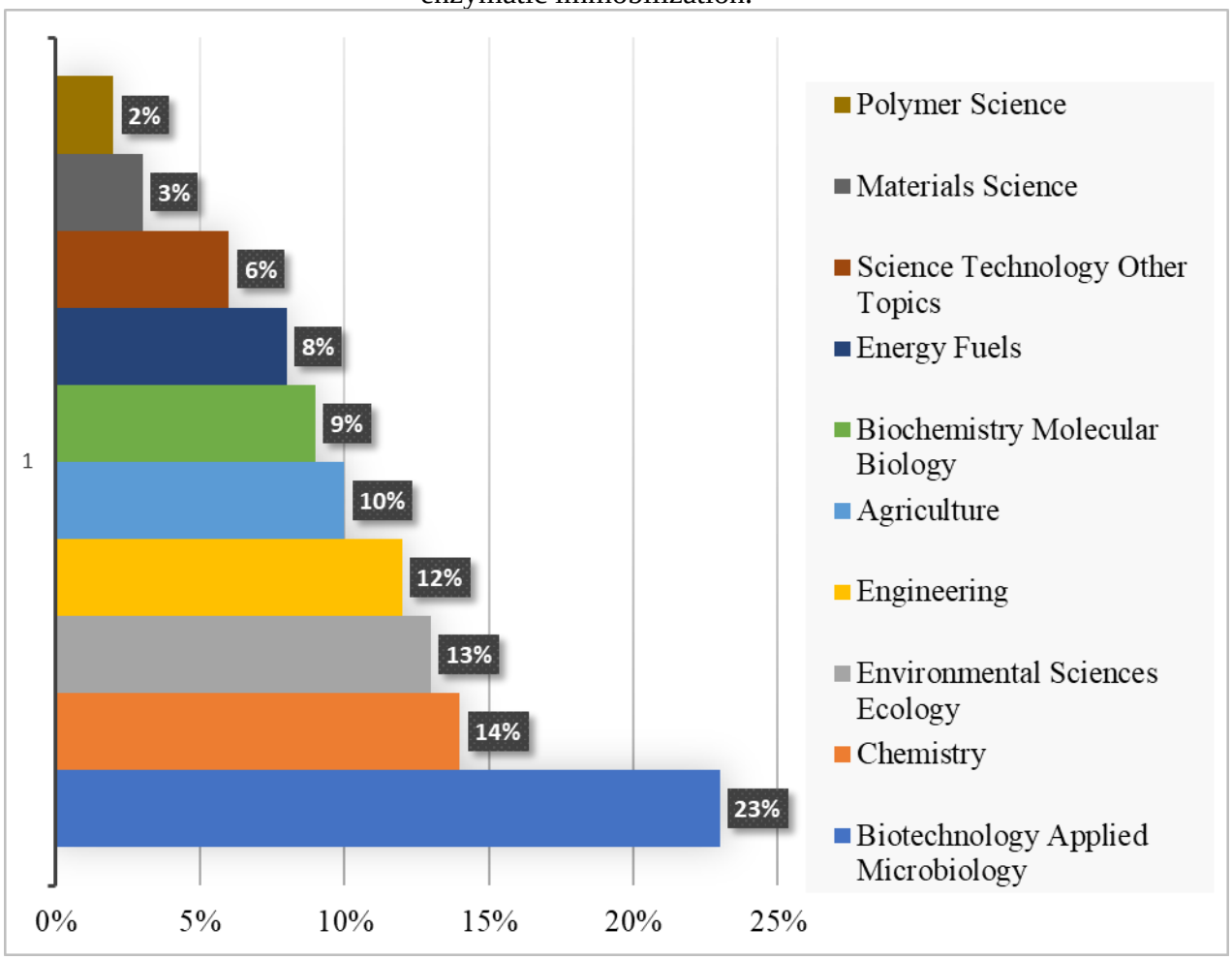
After searching the database, we found 40 research areas on lignocellulosic biomass as support for enzyme immobilization among the 1,211 articles published from 2000 to 2022. We highlight the principal research lines in Figure 3.8. The journal Biotechnology Applied to Microbiology stood out the most, with 426 occurrences (23% of the manuscripts). In the sequence, the area of Chemistry obtained 262 records, occupying the second place in this category and representing 14% of the total articles analyzed.

It is possible to verify that research is not centralized in a few areas, but well distributed in several others. Such distribution is favorable because it ensures greater applicability of immobilized enzymes and increased demand for this material. As a reflection of the demand for a certain material, it reduces its market value because of the supply and demand theory. Another reflection of the large-scale use of these immobilized enzymes ensures a significant reduction in the impacts caused to the environment by conventional chemical

processes.

Finally, we must clarify that there was a delimitation in the database, excluding some manuscripts that were not relevant to the topic of this study. Therefore, only papers that fell into the category of article, review article, and conference paper were considered in this analysis.

Figure 3.8 — Distribution of research areas related to lignocellulosic biomass as support for enzymatic immobilization.



Source: Author (2023).

3.4.2. Hot Research topics

3.4.2.1. Quantitative analysis of frequent keywords

The analysis of the keywords determines the amount of research developed in certain areas, thus projecting the growth trends of discussions in these areas and which parameters are followed by the authors. This information is necessary because together it confirms the development of the area studied. Table 3.4 ranks the 20 most relevant keywords among the manuscripts present in the database of this study. Another point addressed in this

table is the strength of the link that each word has.

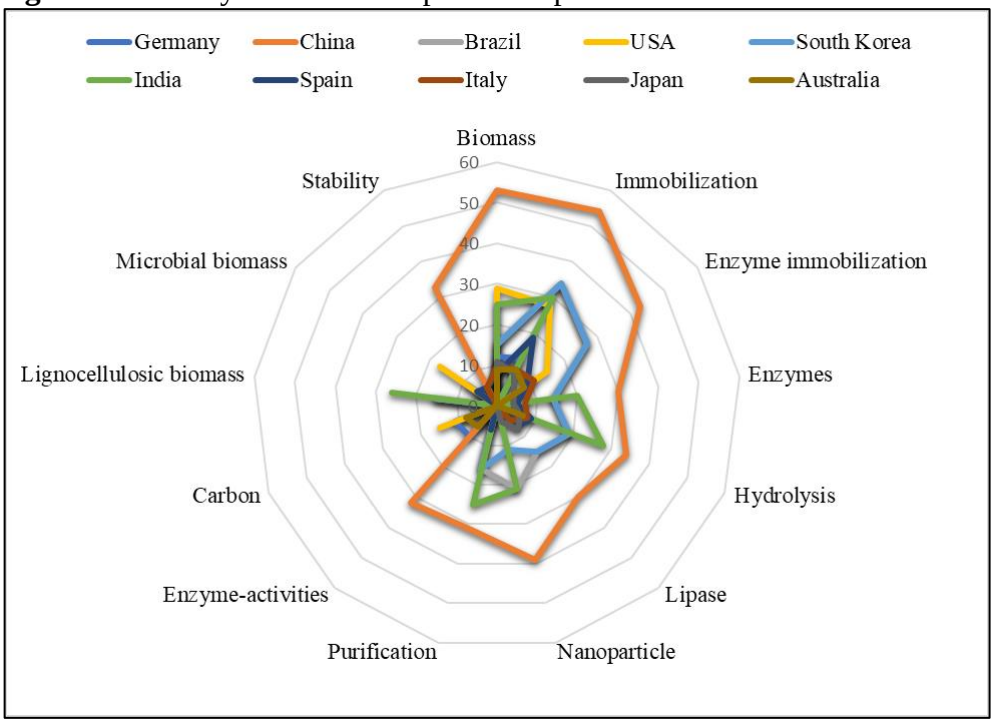
Table 3.4 — Top twenty most prominent keywords in articles search.

Rank	Keyword	Frequency	Total Link Strength	Rank	Keyword	Frequency	Total Link Strength
1	Immobilization	385	622	11	Lignocellulosic biomass	80	170
2	Biomass	192	290	12	Optimization	80	169
3	Enzyme immobilization	125	234	13	Biodiesel	78	108
4	Hydrolysis	116	262	14	Enzyme-activities	76	64
5	Lipase	107	220	15	Fermentation	69	126
6	Purification	106	225	16	Enzyme	67	130
7	Enzymes	106	184	17	Adsorption	66	123
8	Nanoparticles	91	191	18	Pretreatment	65	160
9	Stability	89	236	19	Microbial biomass	64	54
10	Cellulase	80	237	20	Degradation	62	93

Source: Author (2023).

Furthermore, from the keyword analysis, you can see in Figure 3.9 of the radar map the difference in the search direction of the top 10 producing countries. Studies in the United States, China, Brazil, Spain, Australia, India, South Korea, Italy, and Germany show that there are studies on different topics in this field.

Figure 3.9 — Keyword radar map of the top 10 countries from 200**0** to 2022.

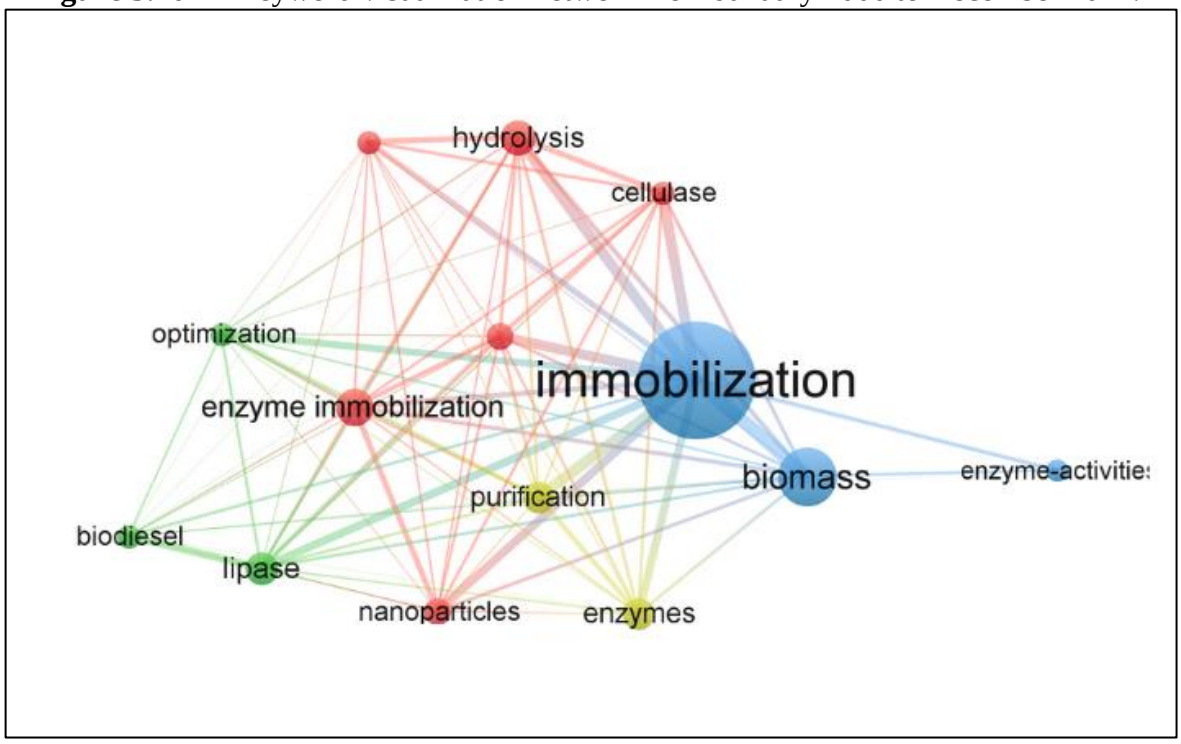


Source: Author (2023).

Figure 3.10 shows the relationship and application trend of the main keywords present in the database. This analysis allowed us to conclude that in the period from 2000 to 2022, the word that had the greatest impact was "immobilization" (385 occurrences). The result

of this highlighted word represents the direction of research for this area. In addition, other prominent words follow the same application logic (i.e., are linked to immobilization), such as the keywords "biomass" and "immobilized enzyme", which have 192 and 116 occurrences in this connection network, respectively. The size of the links represent the relationships between words; therefore, the larger the link, the greater the interaction between them.

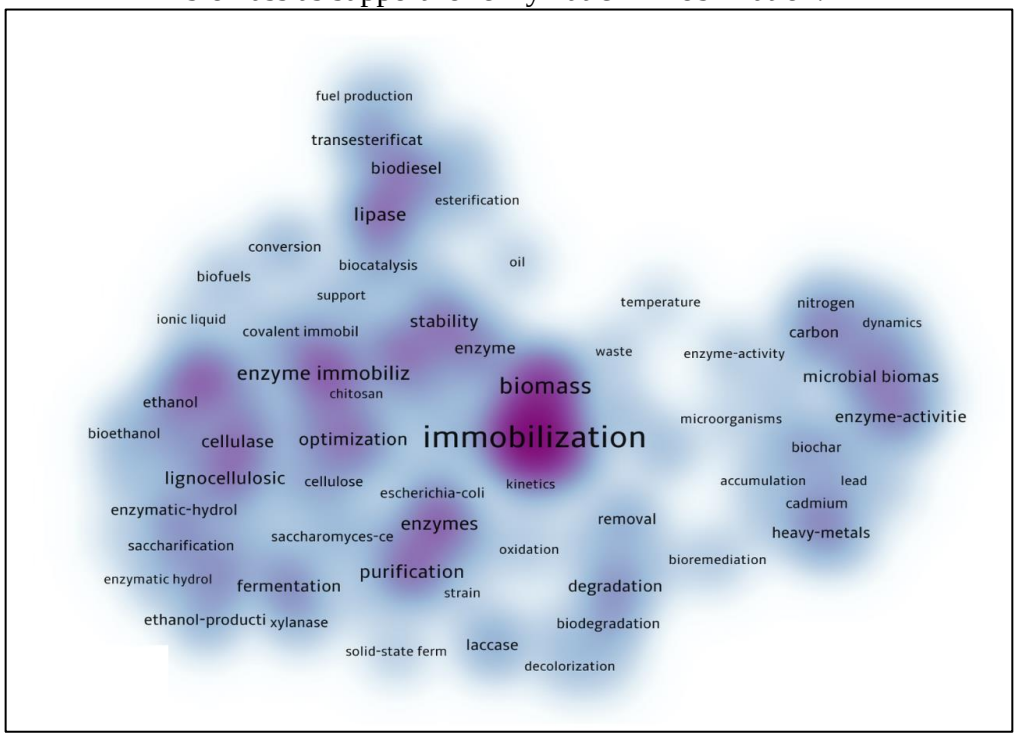
Figure 3.10 — Keyword visualization network from January 2000 to December 2022.



Source: Author (2023)

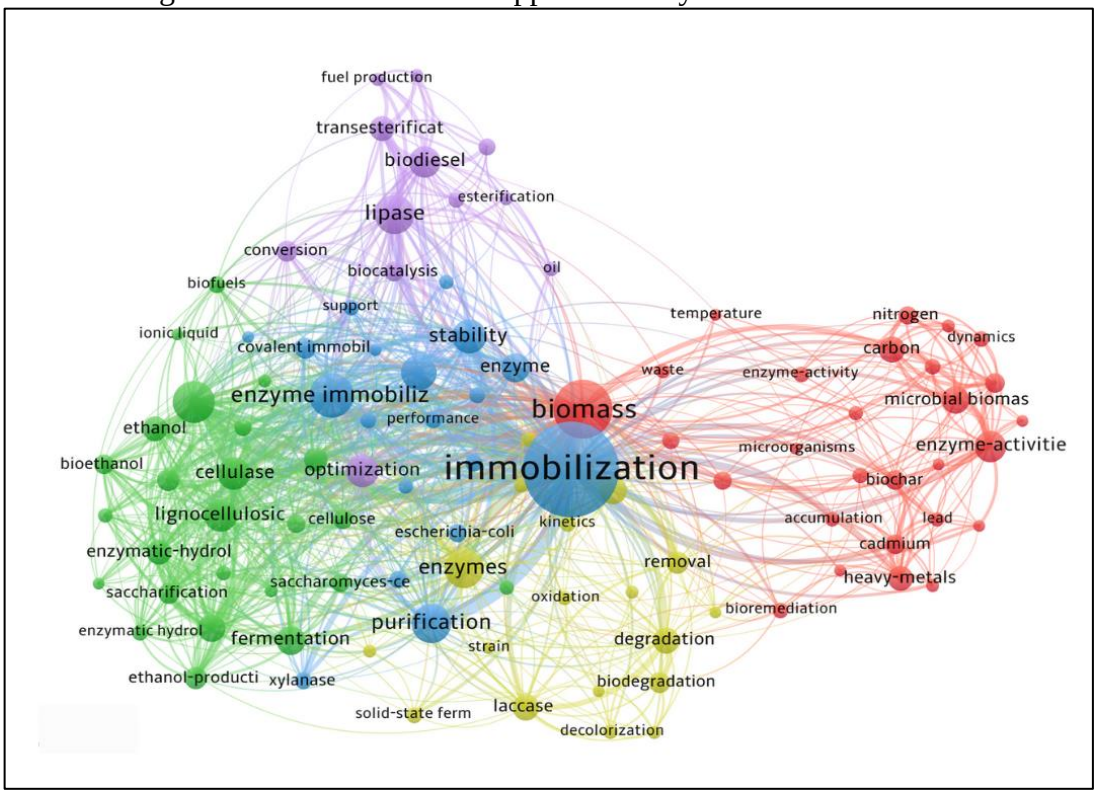
Figure 3.11 shows the keyword density map, points with more intense colors show keywords with a higher number of occurrences. The delimitation for this network was at least 20 occurrences in different articles, which resulted in a set of 100 words. In addition, Figure 3.12 results from another analysis of the keywords, by which it was possible to obtain a broader network of the use of these words. However, in Figure 3.12, they relate to a wider range of areas represented by the words. The larger number of items in the network allows the generation of islands of words that are part of the same search field, where, in the image, a different color represents each field. This image follows the same criteria used previously.

Figure 3.11 — Co-citation density map of keywords in the database related to lignocellulosic biomass as support for enzymatic immobilization.



Source: Author (2023).

Figure 3.12 — Visualization map of keyword co-citation network in research related to lignocellulosic biomass as support for enzyme immobilization.



Source: Author (2023).

The cluster symbolized by the blue color is the most relevant because it contains

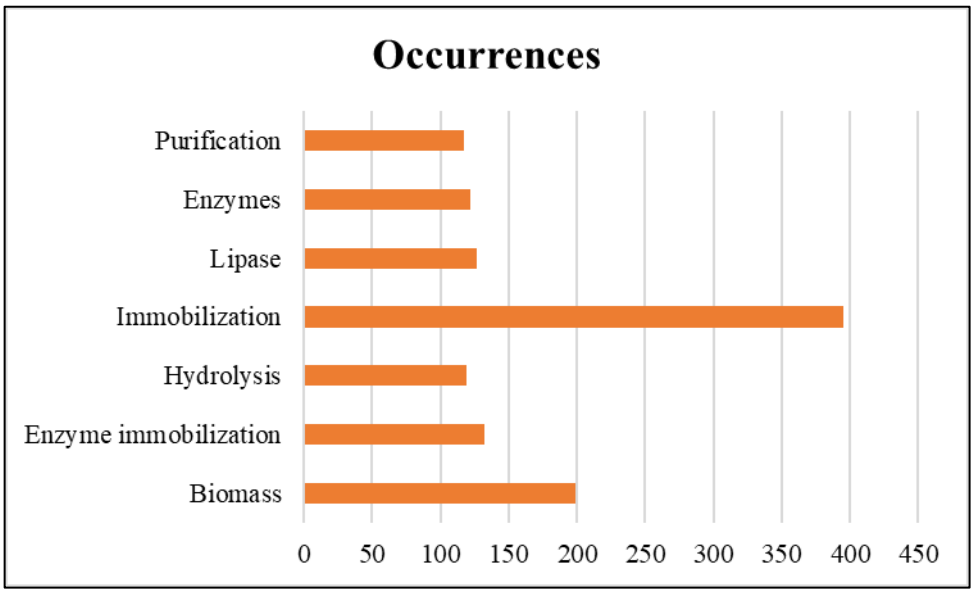
the most frequent keyword—“Immobilization”—which is directly in line with the theme of this study. Still in this cluster are the keywords “enzyme immobilization”, “lipase”, and “purification”, which together will represent a research field. The keyword “biomass” is the highlight of the red cluster and thus adds relevance to this group since its percentage of occurrence is perceived in the second position of the keywords ranking. The words “waste” and “microbial biomass” are highlights that are linked to “biomass” and thus define another research field.

The keyword “optimization” links the green and purple clusters, highlighted among the 20 most cited. However, these clusters differ in their application. The green cluster is linked to the keyword “fermentation”, which relates to “ethanol” and “bioethanol”, forming one research field. The purple cluster has “lipase” as a highlight, relating strongly to “biodiesel” and “esterification”, resulting in another distinct research field.

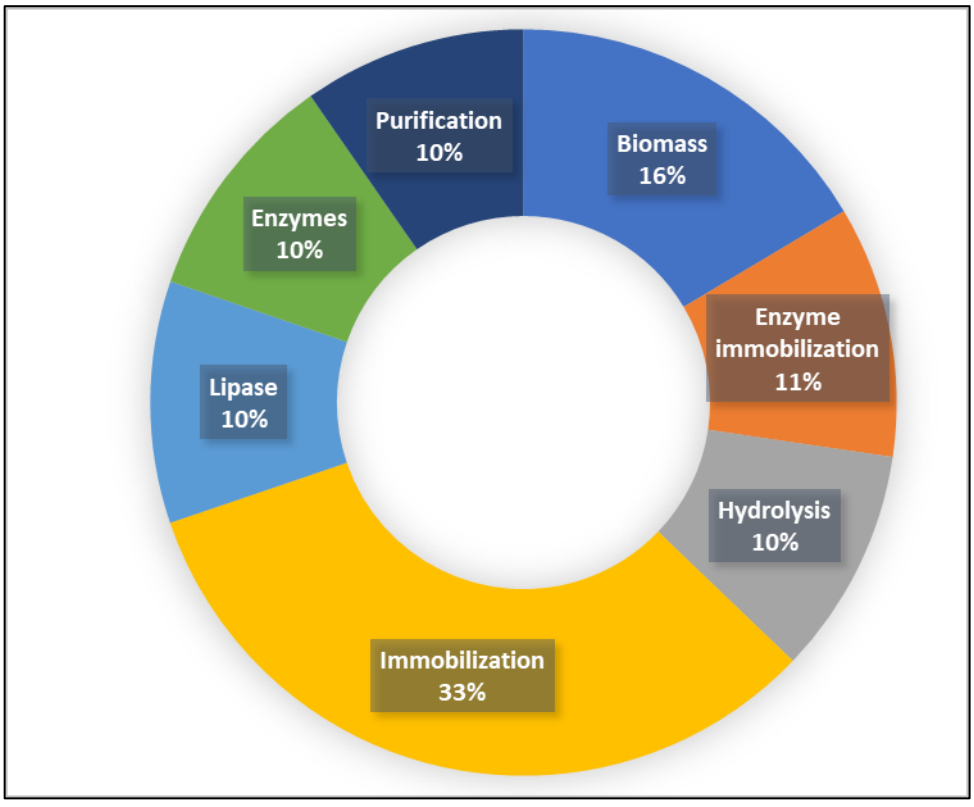
The yellow cluster focuses on issues related to the environment, using the keyword “enzyme” as a highlight and relating it to the keywords “biodegradation” and “removal”. These words are linked to the purification and selectivity promoted by enzymes, where such characteristics reduce the environmental impacts associated with conventional production routes (DA S. MOREIRA et al., 2022).

Finally, Figure 3.13 will demonstrate the occurrence relationship between some of the keywords used in works involving lignocellulosic biomass, which resulted in the analysis of 1,211 publications. By analyzing this graph, it is clear that Immobilization and Biomass, as mentioned earlier, occupy a prominent place among the articles. Hydrolysis is the word with the lowest number among these, as it is still in the process of developing research with the use of lignocellulosic biomass and may be an emerging trend in the future of scientific literature.

Figure 3.13 — List of most cited keywords (A) and the percentage of articles on the occurrence of keywords (B) presented in the titles of the analyzed documents.



(A)



(B)

Source: Author (2023).

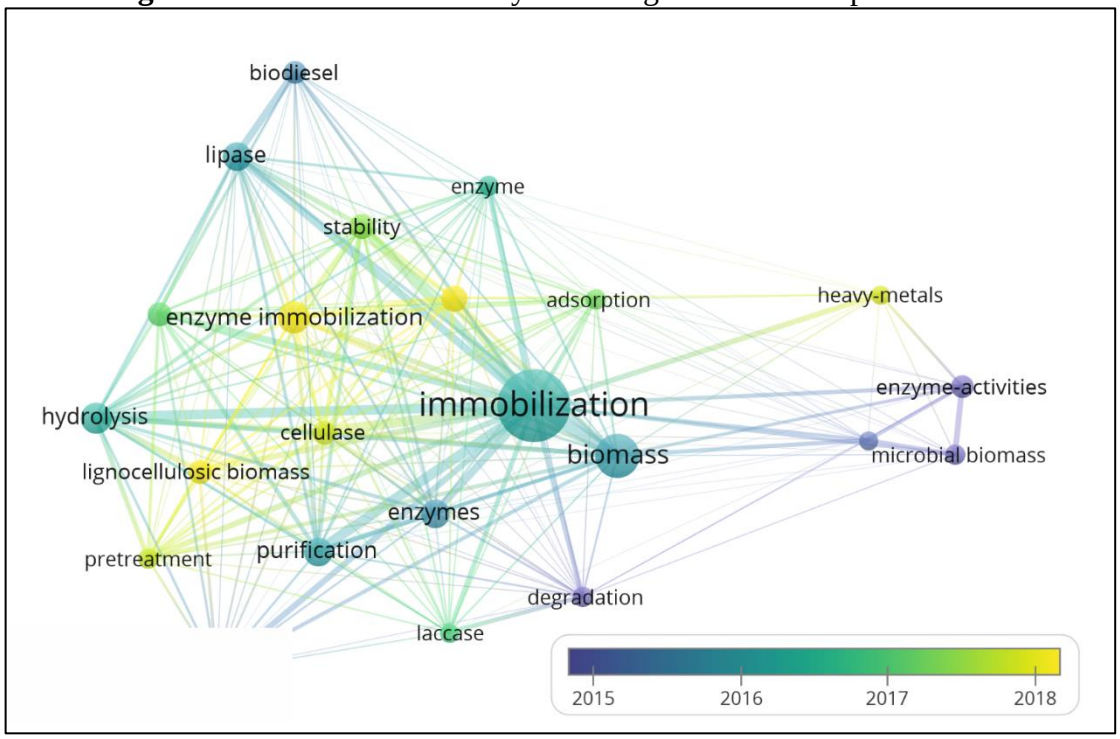
3.4.2.2. Research hotspots

We use CiteSpace software, which acts on the database analysis to delineate which

trends stand out and thus define which ones emerge in the topic of the present research. CiteSpace is used to facilitate the analysis of the intellectual structure and emerging trends (CHEN et al., 2012), as well as to improve the understanding of platform research and enable future developments for theorists and practitioners (DING; YANG, 2022).

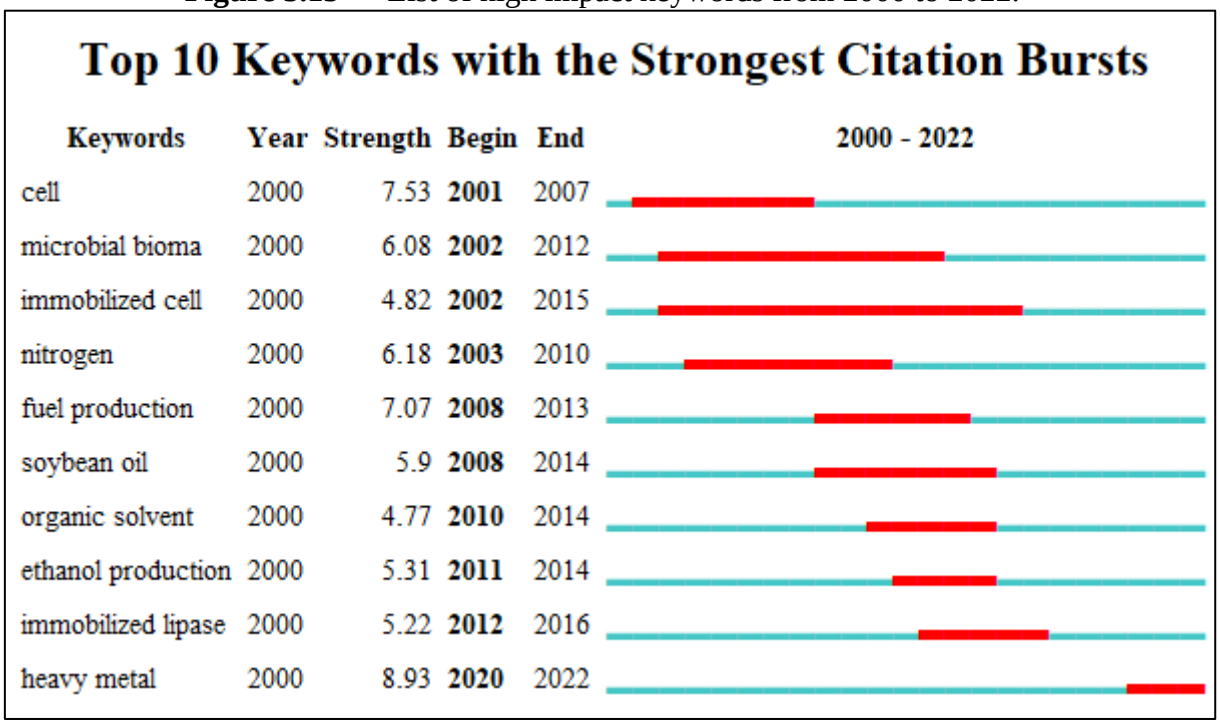
In the visualization of Figure 14, it is clearly shown that the three main categories of subjects are Immobilization, Biomass, and Enzymes. In recent years, as can be seen in Figure 3.15, the four keywords with the highest intensity of emergence are heave metal (8.93), cell (7.53), fuel production (7.07), and nitrogen (6.18). In addition, the keywords Ethanol Production and Immobilized Lipase during 2011-2014 and 2012-2016, respectively, indicate that this area of research has become very prevalent in recent years, and it can be assumed that research on these topics has been encouraged and widely developed. The words involving immobilization and enzymes reinforce the importance of this area of research and, consequently, strengthen the need for new materials for study and application.

Figure 3.14 — The relevant keyword usage evolution map over time.



Source: Author (2023).

Figure 3.15 — List of high impact keywords from 2000 to 2022.



Source: Author (2023).

3.4.2.2.1. Constant fields of investigation

The finding of the increase in the relevance of an area is directly related to the growth in the number of publications that this area receives. Thus, the keywords will be used as guides to determine the path of the next trends of future studies that will be linked to using lignocellulosic biomass for enzyme immobilization. Table 3.5 presents the top six sets of co-citations among the manuscripts present in the study database that will refer to the same subject.

Table 3.5 — Top six co-citation clusters on raw in the area of lignocellulosic biomass as supports for enzymatic immobilization based on CiteSpace analysis.

Cluster ID	Label	Node Size	Mean	Top Five Terms	Representative Articles
#0	Microbial activity	159	2010	Microbial activity; agricultural soil; microbial biomass; soil microorganism; temperate forest soil	(HUANG et al., 2020; ZHENG et al., 2010)
#1	Enzymatic hydrolysis	145	2014	Enzymatic hydrolysis; lignocellulosic biomass; situ saccharification; thermal stability; mesoporous silica	(JIA et al., 2017; R. et al., 2022)
#2	White-rot fungi	63	2009	White-rot fungi; ligninolytic enzyme; *Phanerochaete chrysosporium*; white rot; dye decolorization	(CEN et al., 2022; PANT; ADHOLEYA, 2007)
#3	Chromatographic	55	2006	Chromatographic behavior; metal chelate;	(LEE-PARSONS;

	behavior			continuous production; tomato pomace; hydrophilic polyurethane foam	SHULER, 2005)
#4	Biodiesel production	53	2012	Biodiesel production; enzymatic production; solvent free system; recombinant *Rhizopus oryzae* lipase; biotechnological production	(ALAVIJEH et al., 2015; WANG et al., 2010)
#5	Microbial growth	52	2008	Microbial growth; yarrow lipolytic; biobased production; lignocellulosic hydrolysate; lip2 lipase	(PALMQVIST; HAHN-HÄGERDAL, 2000; YUZBASHEVA et al., 2011)
#6	Semiarid area	34	2006	Semiarid area; process engineering aspect; *Acremonium chrysogenum*; complex media; *Cephalosporin c*	(SCHOEBITZ; MENGUAL; ROLDÁN, 2014; SEIDEL et al., 2002)

Source: Author (2023).

Cluster #0 has the keyword "Microbial activity", which, like the other words, refers to microorganisms. Microbial biomass is one of the potential materials used as enzyme support and fits within lignocellulosic biomass (THAPA et al., 2020). The article "Role of biochar and Eisenia fetida on metal bioavailability and biochar effects on earthworm fitness" is one of the representatives of this cluster and conducts studies on the individual and combined effects of biochar and earthworms *(Eisenia fetida*) on soil properties, bioavailability, and earthworm fitness in soils historically contaminated with heavy metals (HUANG et al., 2020). Cluster #5 is closely related to this cluster.

The keyword "Enzyme hydrolysis" represents cluster #1 and, together with the other highlighted words, defines this group as the representative enzyme immobilization. Enzymes can be used in the synthesis reactions of many products; thus, different ways to improve these enzymes are studied, such as improving thermal stability (as highlighted in the cluster), selectivity, and resistance to denaturation (FERREIRA MOTA et al., 2022). Here, the immobilization is on lignocellulosic biomass, but the closest can be immobilized on mesoporous silica (ZHANG; SUN, 2018). The manuscript "Novel Magnetic Cross-Linked cellulase Aggregates with a Potential Application in Lignocellulosic Biomass Bioconversion" is one representative of this cluster and explains that the stabilization technique can improve the stability and recyclability of enzymes and thus a novel cross-linked cellulase magnetic complex was developed and applied to biomass biotransformation (JIA et al., 2017).

Cluster #2 is headed by the keyword "White-rot fungi", which is directly related to

the other highlighted words. White-rot fungi can synthesize enzymes and are related to the topic of this paper. An example of an enzyme obtained from white-rot fungi is the enzyme lignin peroxidase, obtained from the fungus *Phanerochaete chrysosporium* (DOMÍNGUEZ et al., 2001). This cluster is represented by the manuscript "Green production of a yellow lacase by coriolopsis gallica for phenolic pollutants Removal" which addresses research on a new effective green production strategy for lacase fungi and showed that the resulting purified lacase from *C. gallica* showed good enzymatic properties and catalytic potential for phenolic pollutants' removal (CEN et al., 2022).

Cluster #3 has the keyword "Chromatographic behavior" as a representative. In this cluster, the multidisciplinarity of the theme of this work was evident, since this cluster presents from lignocellulosic biomass, such as tomato pomace, to examples of chemical analyses performed, such as chromatography. The article "Sparge gas composition affects biomass and ajmalicine production from immobilized cell cultures of *Catharanthus roseus*" represents this cluster and addresses the effects of O_2 and CO_2 on secondary development and metabolism that were investigated using ajmaline production from *Catharanthus roseus* cultures (LEE-PARSONS; SHULER, 2005).

Cluster #4 is represented by the keyword "Biodiesel Production". This word represents this cluster well, as all the highlighted words are related to biodiesel production. This area is another possibility for the application of enzymes, especially immobilized enzymes. Here, we can already see that the environmental issue is being emphasized in the theme of this study because some alternative biofuels to petroleum derivatives have already been cited. The manuscript "Enzymatic production of biodiesel from microalgal oil using ethyl acetate as an acyl acceptor" is the highlight of this cluster and presents a study on the application of ethyl acetate in the enzymatic production of biodiesel, in a pioneering way, using the microalgae *Chlorella vulgaris*, as a source of triglycerides. In this manuscript, the enzymatic conversion of fatty acids to biodiesel was catalyzed by Novozym 435, which is an efficiently immobilized lipase for biodiesel production (ALAVIJEH et al., 2015).

3.5 Future Trends

Future prospects in this area are:

- Increase and strengthen cooperation between emerging countries to enhance research with lignocellulosic biomass;
- Strengthening of relations between organizations with researches that follow the same objective, guaranteeing an exchange of data and information that will be significant for the development of this area, where enzymatic immobilization in lignocellulosic biomass is carried out;
- The highlighted keywords analyzed showed the future trend for this area, where these new biomaterials will be applied in the most diverse research;
- Finally, an increase in the applicability of these materials is expected to reduce the impacts involved in incorrect disposal in the environment.

3.6 Conclusions

With the advanced bibliometric analysis, it is possible to highlight that the application of lignocellulosic biomass as enzyme support has, as its focus, concerns with environmental problems caused by production lines that use the chemical route. In addition, many methodologies aim to circumvent such mishaps to ensure a more sustainable market for lignocellulosic biomass.

Regarding the origin of the periodicals that address this theme, the large role of European periodicals was evident. A large number of journals leads to the conclusion that there are many application possibilities for the theme in question, as each journal has its aims and scopes. Based on this data, an analysis of the countries that host these journals was carried out, which leads to the understanding that some countries stand out to the detriment of others. We believe that this is due to the economic scenario of these countries. Countries such as the USA, China, India, and Brazil are frequently cited in the analyses. These countries have well-developed economies or stand out among developing economies in common.

Concerning the numbers, Chinese prominence is unquestionable. China produces 28.07% of the articles on lignocellulosic biomass for enzyme immobilization. This achievement guarantees it a high-impact position in this area and shows its prospects in this research line. In addition, China has the highest percentage of citations among all countries in the database.

Another interesting point concerns the collaboration that exists between countries. From the analyses performed, it is possible to conclude that cooperation exists within each country—being carried out between internal organizations—with promising results regarding academic production. It also exists between countries, where borders are no longer barriers and become bridges for exchanging information and knowledge. This exchange of information, research, and technology is essential for developing countries and research areas.

The lignocellulosic biomass as a support for enzyme immobilization is an alternative to be considered because it composes a sustainable method and meets problems related to storing and handling these residues. This theme has been highlighted in scientific research in several areas, such as the chemical, pharmaceutical, biotechnological, food, and environmental industries. Therefore, it is possible to determine that biocatalysts on lignocellulosic supports represent an environmentally friendly method and aim to make bio-processes in this area more sustainable.

CHAPTER 4

CHAPTER 4 – THEORETICAL AND EXPERIMENTAL STUDY FOR THE ENZYMATIC PRODUCTION OF GERANYL BUTYRATE USING LIPASE EVERSA®

4.1 Abstract

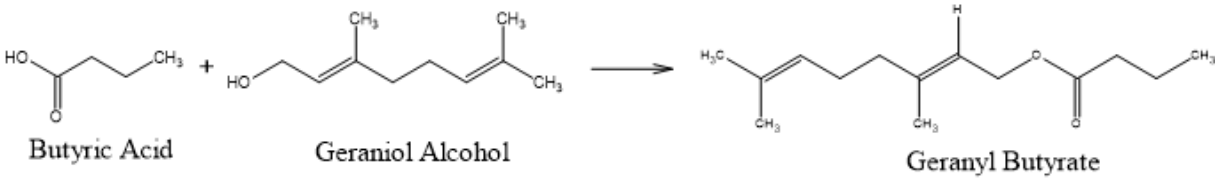
Aromatic esters are organic compounds with wide application in the perfumery industry to impart specific odors to products. These aromas can be synthesized from enzymatic catalysis, the process of using enzymes to speed up chemical reactions. In this sense, the Eversa® enzyme is a lipase that can be applied in this industry because it is resistant and capable of operating under high pressures and temperatures. This process requires experimental planning, where factors that affect the reaction, such as temperature, reagent concentration, reaction time, etc., are evaluated and optimized. In terms of theoretical research, molecular docking technology stands out, which is a technique for predicting interactions between molecules, such as proteins and reagents. Therefore, the objective of this work is to optimize the synthesis of the aromatic ester geranyl butyrate using the Taguchi method, to use chromatographic techniques in the characterization of these products, and to evaluate the interaction between biocatalyst and ligand applying computational tools such as docking and molecular dynamics. Taguchi's method optimized the reaction system (1:5 v/v, 15% biocatalyst, 50 °C and 6 h), with a yield of 93.05±0.45%, where time was the parameter that most influenced the reaction. In the theoretical study, MarvinSketh was used to analyze the protonation state of the ligand and, for the enzyme, the H++ server. The conformational changes involved in the forming complexes, the conformational interactions, and the energy required for this process were analyzed using molecular dynamics, to align theoretical and experimental processes. Ligand/Eversa complexes were submitted to Molecular Dynamics (MD) and evaluated by mean values of Root Mean Square Deviation (RMSD) and interaction energy, the smallest and most stable being equivalent to -104.756 kj.mol -1. Theoretical results agree with the experimental ones, confirming the relevance of the work.

Keywords: Aroma. Eversa®. Taguchi planning. Molecular Docking. Molecular Dynamics.

4.2 Introduction

Flavor esters are organic compounds widely used in the fragrance industry to give products a pleasant aroma (CHEN et al., 2022a). They are produced by reacting fatty acids with alcohols and are known for their wide range of fragrances, including fruits, flowers, and spices (COSTA et al., 2022). As such, they are used in a wide range of products, including soaps, deodorants, perfumes, food, and beverages (GUCKENBIEHL et al., 2022). They also play an important role in the food industry, where they are used as natural and synthetic flavoring agents (WU et al., 2023). These aromas can be obtained by chemical route or through enzymatic catalysis (YU et al., 2023). Figure 4.1 shows the reaction for the synthesis of Geranyl Butyrate.

Figure 4.1 — Esterification reaction for the production of geranyl butyrate.



Source: Author (2023).

Enzymatic catalysis is the process of accelerating chemical reactions through the use of enzymes (TANG et al., 2019; ZHANG et al., 2021). These biomolecules work as natural catalysts, increasing the speed of chemical reactions without being consumed or altered, and each one of them is specific for a certain chemical reaction and binds to the substrates involved in the reaction to accelerate them (BHATTACHARJEE; MORYA; GHOROI, 2020; BILAL et al., 2018; CHUNG et al., 2018). Enzymatic catalysis is important for some industrial processes as it ensures that they occur efficiently and quickly (MONTEIRO et al., 2020). These applications include the production of food, beverage, medicine, and other products (OLIVEIRA et al., 2019). Enzymes are divided into classes and one of them is lipases, which are widely used in enzyme catalysis (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; CHAPMAN; ISMAIL; DINU, 2018; GUO et al., 2023; LI et al., 2012).

Lipases are enzymes that catalyze the hydrolysis of lipids, such as fats and oils, into glycerol and fatty acids (BILAL et al., 2021; FERREIRA MOTA et al., 2022; LAI et al., 2019). They are found in many and synthesized by many microorganisms. In addition, lipases are also widely used in industrial applications, including the production of biodiesel, detergents, food, and other products (LEE et al., 2019; SONI; DWIVEDEE; BANERJEE, 2018; WAFTI et al., 2021). The high specificity of lipases for different types of lipids and their ability to function in acidic or alkaline conditions make these enzymes valuable in many applications (CHOI et al.,

2022; SALIHU; ALAM, 2015). Within this class of enzymes, there is Eversa®, which is a lipase from *Aspergillus oryzae* (GUIMARÃES et al., 2021b).

The Eversa® enzyme is an extremophilic lipase that is capable of functioning under extreme temperature and pressure conditions (ALVES et al., 2022; FRAGA et al., 2019). It was first isolated from thermophilic bacteria and is known for its ability to hydrolyze lipids at high temperatures (CAVALCANTE et al., 2022; GUIMARÃES et al., 2021a). Eversa® is widely used in industrial applications, including the production of biodiesel and other products of interest (SUN; GUO; CHEN, 2021). In addition, its structure and mechanism of action have been the subject of intensive study in enzyme research, as they help to understand the ability of proteins to function under adverse conditions (FRAGA et al., 2019). Based on its thermophilic capacity, it is possible to organize an experimental design for the study, aiming at greater efficiency in the synthesis of Geranyl Butyrate (GUCKENBIEHL et al., 2022).

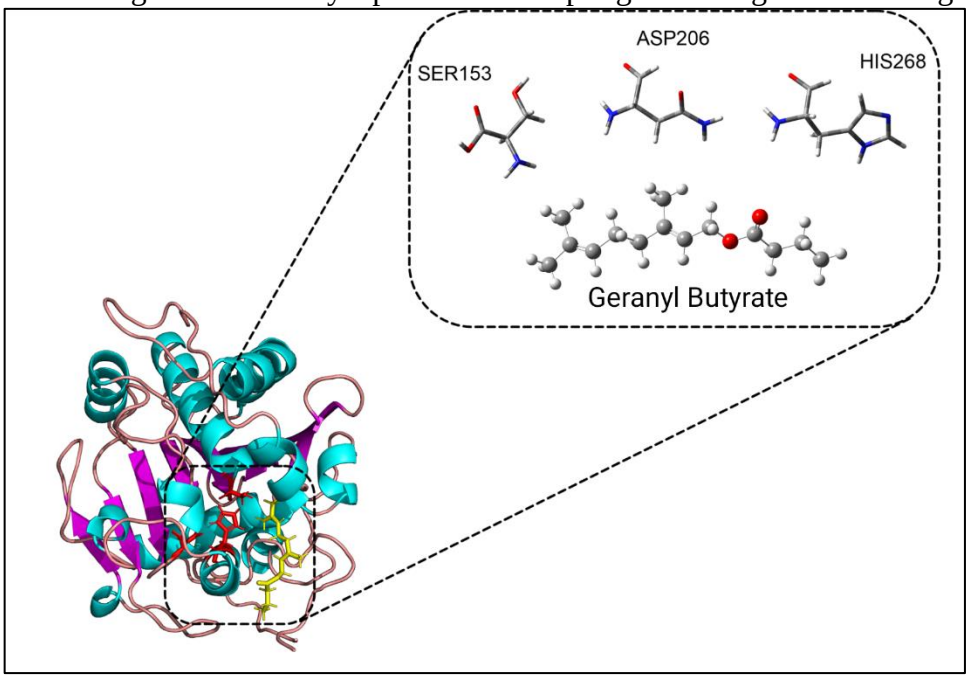
Experimental planning is a critical step in conducting chemical reactions, as it allows for determining the best conditions for obtaining desired products efficiently and at a low cost (MOULA ALI; BAVISETTY, 2020; RAVURI; SHIVAKUMAR, 2020). During the experimental planning, the factors that affect the reaction, such as temperature, the concentration of reagents, and reaction time, among others, are evaluated and optimized (CHEN et al., 2022b; VENKATARAGHAVAN; THIRUCHELVI; SHARMILA, 2020). In addition, experimental planning may include the choice of solvents, reagents, methods of purifying products, and other relevant aspects (MAAMOUN; ELJAMAL; ELJAMAL, 2023; MOHAMMAD MIRZAIE et al., 2016). Once the best conditions are established, the experiment can be run in a safe and controlled manner, maximizing reaction efficiency and minimizing waste of resources (RENJITH; DEVASENA; ABEENS, 2023). Experimental design is therefore a fundamental part of chemical research and the production of chemical compounds.

Following, the Taguchi methodology is an experimental planning and process organization technique that aims to improve product quality and production efficiency (CHEN et al., 2022b). It was developed by Japanese engineer Genichi Taguchi and is based on the statistical analysis of experiments to identify the most important factors that affect the performance of a process (TAGUCHI, 1995). This design is widely used in industrial applications, including the production of consumer goods, automobiles, electronics, and other items (MONTEIRO et al., 2022). It allows the evaluation of various combinations of process conditions to determine the best conditions to achieve the quality objective (MOREIRA et al.,

2020). The Taguchi methodology is a valuable tool for continuously improving processes and maximizing production efficiency and profitability (DA S. MOREIRA et al., 2022).

With regard to theoretical studies, molecular docking stands out, which is a technique used to predict the interaction between molecules, such as proteins and reagents (CULLETTA; ALMERICO; TUTONE, 2020; SÁNCHEZ-CRUZ, 2023). It simulates the way two molecules fit together, assessing bond strength and three-dimensional configuration (AVIZ-AMADOR; CONTRERAS-PUENTES; MERCADO-CAMARGO, 2021; GUO et al., 2023). This technique is widely used in industry to identify compounds that may be candidates for pharmaceutical line drugs, as well as to study protein-protein or protein-ligand interactions in the process industry (ALMEIDA-NETO et al., 2020; CHEDADI et al., 2021; IQBAL et al., 2023). Molecular docking can be performed with several algorithms, each with its features and limitations (GOVINDARASU et al., 2021; SARKAR; SRIVASTAVA; AILANI, 2022). The combination of different techniques, such as dynamic simulation and structure modeling, may be necessary to accurately predict the interaction between molecules. (BARREALES et al., 2021; BRELA et al., 2022; PAHLANI; SCHWARTZENTRUBER; JAMES, 2023) Examples of these interactions are shown in Figure 4.2.

Figure 4.2 — Representation of the catalytic triad of the Inverse Transform 2.0 interacting with the ligand. The catalyst pocket and coupling are enlarged for viewing.



Source: Author (2023).

Thus, the objective of this work is to improve the synthetic routes used to obtain structured lipids, characterize them from chromatographic techniques and finally, evaluate the

behavior of the biocatalyst and ligands using computational tools, such as molecular docking and molecular dynamics. These tools will allow an understanding of the interaction energies involved in the esterification of Geraniol with Butyric Acid catalyzed by Eversa ® Transform 2.0, where the Geranyl Butyrate obtained in the experimental part will be evaluated together with the catalytic triad of the enzyme. Furthermore, bringing the theoretical model closer to the experimental one, molecular dynamics takes into account the effects of solubility and entropy and thoroughly explores the binding and interaction of its receptors from the perspective of energy and mechanism.

4.3 Methodology

4.3.1 Materials

The commercial lipase Eversa® transform 2.0 from *Aspergillus oryzae* was purchased from Sigma-Aldrich Brasil Ltda (Cotia, São Paulo, Brazil). The chemical reagents used were analytical grade from Synth (São Paulo, Brazil) and Vetec (São Paulo, Brazil). The Statistica®10 software (Statsoft, USA) was used for the experimental design based on the Taguchi method.

4.3.2 Methods

4.3.2.1 Biocatalysis

The samples were distributed to promote greater interaction between the reagents, following the experimental design, using different ratios of alcohol, biocatalyst, and acid. The containers with the reagents were orbitally shaken at 200rpm, varying temperature, molar ratio, biocatalyst, and time, for different samples.

After performing the esterification process (GERMANO DE SOUSA et al., 2022), the samples were analyzed in duplicate, in two Erlenmeyer flasks with 0.2g of the sample, 5mL of standardized ethyl alcohol, and 3 drops of phenolphthalein, each. It was titrated with 0.1 molar NaOH solution until the color changed to a subtle pink. After the titration, the volume values used were applied in Equation 4.1 to obtain the acidity index (AI).

$$\boldsymbol{IA}\left(\frac{\boldsymbol{mgNaOH}}{\boldsymbol{g}}\right) = \frac{\boldsymbol{MM_{NaOH}}.\boldsymbol{M_{NaOH}}.\boldsymbol{f}.\boldsymbol{V_{NaOH}}}{\boldsymbol{m}} \quad (4.1)$$

Where, MM_{NaOH} (g/mol) is the molar mass of NaOH; M_{NaOH} (mol/L) is the molarity of the NaOH solution; f is the correction factor determined by NaOH standardization; V_{NaOH} is the volume of NaOH used during the titration, and m (g) is the mass of the sample to be studied.

The conversion of butyric acid to the aroma ester of geranyl butyrate is given by Equation 4.2, where IA_i represents the initial acidity value given by the amount of acid added in the reaction, and IA_f to the final acidity value, equivalent to the remaining acids in the solution, not used by the enzyme.

$$\boldsymbol{Conversion}\ (\%) = \frac{\boldsymbol{IAi - IAf}}{\boldsymbol{IAi}}\boldsymbol{x100} \quad (4.2)$$

4.3.2.2 High performance liquid chromatography analysis

The sample obtained from the optimal conversion point was analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu LC-18 column at a temperature of 25°C and equipped with a UV detector. The eluent used was a mixture of acetonitrile and water in a ratio of 4:1 (v/v), and the flow rate was 3.0 ml/min for 5 minutes. For the analysis, a wavelength of 220 nm was chosen, which showed the highest absorption after scanning.

4.3.2.3 Characterization of the glycerides

The one-dimensional spectra of Hydrogen Nuclear Magnetic Resonance (RMN ^{1}H) and Carbon (RMN ^{13}C) were obtained in a Bruker spectrometer, model Advance DRX-300, belonging to the Northeast Center for Application and Use of Nuclear Magnetic Resonance, located at the University Federal do Ceará (CENAUREMN-UFC). The experiment was carried out at the hydrogen frequency at 300 MHz and at the carbon frequency at 75 MHz. The solvent used to dissolve the samples was deuterated chloroform ($CDCl_3$) and they were analyzed in 5 mm tubes.

4.3.2.4 Experimental design and statistical analysis (Taguchi Method)

For these experiments, an experimental design based on the Taguchi method was used with a standard L9 orthogonal matrix (the "L" and "9" represent the Latin square and the number of experiments, respectively) to distribute four factors in three levels, to maximize ester conversion. Table 4.1 shows the four independent factors (Molar ratio between acid and alcohol, biocatalyst, temperature, and time) and their corresponding levels, emphasizing that the percentage of biocatalyst was calculated from the reaction volume after calculating the molar ratio.

Table 4.1 — Determination of experimental procedure levels and range of independent parameters.

Levels	Molar ratio	Biocatalyst (%)	Temperature (°C)	Time (h)

Level 1 (L1)	1:1	5	30	2
Level 2 (L2)	1:5	10	40	4
Level 3 (L3)	1:9	15	50	6

Source: Author (2023).

The values of the S/N ratios (Signal-to-Noise) corresponding to the conversions were calculated using the characteristics of the "bigger is better" function since the objective of this study is to maximize the response (fatty acid conversion). In the Taguchi method, the S/N ratio is the measure of quality characteristics and the deviation from the desired value. Using the signal-to-noise (S/N) ratio to analyze the results reduces the sensitivity of the system to sources of variation, resulting in a good performance. The value of the S/N ratio for each experiment was calculated according to Equation 4.3.

$$\frac{S}{N} = -10 \log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}\right) \quad (4.3)$$

Where *y* is the fatty acid conversion for the corresponding sample, *i* is the number of replicates, and *n* is the number of responses for the combination of factor levels in any given parametric combination. The predicted S/N ratio under optimal conditions for the process of obtaining the maximum conversion was estimated by Equation 4.4.

$$\frac{S}{N_{predicted}} = \frac{\bar{S}}{N} + \sum_{j=1}^{n}\left(\frac{S}{N_j} - \frac{\bar{S}}{N}\right) \quad (4.4)$$

Where $\bar{S}/N$ is the arithmetic mean of all S/N ratios, S/ Nj is the S/N ratio at the sweet spot for each factor, and n is the number of factors that significantly affect the process.

4.3.2.5 Computational study of the reaction system

4.3.2.5.1 Obtaining the structure of Eversa lipase

The homology technique was applied to model Eversa® Transform 2.0 lipase. The amino acid CAS number 9001-62-1 was purchased from Sigma-Aldrich [4]and the Basic Local Alignment Search Tool (BLAST [5]) software was used to perform the homology analysis, as well as the PDB database. Next, the target protein was identified with the code 5XK2 on the Protein Database [6], where it is represented by *Escherichia coli-Pichia*. The microorganism that represents it is Aspergillus oryzae and its classification is hydrolase.

[4] https://www.sigmaaldrich.com/catalog/product/sigma/sae0065?lang=pt&region=BR
[5] https://www.ncbi.nih.gov/BLAST
[6] https://www.rcsb.org/

The Modeller software [7]was applied in the alignment of the primary structures and the development of the model, which resulted in obtaining a new protein, named Eversa® Transforme 2.0. The evaluation of this new protein was based on stereochemical parameters and objective functions. Stereochemical, conformational, and energetic terms were used to validate the model and the Ramachandran graph was limited to validating the performance of the model developed in the PROCHECK program.

4.3.2.5.2 Optimization of the ester structure

The construction of geranyl butyrate was elaborated using the GaussView 6.0 software, in which a model was obtained taking into account the possibilities of altering the structure of the ligands. The method Density Functional Theory (DFT) was applied to optimize the structures with the three-parameter hybrid functional of B3LYP Becke with the Lee-Yang-Parr correlation functional together with the basis set 6–31+G (d,p) implemented in Gaussian 9 package. To verify if the geometries are transition states or real minima, the vibrational modes of the optimized geometries were used. The calculations were based on the Polarizable Continuous Model, where this model considered the reaction in a vacuum and the formalism of integral equations. The CENAPAD-UFC computers were used to host the optimization and frequency of structures, where these calculations aim to define conformations of each ligand with more quality, which results in the definition of lower energies. In addition, the ChemAxon software MarvinSketh 22.16.0 was used to define their protonation states.

4.3.2.5.3 Molecular docking

After the homology technique was used for the development of the Eversa® Transform 2.0 protein, it was optimized in the AutoDock Tools software to perform charge correction from the addition of hydrogen atoms in the H++ server [8], where the hydrogen atoms were added from the automation of the system that obtains pKa results of ionizable groups in macromolecules. In addition, missing hydrogen atoms were added based on the pH of the reaction.

Docking was elaborated from the AutoDockVina software, from the consideration of rigid enzymes and flexible ligands. The grid configuration was applied to design the coupling perimeter, with the enzyme catalytic site (SER153, ASP206, HIS268) as the center and the alpha carbon (Cα) of histidine as the anchor point. The anchored poses were highlighted by the

[7] https://www.salilab.org/modeller/
[8] http://newbiophysics.cs.vt.edu/H++/index.php

AutoDock Tools software and the analysis of the energy profiles of ligand-receptor interactions was also performed.

4.3.2.5.4 Molecular dynamics simulations

Initially, it was defined that molecular dynamics would be carried out in a vacuum. To achieve the systems neutralization, seven sodium counter-ions were added and the Leap-Frog algorithm was applied to include movement in the equation every 2.0 fs. The particle mesh Ewald sum (PME) was applied to measure long-range interactions with a cutoff of 1.2 nm. For equilibrium, two 1ns steps were used, as well as an NVT (Particle Numbers, Volumes, and Temperatures) set with the modified Nosé-Hoover thermostat.

The simulation of molecular dynamics production of 100 ns added to an NVT set was performed in each system to determine its interaction with the ligand. The MarvinScketch software was applied to verify the micro spatial distributions and the protonation states, based on the pH 4 of the reaction. At the end of the molecular dynamics simulations, the most stable interaction complex was evaluated using Equation 4.5 (OLIVEIRA et al., 2022), which concerns the Potential Energy of Interaction (EPI) and results from the sum of intermolecular van der Waals interactions and electrostatic contributions. Finally, the conformational changes of the protein were obtained using mean square deviations (RMDS), based on Equation 4.6 (DE MEDEIROS et al., 2021)

$$\boldsymbol{IPE_{i,j}} = \sum_{\boldsymbol{i}}^{\boldsymbol{Ni}} . \sum_{\boldsymbol{j \neq i}}^{\boldsymbol{Nj}} \boldsymbol{VvdW}(\boldsymbol{r_{ij}}) + \boldsymbol{V_{elec}}(\boldsymbol{r_j}) \qquad \textbf{(4.5)}$$

Where $IPE_{i,j}$ is the potential energy of interaction between a group of atoms of *I* with a group of atoms of *j*, *Ni* and N_j is the total numbers of atoms in groups *i* and *j*, $V_{elec,}$ and *VvdW* are the variables corresponding to the electrostatic contribution and van der Waals type interactions, respectively.

$$\boldsymbol{RMSD} = \sqrt{\frac{\boldsymbol{1}}{\boldsymbol{N}} \sum_{\boldsymbol{i}}^{\boldsymbol{N}} [r_i\,(t) - r_i(0)^2]} \qquad \textbf{(4.6)}$$

Where $r_i(t)$ and $r_i(0)$ are the coordinates of the *i*-th atom at time *t* and 0, respectively, and *N* is the number of atoms in the domain of interest (usually Cα or main chain atoms).

4.4 Results and discussion

4.4.1 Parameter optimization using the Taguchi method

The optimization was carried out using the Taguchi method, which allowed the reduction of experimental steps in the process. Thus, a variation in the interaction between the reaction parameters was generated to determine the best level of each parameter to guarantee a greater conversion of the aroma esters. Table 4.2 presents, in an expanded form, the result of the experimental planning, as well as the conversion and S/N values for each analyzed point. Such points were performed in triplicate to ensure that the margin of error of the process was within the desired standards.

Table 4.2 — Experimental Design of the Taguchi L9 Plan.

Reaction	Molar ratio	Biocatalyst (% w/w)	Temperature (°C)	Time (h)	Conversion (%)	S/R
1	1:1	5	30	2	15.2±0.47	23.61
2	1:1	10	40	4	56.5±0.63	35.04
3	1:1	15	50	6	90.1±0.19	39.09
4	1:5	5	40	6	75.8±0.51	37.59
5	1:5	10	50	2	50.9±0.21	33.95
6	1:5	15	30	4	53.8±0.22	34.61
7	1:9	5	50	4	66.1±0.73	36.40
8	1:9	10	30	6	64.6±1.47	36.20
9	1:9	15	40	2	33.8±0.14	30.59

Source: Author (2023).

From the analyzes carried out after the experiments, it was possible to determine the parameters that most influenced the reaction, with time and temperature being the main observed highlights. Time, among all the parameters, was the greatest determinant of the process and as this variable increased, the conversion percentages also increased. In addition, it is worth highlighting points 3, 4, and 8 in Table 2, which represent two of the three highest yields and are based on a time of 6 hours.

Next, the temperature at its highest level also contributed to greater conversions into esters, as observed in points 3 and 7 which have the highest conversion rates and are based on a temperature of 50° C. With these results that emphasize the significance of temperature, it is possible to determine that the biocatalyst used did not present denaturation due to the increase in temperature since the conversions remained high with the variation of this parameter.

Thus, the parameters of the molar ratio between acid and alcohol and the variation in the percentage of biocatalysts were the factors that least influenced the conversion values of the esters. Its importance for the process is not discarded, however, other factors were more determinant. Regarding the molar ratio, an increase in alcohol can cause denaturation and/or inactivation of the biocatalyst, thus reducing the reaction yields, therefore, point 3 with the highest conversion value is highlighted using the ratio of 1:1. This possibility is in line with the increase in the concentration of enzymes, which at a given concentration may be affected by the molar ratio, or maybe saturating the reaction medium with the increasing amount of biocatalyst in the medium. The reduction in the number of enzymes to be used impacts the cost reduction of the process, being in this case a positive point to be considered.

4.4.1.1 Analysis of the S/N ratio

To determine the significance of the analyzed parameters, the Taguchi method provides S/N ratio values. Thus, the present study applied the "bigger is better" function to identify these ratios from the conversion values, as it is linked to the S/N ratio. Table 4.3 shows the information on the average S/N of all levels of each factor, in addition to the delta values. The results represented by delta are obtained from the subtraction between the factor with the highest S/N value and the lowest value. The results of these subtractions will be used to rank the factors and thus define their significance for the process.

Table 4.3 — Response to mean S/N ratios and ordering of variables.

Factors/Levels	**Molar ratio**	**Biocatalyst**	**Temperature**	**Time**
1	32.58	32.53	31.47	29.38
2	35.38	35.07	34.40	35.35
3	34.39	34.76	36.48	37.63
Delta	2.80	2.53	5.01	8.25
Ranking	3	4	2	1

Source: Author (2023).

From the analysis of Table 3, it is possible to determine that time and temperature stood out among the analyzed parameters, obtaining deltas of 8.25 and 5.01, respectively. Such results are in line with the experimental data presented and already discussed in Table 2, which indicated the same parameters as more significant for the process. Concerning time, the S/N averages expressed a significant gain as the levels increased, thus the variation from 2 hours

(Level 1) to 6 hours (Level 3) proved to be relevant. Added to this, increasing the temperature from 30 °C (Level 1) to 50 °C (Level 3) maintained the gain in the S/N ratio.

Relating the best parameters with those that were less significant, it is possible to conclude that the biocatalyst remained active for the longest time and at the highest temperature, thus showing the stability of the enzyme in the process and its thermal resistance. However, increasing the percentage of biocatalysts from 5% (Level 1) to 15% (Level 3) may have generated saturation in the system, leading to the inhibition of one enzyme by another, thus the best S/N ratio showed in Level 2 (10%). Finishing with the analysis of the molar ratio, the variation from 1:1 (Level 1) to 1:9 (Level 3), can result in an excess of alcohol and the denaturation of the catalytic sites of the enzymes, so Level 2 (1:5) is the most recommended for these reactions, based on the average S/N results.

4.4.1.2 Analysis of variance (ANOVA)

After applying the experimental design, the Analysis of Variance (ANOVA) was performed using statistical methods. The values obtained in this analysis are presented in Table 4.4, where the main highlight is given to the p-value which, according to the literature, is the value that determines the significance of the factors during the studied reaction.

Table 4.4 — Results of analysis of variance (ANOVA) for parameters that affect the esters production.

Factors	SS	DF	IN	*F value*	*p-value*	Contribution (%)
Molar ratio	60,620	2	30.310	0.05	0.8351	0.03
Biocatalyst	73.607	2	36,803	3.01	0.1575	1.84
Temperature	879,167	2	439,583	36.95	0.0037	23.49
Time	2916,847	(2)	1458,423	127.71	0.0004	74.64
Total	3,930,241	6	-	-	-	100

Source: Author (2023).

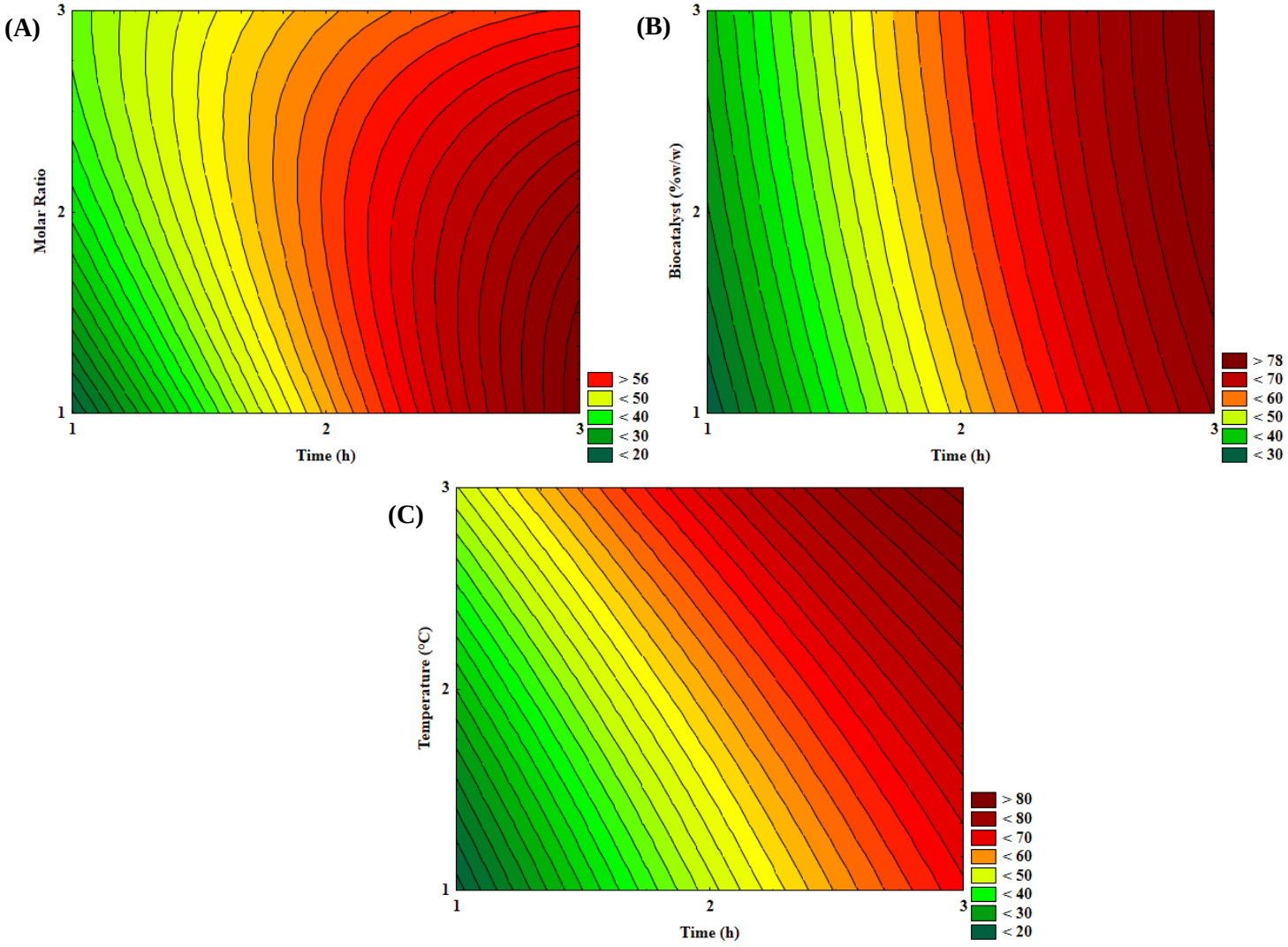
The significance of a parameter is linked to the p-value that can guarantee up to 95% confidence, provided that this value is less than 0.05. Thus, time and temperature showed significance within the best reliability range, as they presented p-values equal to 0.0004 and 0.0037, respectively. However, the contribution of time overlapped that of temperature, presenting 74.64%, against 23.49%. From the analyzes carried out, it was possible to define the optimized reaction conditions, which could guarantee higher conversion values in the reaction, thus it was defined that the best time was presented in Level 3, with 6h (L3), the best temperature was 50 °C (L3), the percentage of biocatalyst was defined as 15% (L3) and finally,

the molar ratio stood out in Level 2, in the ratio of 1:5 (L2). In theory, the conversion shown at this optimized point would be 95.98%, but after applying this point experimentally, the conversion was 85.17 ± 0.06%.

4.4.2 Statistical conversion analysis

From the statistical conversion analysis, contour surface graphs were prepared that relate time, the main parameter of influence, with the other planning parameters. The graphs in Figure 4.3 represent the conversion profile related to these combinations.

Figure 4.3 — Contour surfaces for the production of Geranyl Butyrate. (A) Biocatalyst (%) versus Time (h). (B) Temperature (°C) versus Time (h). (C) Molar ratio versus Time (h).

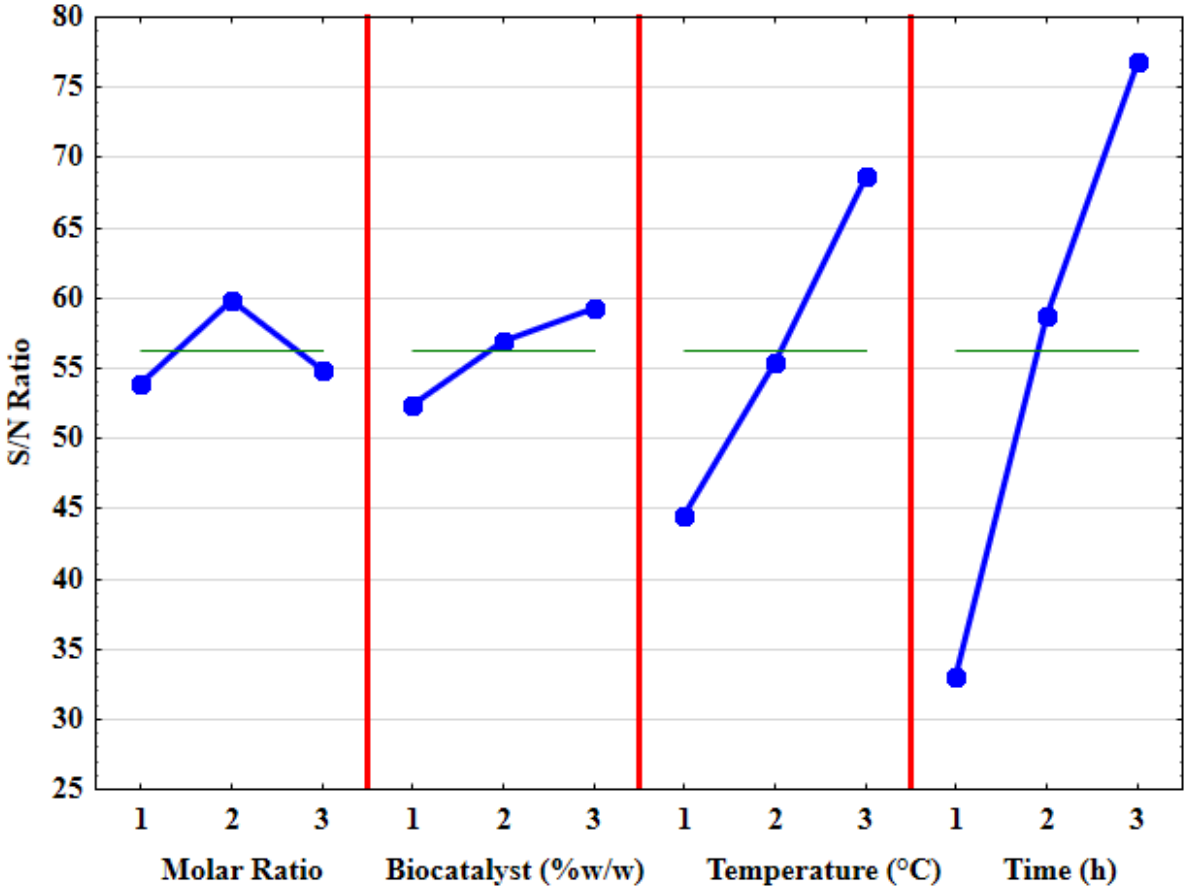


Source: Author (2023).

It is also possible to observe which parameters vary more and thus have a greater impact on the transformation from the graph of averages shown in Figure 4.4. Time again proved to be the most important factor in obtaining the reaction products, representing greater

mean variations, greater slopes, and more significant shifts between the graph points. The percentage of biocatalysts remains a low-impact parameter, as evidenced by the low slope and small mean change in the graph.

Figure 4.4 — Graphic of the averages of the parameter's variations.



Source: Author (2023).

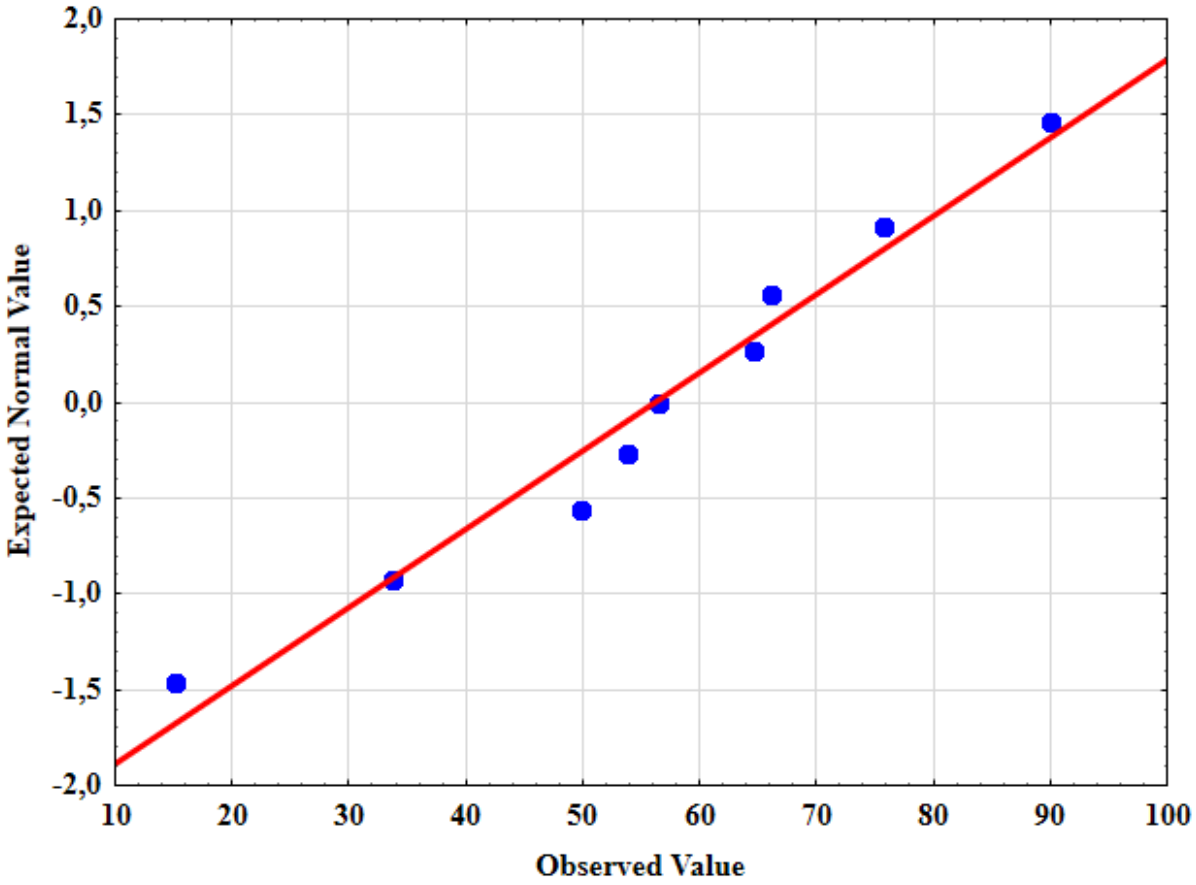
Based on the data obtained in the Taguchi method, it is possible to determine a regression equation, determining four dependent variables and estimating that the conversion follows the parameters of Equation 4.7.

$$\boldsymbol{Y}\,(\%) = \mathbf{-43,5 + 0,110}\boldsymbol{A} + \mathbf{0,691}\boldsymbol{B} + \mathbf{1,209}\boldsymbol{C} + \mathbf{10,970}\boldsymbol{D} \qquad \textbf{(4.7)}$$

Where *Y* is the conversion for the esterification reaction and *A*, *B*, *C*, and *D* are the encoded values of Molar ratio, percentage of biocatalyst, temperature and time, respectively.

A typical probability plot, shown in Figure 4.5, allows identification of experimental behavior and defines how close the model is to reality.

Figure 4.5 — Regular probability graph obtained as a comparison between theoretical and experimental methods.



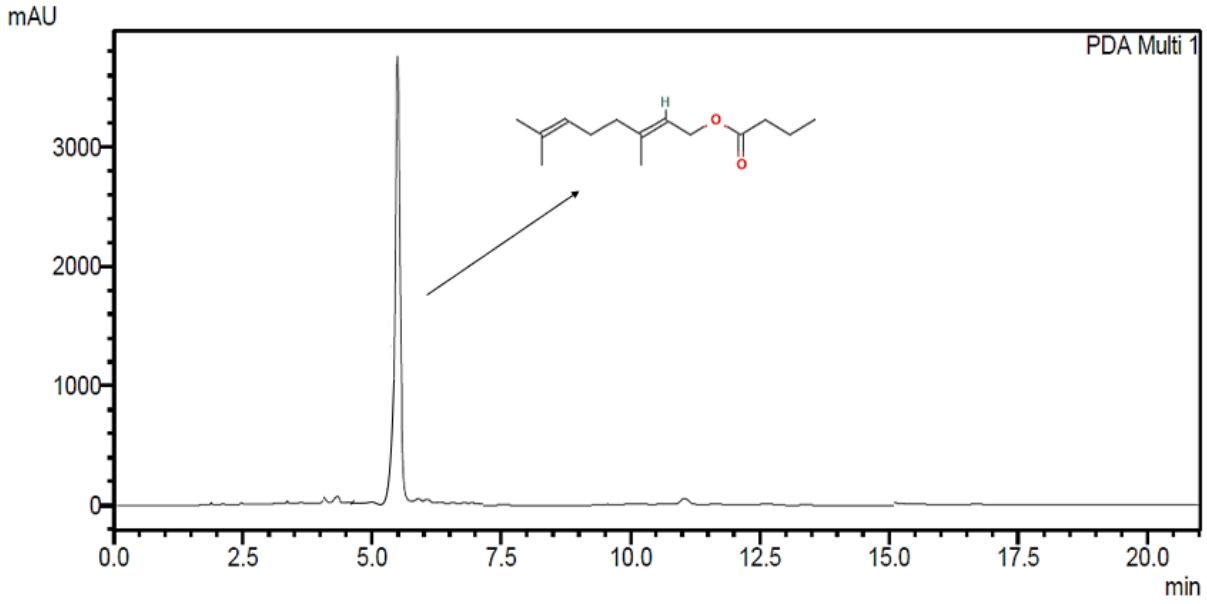
Source: Author (2023).

4.4.3 Analysis and characterization of geranyl butyrate

4.4.3.1 HPLC

The peak observed in the analysis is related to the reaction product, geranyl butyrate. This occurs because the reversed phase column has a greater interaction with products of lesser polarity, while the polar solvent drags products of greater polarity. Therefore, the starting substrate, geranyl butyrate, has higher polarity, and its corresponding peak will be observed for a longer period. The maximum absorption of this product was observed at 220 nm, as evidenced by the scan performed (Figure 4.6). The area under the curve of this peak indicates that geranyl butyrate was produced in significant amounts.

Figure 4.6 — Chromatogram of the peak obtained at a wavelength of 220 nm.

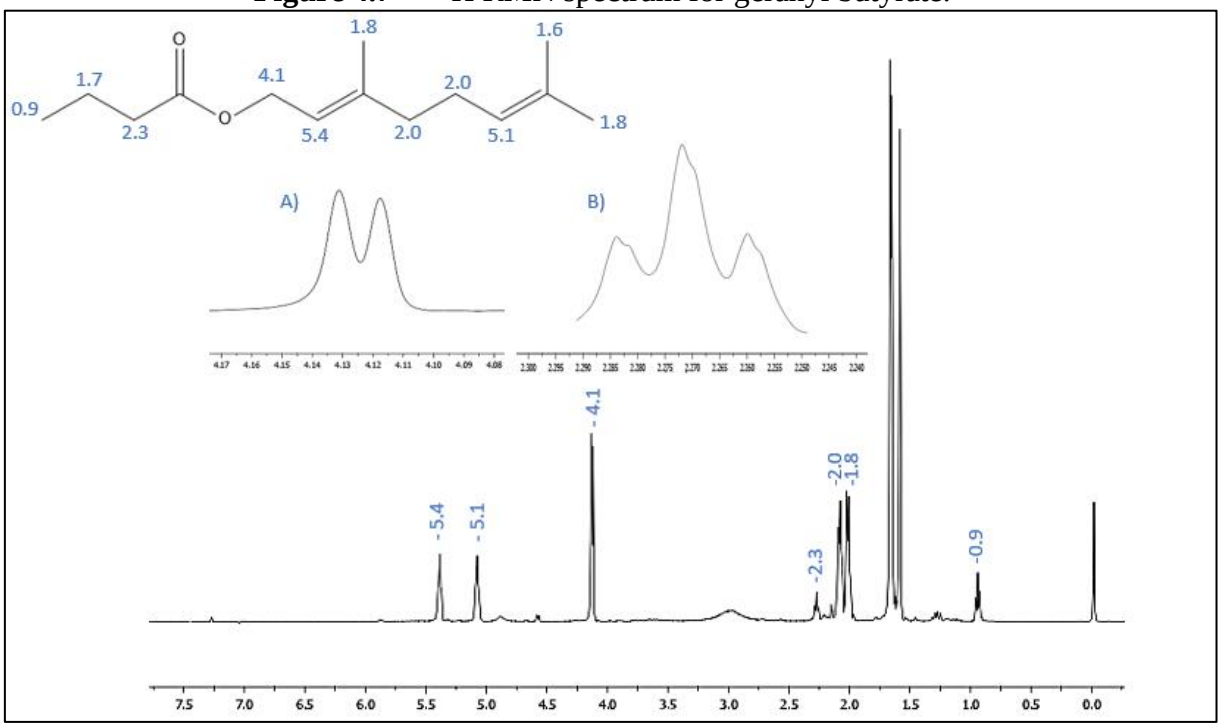


Source: Author (2023).

4.4.3.2 ^{1}H e ^{13}C RMN spectrum

^{1}H NMR spectrum of geranyl butyrate showed that there was substrate conversion catalyzed by the enzyme. In figure 4.7 characteristic peaks are observed for the hydrogens of the ester structure, the signs close to δ 4.1, represented by the expansion A, are related to the methylene hydrogens directly linked to the oxygen of the ester, both the multiplicity, a doublet originated from the coupling with the δ 5.1 hydrogen bonded to the sp^2 carbon, regarding its integration. The nearby peaks at δ 2.3 show a triplet, represented by the B expansion, where the chemical shift is expected for methylene hydrogens bonded to ester carbonyl carbon. The other peaks can be compared by a spectroscopic profile of the couplings to the structure, demonstrating their chemical shifts favorable to the characterization of the structure.

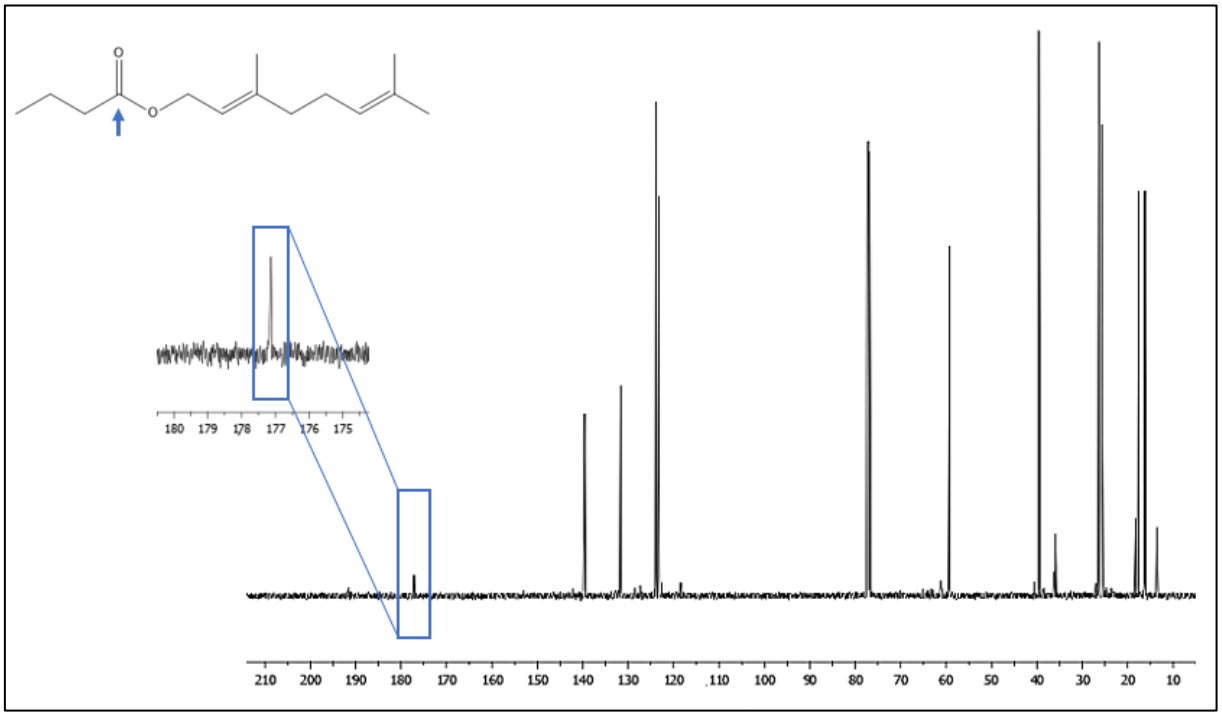
Figure 4.7 — ^{1}H RMN spectrum for geranyl butyrate.



Source: Author (2023).

^{13}C RMN spectrum was used to indicate the conversion of alcohol into ester by the appearance of the ester's carbonyl carbon, which has a signal close to 175 ppm. A low-intensity peak in this region of chemical shift indicates that there has been conversion, as in alcohols, the carbon attached to the hydroxyl has peaks in other regions of the ^{13}C RMN spectrum. In figure 4.8, it is possible to identify the expected profile for geranyl butyrate, where the characteristic peak appears at 177 ppm.

Figure 4.8 — ^{13}C RMN spectrum for geranyl butyrate, indicating the main peak referring to the conversion of the alcohol into the ester.



Source: Author (2023).

4.4.4 Theoretical study

4.4.4.1 Molecular docking

Molecular Docking generated nine poses for the ligand, and among the models, the one with the lowest energy and closest to the catalytic site of the enzyme was selected for visualization in AutoDock Tools. The enzyme pocket is composed of SER 153, HIS 268, and ASP 206, with a serine residue acting as a nucleophile, located within the substrate pocket. Only substrates with adequate conformations and chemical affinity with the catalytic residues can occupy these sites to undergo catalysis.

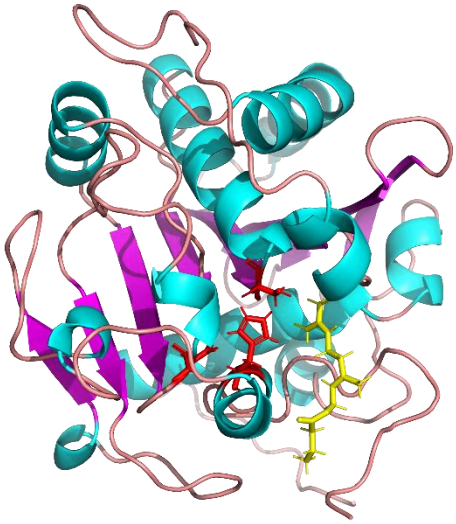
In the first pose, the ligand showed favorable energies and better approximations to the catalytic site when oriented to the alpha carbon of histidine, and for this reason, this pose was defined as the most suitable, as shown in Table 4.5. In Figure 4-9, the structure obtained is shown after molecular docking, where the catalytic triad is represented in red and the ligand in yellow.

Table 4.5 — Energies of approximations of the catalytic site when oriented to the alpha carbon of histidine

Mode	Affinity (kcal/mol)	Distance from the best mode (rmsd lb)
1	-5.9	0.000
2	-5.9	25,956
3	-5.8	25,564
4	-5.8	4,527
5	-5.8	25,570
6	-5.8	1.425
7	-5.7	15,904
8	-5.7	16,728
9	-5.6	3,550

Source: Author (2023).

Figure 4.9 — The structure obtained in molecular pos-docking.

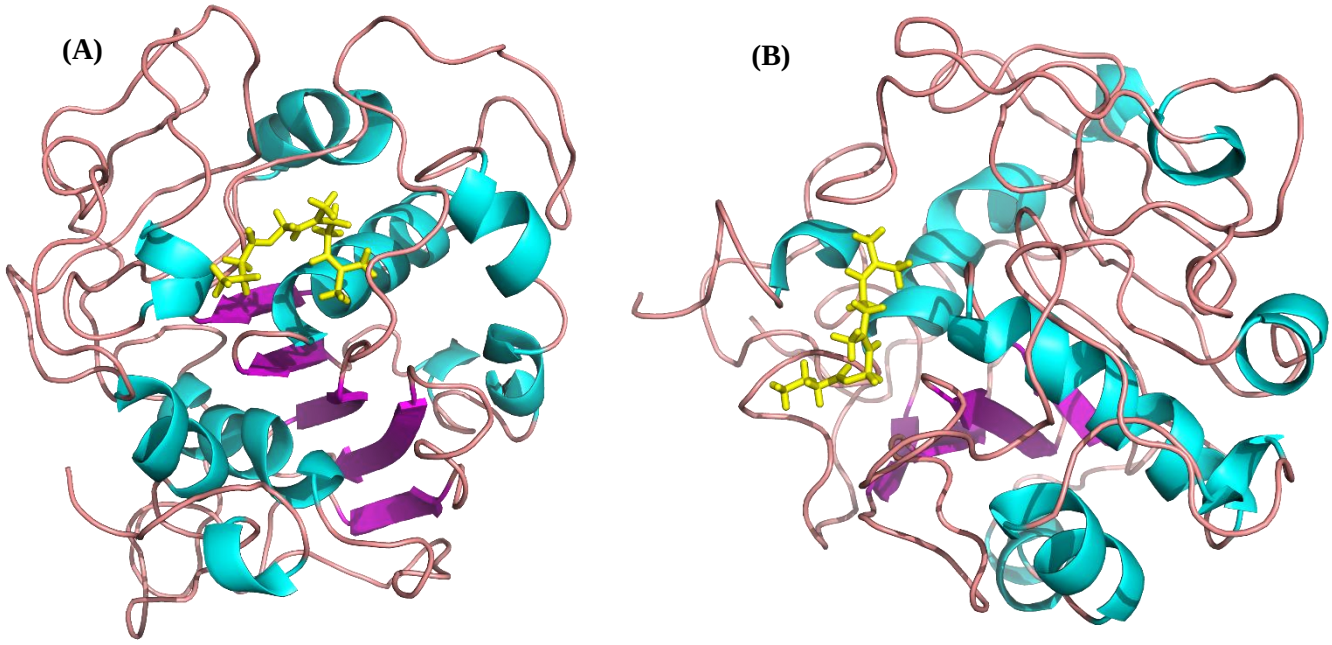


Source: Author (2023).

4.4.4.2 Molecular dynamics

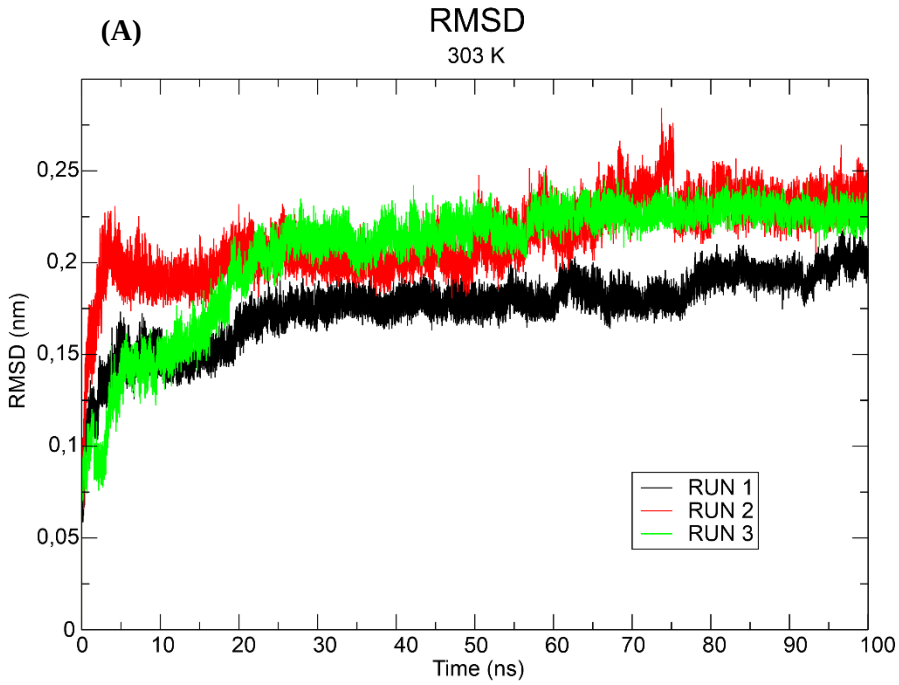
Molecular Dynamics simulations were performed to analyze the time interval in which receptor-ligand complexes reached equilibrium. Figure 4.10 represents the results for the molecular dynamics at the end temperatures 303K and 323K, while the complete results with all triplicates of the dynamics performed at the highlighted temperatures are presented in Annex 4.1 of this chapter. Figure 4.11 illustrates the Root Mean Square Deviation (RMSD) between the Eversa Transform 2.0 enzyme and the ligand performed in triplicate, also at temperatures of 303K and 323K. The analyzed complexes reached equilibrium in the triplicate range of 20-100 ns. Thus, the analyzes of the potential energies of interaction (PEI) were carried out in this time interval.

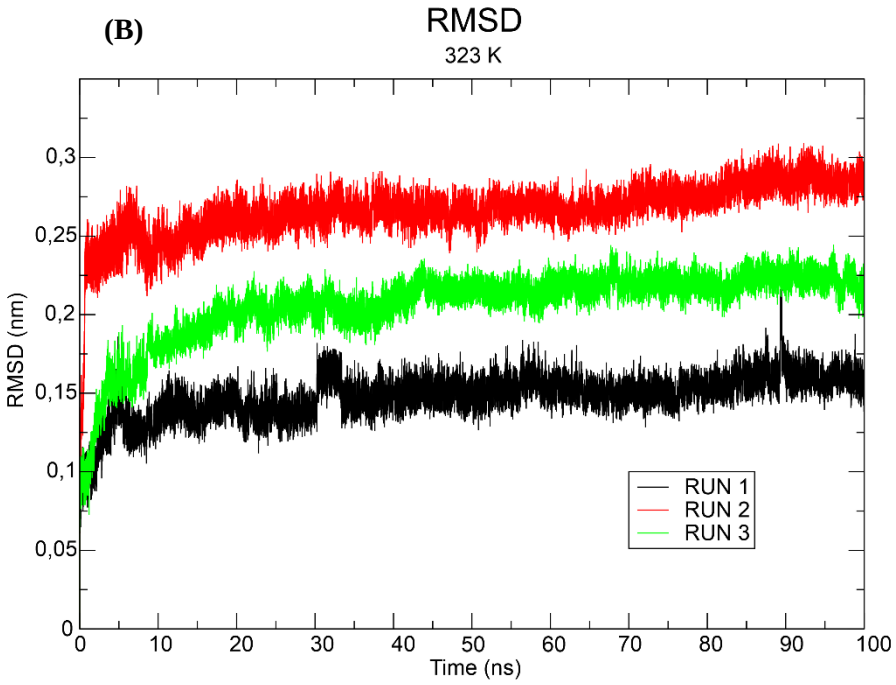
Figure 4.10 — Image generated from the molecular dynamics of the Eversa enzymatic complex with the ligand at a temperature of (A) 303K (30 °C) and (B) 323K (50 °C), obtained and treated in the Pymol program.



Source: Author (2023).

Figure 4.11 — Triplicate of RMSD of the Eversa enzymatic complex with the ligand at the two extreme temperatures (A) 303K (30 °C) and (B) 323K (50 °C), highlighting the energy extremes previously obtained in molecular docking results.





Source: Author (2023).

For each MD simulation, a triplicate was performed to ensure the stability of the interaction between the Eversa enzyme and the ligand. On average, the RMSD results of the triplicated binder showed acceptable variation between one MD simulation and another, demonstrating satisfactory results. Now, analyzing an RMSD from one temperature to another, it is noticed that the most stable variation of the C_α-C_α enzyme backbone is the 303K case (30 °C). This may indicate that the interaction between the Eversa enzyme and the ligand at this temperature is the most favorable. Which goes against the experimental results. However, this result can be justified by the PEI results presented below.

The PEI values for the MD simulations are shown in Table 4. Compared with the statistical results obtained with the aid of the Taguchi method, it is possible to notice the temperature difference that most influenced the theoretical and experimental results. However, this difference between the results can be justified because the energy difference is not high, analyzing only the temperature difference, so it really is only the second most important criterion and thus, the experimental and theoretical results begin to complement each other. An alternative would be to increase the temperature variation to ensure an analysis with points farther apart. Therefore, experimental and theoretical studies indicate that temperature is not the most favorable factor for conversion.

Table 4.6 — EPI values for the binder in the triplicate performed, in kj.mol $^{-1}$.

DM	303K	323K
1	-105,752	-103,949
2	-107,076	-109,872
3	-110,500	-100,447
Total	-323,328	-314,268
Average	-107,776	-104,756

Source: Author (2023).

4.5 Conclusions

Based on the results obtained during the experimental phase, it was possible to statistically determine the parameters that most affected the conversion of Geranyl Butyrate. The time indicated as the most important parameter shows that changes in this domain can affect the enzymatic activity of this reaction system. The values of energy and conformational modes obtained during the molecular docking step were used to justify the selection of the extremum for the molecular dynamics step, as this step requires more computation time in addition to presenting a higher operational cost.

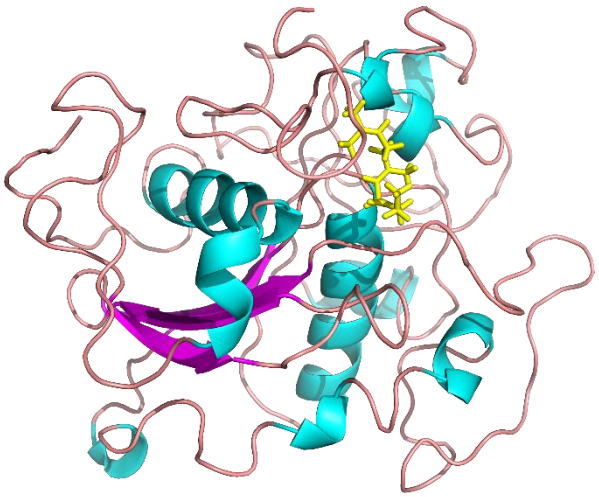
Molecular dynamics determines the optimal configuration and the interaction energy between the bonds generated in the reaction medium, with a temperature of 30 °C being the optimal configuration and the best interaction energy. Therefore, this could be used as a reason for the formation of Geranyl Butyrate in the reaction medium, due to the greater interaction with the Eversa active site in terms of compatibility and energy performance. However, in the experimental analysis, the temperature of 50°C has the greatest influence on the reaction. This mismatch between theoretical and experimental results is justified by the relative proximity of the analyzed temperatures and the limitations of the models used in the analyses. But even so, this mismatch justifies the position of temperature as the second influencing factor.

Finally, the results indicate that the methods used in this work have the potential to be used for greater scientific development, as it is possible to increase the production of studies in this area based on optimizations. Statistical work has proven to be effective in reducing the time and cost of optimized process steps, in addition to theoretical studies that guarantee confirmation of experimental analyzes and enable adjustments aimed at greater efficiency in obtaining the target products. Both studies ensure that the use of enzymes as biological catalysts is an important tool for industrial use and with large-scale potential.

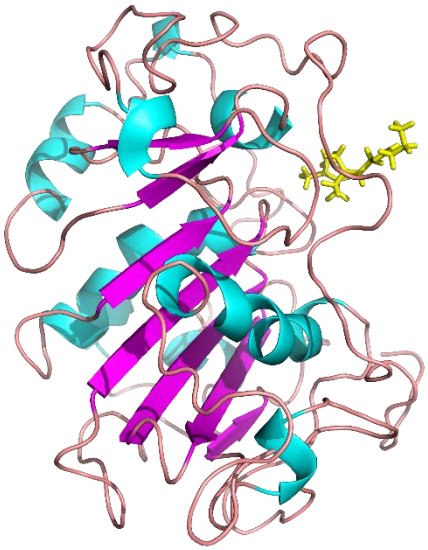
ANNEX 4.1

Molecular Dynamics triplicate results for 303K (30 °C) in the first frame (Start).

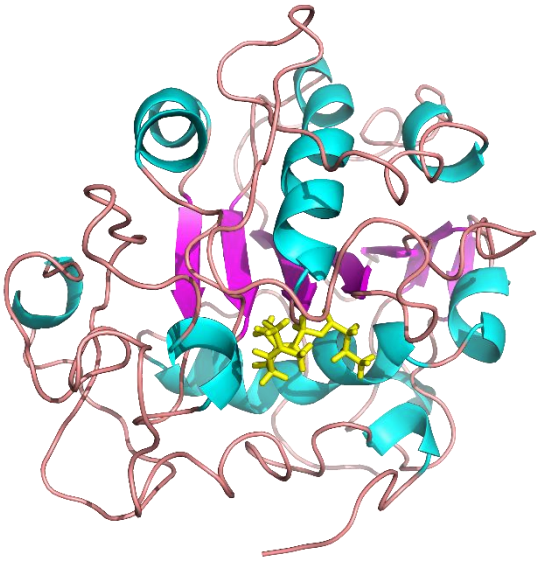
Dynamics 1.



Dynamics 2.

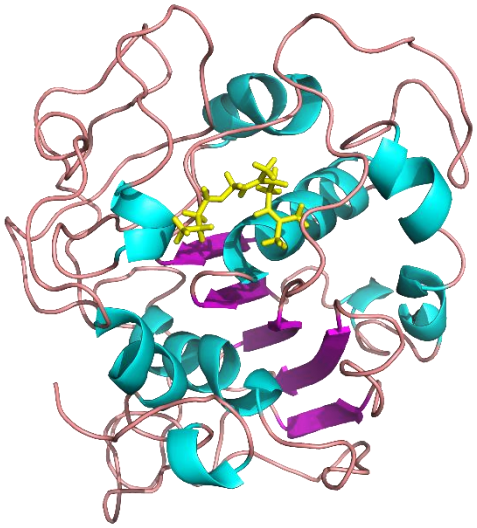


Dynamics 3.

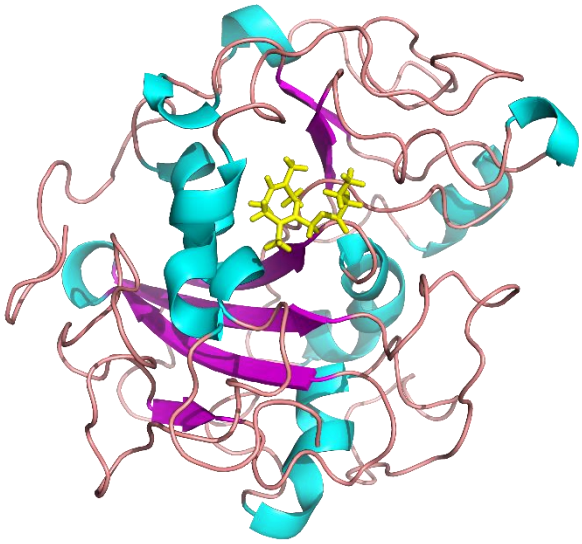


Molecular Dynamics triplicate results for 303K (30 °C) in the last frame (End).

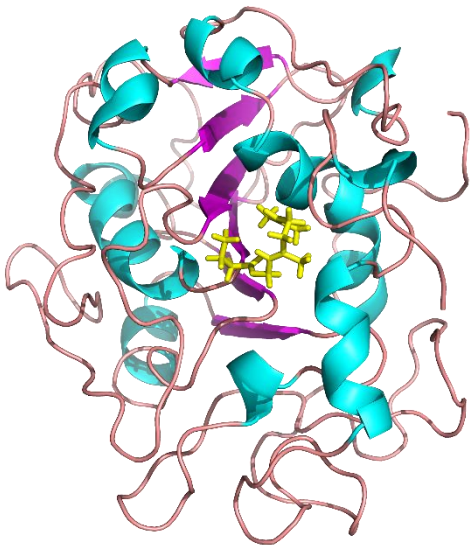
Dynamics 1.



Dynamics 2.

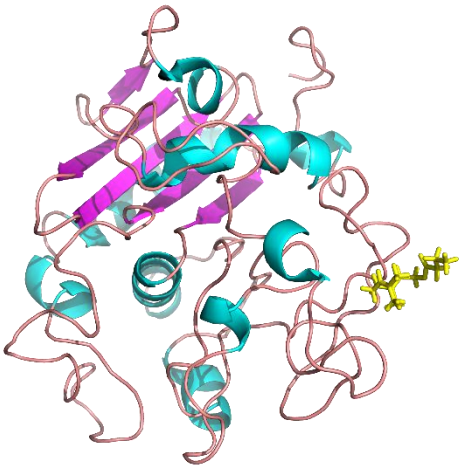


Dynamics 3.

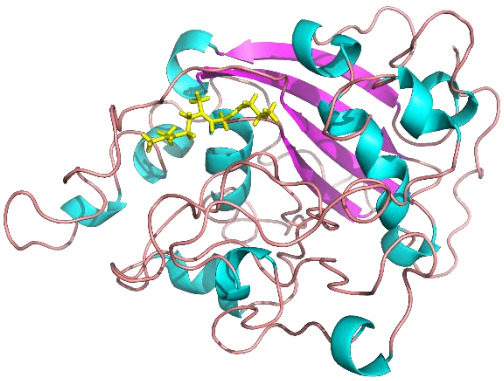


Molecular Dynamics triplicate results for 3 23K (50 °C) in the first frame (Start).

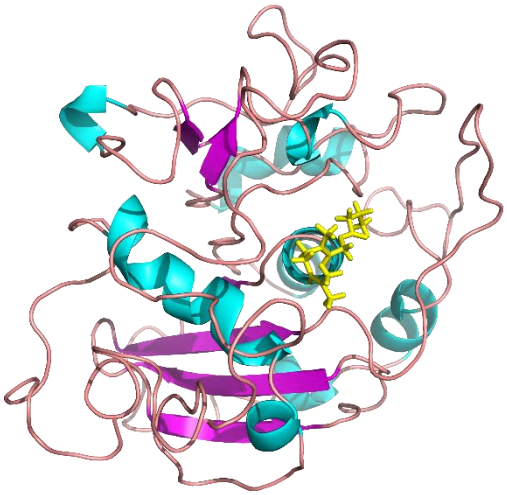
Dynamics 1.



Dynamics 2.

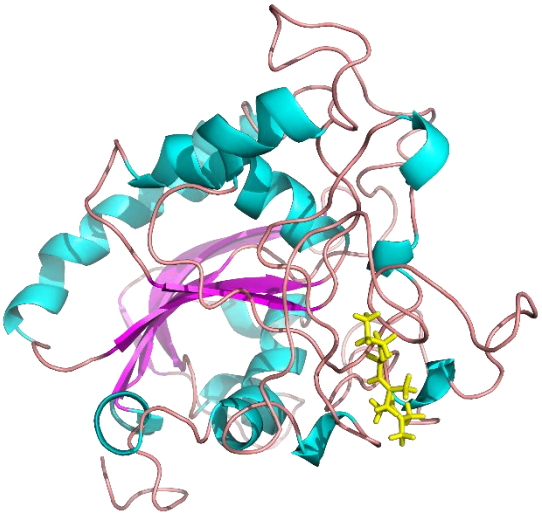


Dynamics 3.

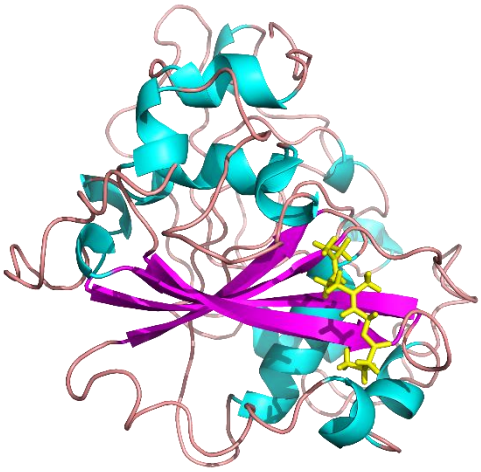


Molecular Dynamics triplicate results for 3 23K (50 °C) in the last frame (End).

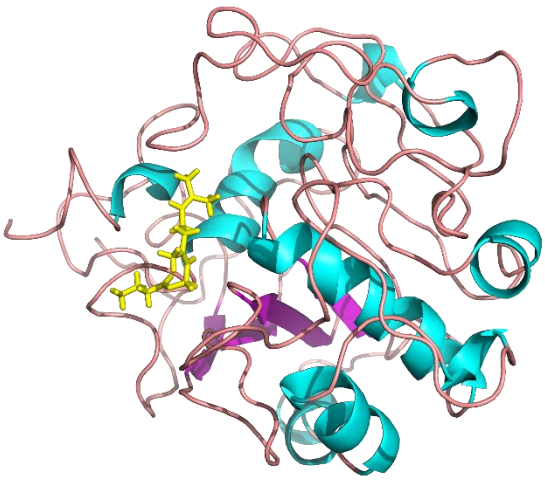
Dynamics 1.



Dynamics 2.



Dynamics 3.



CHAPTER 5

CHAPTER 5 – USE OF AGROINDUSTRIAL WASTE FROM CARNAUBA (*Copernicia prunifera*) IN THE STABILIZATION OF ENZYMES TO CATALYSE THE SYNTHESIS OF INDUSTRIAL SCENT

5.1 Abstract

The use of biocatalysts in catalysis reactions is an alternative to conventional chemical catalysis. Enzymes are biodegradable biocatalysts, which help to reduce unwanted by-products, as their selectivity allows the reaction to form a specific product. Among the enzymes, lipases are the most used in catalysis and the genetically modified Aspergillus oryzae lipase, commercially called Eversa®, was applied in this work. However, the use of soluble enzymes in the industry is limited due to problems related to their stability and difficulty in reuse. Thus, enzymatic immobilization is presented as a promising alternative that will guarantee a better performance of the enzyme. Thus, the present work aims at the use of carnauba straw (*Copernicia prunifera*) in the stabilization of enzymes to catalyze the synthesis of scent ester geranyl butyrate. From the use of experimental planning, it was possible to verify that in the immobilization of lipase, the determining factors were the percentage of biocatalyst and time. The highest immobilization yield obtained was 82.21%. The process optimization highlighted a point with 5% of biocatalyst, a time of 24 h, ionic strength of 5 mM, and 13% of glutaraldehyde in the activation of the support, which resulted in a yield of 82.54%. The application of the biocatalyst was carried out to confirm the effectiveness of the immobilization process used and resulted in 91.68±0.07% conversion of Geranyl Butyrate.

Keywords: Enzymatic immobilization; Eversa® Transform 2.0; Carnauba (*Copernicia prunifera*); Scents.

5.2 Introduction

Biomass is defined as all raw materials used in the generation of energy, whether of plant or animal origin (RAZA et al., 2020; YADAV et al., 2021). This material is considered a renewable source of energy due to its low emission of gases that aggravate the greenhouse effect (AHMAD FARID; ANDOU, 2022; NEIVA; FURTADO; FINZER, 2018). This is due to the carbon cycle since the amount of carbon dioxide that is absorbed in the development of beings that resulted in the formation of biomass is close to the release value of this gas in the process of using this material (ANTAR et al., 2021; CARVALHO et al., 2020a).

Agroindustry residues are formed during the production of food products, such as sugar, fiber, and leather products, such as clothing and fabrics, among other industrial specialties (FUENTES et al., 2021). Its production is usually seasonal, depending on the maturity of the crop or the availability of raw materials (SILVA et al., 2020). These industries also use a considerable volume of water, which can come from steps such as washing, burning, cooking, cooling, and cleaning process facilities (GOMES et al., 2009). Solid waste, which is used as biomass, consists mostly of waste from processes (DE CASTRO COÊLHO et al., 2021).

Among the many available biomasses, it is possible to highlight sugarcane, which is widely used in the synthesis of bioethanol, which results in sugarcane bagasse (CHAGAS et al., 2022). With the growing development of research with this by-product, numerous reapplications have been analyzed for it, such as its application in the production of briquettes, a solid biofuel used in the generation of thermal energy in ovens and boilers (BHATTACHARJEE; MORYA; GHOROI, 2020). Another application of this bagasse is in the diet of livestock and even in the cosmetic industry. All the possibilities of reuse of these by-products are responsible for reducing incorrect disposal and consequent environmental pollution, reducing production costs, since a new application means greater financial use of the material, thus guaranteeing an increase in profit and appreciation. commercial biomass (CHAGAS et al., 2022).

With the advancement of research focused on reuse, it was concluded that agro-industrial waste had the potential for reuse (PALMQVIST; HAHN-HÄGERDAL, 2000; RODRÍGUEZ-RESTREPO; ORREGO, 2020). Within this class of residues, there are residues of lignocellulosic materials, which have great potential for use in the most diverse lines of research (BASSAN et al., 2016; JIA et al., 2017; THAPA et al., 2020). These materials are represented by different residues, such as sugarcane bagasse (BHATTACHARJEE; MORYA; GHOROI, 2020), used in the production of ethanol, cashew bagasse, carnauba straw, among

others (CHAGAS et al., 2022; DE SOUZA et al., 2016; SUSMITA DEVI et al., 2022). In studies of potential applications for these materials, the possibility of using them as a support for enzymes was seen.

Enzymes are macromolecules that have the potential to catalyze the most diverse reactions (BRENA; GONZÁLEZ-POMBO; BATISTA-VIERA, 2013; LI et al., 2012; LIVAGE; CORADIN, 2018). These macromolecules are obtained from different microorganisms in different ways, thus resulting in the classification as a biocatalyst (CHAPMAN; ISMAIL; DINU, 2018; ZHU; WU; HUA, 2019). These biocatalysts have a high degree of selectivity and purity, thus reducing the generation of by-products in the reactions where they are applied (MOHAMAD et al., 2015; RUFER, 2021; SECUNDO, 2013). However, these biocatalysts may suffer interference from the reaction medium, as they are susceptible to denaturation by organic solvents and damage to their catalytic site (MEGHWANSHI et al., 2020; PIERRE, 2004; SCHMID; VERGER, 1998). In addition to these points, another disadvantage is the impossibility of reusing these enzymes (DROUT; ROBISON; FARHA, 2019; LIU; CHEN; SHI, 2018; MARIZ et al., 2021).

Because they are materials that require very specific technology and still little industrial demand, these bioproducts have a high market value (FARHAN et al., 2021; HOMAEI et al., 2013; PINHEIRO et al., 2019; ROCHA et al., 2021). Adding the economic factor and the technical factor of not enabling reuse, there is the main negative point in the use of these catalysts (ADLERCREUTZ, 2013; CABRERA et al., 2018; MONTEIRO et al., 2020). Soon, a new area of research emerged, where studies are carried out to enable enzymatic immobilization.

This immobilization results in the potentization of the catalytic capacities of these enzymes to improve stability and resistance to denaturation in an organic medium and enable the separation of the reaction medium, thus allowing the reuse of this biocatalyst (BEZERRA et al., 2020; CUI et al., 2018; DATTA; CHRISTENA; RAJARAM, 2013; DURAIARASAN et al., 2016; MONTEIRO et al., 2021, 2022; VERMA; BARROW; PURI, 2013). In addition, in the studies, new materials are analyzed to be used as a support and this resulted in the possibility of applying lignocellulosic materials (BINHAYEEDING et al., 2020; CARVALHO et al., 2020b; MONTEIRO et al., 2020). Thus, given the need to apply these biocatalysts formed by lignocellulosic materials, the synthesis of industrial aromas and fragrances was selected.

Thus, the present work aims at the use of carnauba straw in the stabilization of enzymes to catalyze the synthesis of industrial aromas and fragrances. This justifies the need to

reduce environmental impacts by polluting industrial waste and producing by-products that donate to the environment from the chemical synthesis of aromas.

5.3 Methodology

5.3.1. Materials

The *Aspergillus oryzae* lipase, commercially known as Eversa® transform 2.0, was obtained from Sigma-Aldrich Brazil Ltda (Cotia, São Paulo, Brazil). The chemical reagents are analytical grade and were purchased from Synth (São Paulo, Brazil) and Vetec (São Paulo, Brazil). The experimental planning based on the Taguchi method was elaborated in the Statistic®10 software (Statsoft, USA).

5.3.2. Methods

5.3.2.1. Support preparation and activation with glutaraldehyde (GLU)

Carnauba straw (*Copernicia prunifera*) (CAR) was pretreated with alkaline hydrogen peroxide (PHA) at a concentration of 4.3% v/v. The pH of the reaction was 11.5 and a 5 mol/L NaOH solution was used to adjust the pH. To carry out the pre-treatment, an orbital shaker with rotation of 200 rpm was used, being maintained for 6 hours at a temperature of 35 °C. In this step, a reaction volume of 100 mL was used for a solid's ratio of 5% m/v. The next step concerns the washing of the support, where vacuum filtration was used in addition to a battery of three washes with distilled water.

Following the treatment of the support, drying was carried out for 24 h at a temperature of 60 °C. Finally, this support was crushed followed by sieving, where 0.16 mm sieves were used to standardize the particles. The product of this pre-treatment process is support for enzymatic immobilization and from this stage will be called CAR.

For immobilization, it is necessary to activate the support. Thus, CAR was activated with Glutaraldehyde (CAR@GLU), based on the method found in the literature with some modifications (MOREIRA et al., 2020). In this step, 100 mg of CAR and 1 mL of a 25 mM sodium phosphate buffer solution at pH 7.0 are used. 100 uL of GLU will also be added and the concentration used in each sample will be defined by the experimental design and may vary between 1, 13, and 25% (v/v) for 2 h at a temperature of 25 °C.

5.3.2.2. Experimental design for lipase immobilization

For the development of this study, an advanced experimental design using the Taguchi method that has a standard L9 orthogonal matrix, in which "L" represents the Latin square and "9" the number of experiments, in its base, was used to investigate four factors (glutaraldehyde concentration, ionic strength, time and enzymatic load) in three levels to determine the best conditions for the immobilization of lipase Eversa® Transform (EVE) in CAR@GLU, abbreviated CAR@GLU-EVE, by the covalent method. For this, 0.1 g of CAR@GLU was suspended in 1 mL of buffer with varying ionic strength (5, 50, and 95 mM) and sodium phosphate pH 7.0, containing a load of EVE protein (1, 3, and 5 mg/g), in the presence of 10uL Triton X-100 0.01%. The system was maintained under constant moderate agitation at 25 °C for the time determined in the planning (6, 15, and 24 h).

After immobilization, the new biocatalysts were separated by decantation and then washed with distilled water until neutral, vacuum dried, and stored. Table 5.1 presents the levels for the variation of the four independent factors (glutaraldehyde concentration, ionic strength, time, and enzymatic load). To carry out the experimental planning and statistical analysis, the Statistic ® 10 software (StatSoft, USA) was used.

Table 5.1 — Determination of experimental procedure levels and range of independent parameters for immobilization.

Levels	Glutaraldehyde (%)	Ionic Strength (mM)	Time (h)	Biocatalyst (%)
Level 1 (L1)	1	5	6	1
Level 2 (L2)	13	50	15	3
Level 3 (L3)	25	95	24	5

Source: Author (2023).

The values of the S/N ratios (Signal-Noise) corresponding to the conversions were calculated, using the "bigger is better" function for the characteristics. This occurred because the objective of this study is to maximize this response (Immobilization Yield). In the Taguchi method, the S/N ratio is used to measure quality characteristics and deviation from the target value. By using the S/N ratio to analyze the results, the system's sensitivity to sources of variation is reduced, resulting in a good performance. For each experiment, the value of the S/N ratio was calculated according to Equation 5.1.

$$\frac{S}{N} = -10 \log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}\right) \quad (5.1)$$

Where y is the fatty acid conversion for the corresponding sample, i is the number of replicates, and n is the number of responses for the combination of factor levels in any given

parametric combination. The predicted S/N ratio under optimal conditions for the process of obtaining the maximum conversion was estimated by Equation 5.2.

$$\frac{S}{N_{predicted}} = \frac{\bar{S}}{N} + \sum_{j=1}^{n}\left(\frac{S}{N_j} - \frac{\bar{S}}{N}\right) \quad (5.2)$$

Where $\bar{S}/N$ is the arithmetic mean of all S/N ratios, S/ Nj is the S/N ratio at the sweet spot for each factor, and n is the number of factors that significantly affect the process.

5.3.2.3. Determination of enzymatic activity and protein concentration

the hydrolytic activity of the soluble and immobilized Eversa® enzyme was determined following the methodology described in the literature, with some changes (Rios et al., 2016). To measure the activity, the substrate used was *p*-nitrophenyl butyrate (50 mM *p*-NPB) in acetonitrile, which was hydrolyzed for 90 seconds under magnetic stirring. The concentration of p-nitrophenyl butyrate produced during hydrolysis was quantified through the increase in absorbance, measured spectrophotometrically at 348 nm.

Activity measurements were performed at 25°C, in a sodium phosphate buffer solution (25 mM, pH 7), where ε under these conditions is 5150 $M^{-1}.cm^{-1}$. To start the reaction, 50 µL of a suspended lipase solution was added to 50 µL of *p*-NPB and 2.5 mL of the buffer solution. In this context, an international unit of activity (U) was considered equivalent to the amount of enzyme that is used to hydrolyze 1 mol of *p*-NPB every minute. To determine the protein concentration, the method described by Bradford was used, with bovine serum albumin being used as a reference (BRADFORD, 1976).

5.3.2.4. immobilization parameters

To evaluate the yields of the proposed methodology for immobilization, the parameters were delimited following the methodology proposed in the literature (Pinheiro et al., 2019a). The immobilization yield (IY) was calculated as the percentage of enzymatic activity presented by the supernatant before and after a certain period. It is worth mentioning that this method is only applicable if the reference activity is maintained during the immobilization time, as in the present study. The immobilized enzyme activity (AtT) was obtained from the immobilization yield (IY) and the amount of protein present in each gram of support. Finally, the recovered activity (AtR) was obtained with the ratio between the biocatalyst activity (AtD) and the theoretical activity (AtT).

5.3.2.5 Application of the biocatalyst

From the analyzes carried out in Chapter 04 of this work, it was possible to define the optimized reaction conditions, which could guarantee higher conversion values in the esterification reaction for the production of Geranyl Butyrate, an aroma ester. Thus, the following conditions were defined: time of 6 h, temperature 50 °C, percentage of biocatalyst at 15%, and finally, the molar ratio of 1:5. In theory, the conversion shown at this optimized point would be 95.98%, but after the experimental application of this point using free lipase, the conversion was 85.17 ± 0.06%. For the esterification, the containers with the reagents were orbitally shaken at 200rpm, with a variation of the reaction parameters, for different samples. After carrying out the esterification process (ROCHA et al., 2021), the samples were analyzed in triplicate in Erlenmeyer flasks containing 0.1 g of the sample, 2.5 mL of standardized ethyl alcohol, and 3 drops of phenolphthalein. The 0.1 molar NaOH solution was added gradually until the color change to a subtle pink occurred. After titration, the volume values used were inserted into Equation 5.3 to obtain the acid number (AI).

$$\boldsymbol{IA}\left(\frac{\boldsymbol{mgNaOH}}{\boldsymbol{g}}\right) = \frac{\boldsymbol{MM_{NaOH}.M_{NaOH}.f.V_{NaOH}}}{\boldsymbol{m}} \quad (5.3)$$

Where, MM_{NaOH} (g/mol) is the molar mass of NaOH; M_{NaOH} (mol/L) is the molarity of the NaOH solution; f is the correction factor determined by NaOH standardization; V_{NaOH} is the volume of NaOH used during the titration, and m (g) is the mass of the sample to be studied.

The conversion of butyric acid into aroma esters from the application of the new biocatalyst is given by Equation 5.4, where IA_i represents the initial acidity value given by the amount of acid added in the reaction, and IA_f to the final acidity value, equivalent to the remaining acids in solution, not used by the enzyme.

$$\boldsymbol{Conversion}\ (\%) = \frac{\boldsymbol{IAi-IAf}}{\boldsymbol{IAi}}\boldsymbol{x100} \quad (5.4)$$

5.4 Results

5.4.1 Parameter optimization using the Taguchi method

The Taguchi method was used to optimize the process, allowing the reduction of experimental steps. In this way, it was possible to vary the interaction between the reaction parameters and determine the best level of each one to increase the immobilization yield. Table 5.2 presents the results of the experimental design, including the conversion and S/N values for each analyzed point. Each point was tested in triplicate to ensure that the margin of error was within the desired standards.

Table 5.2 — Experimental design of Taguchi L9 Planning for immobilization.

Reaction	Glutaraldehyde (%)	Ionic Strength (mM)	Time (h)	Biocatalyst (%)	IY[9] (%)	AtT[10] (%)	AtR[11] (%)	AtD[12] (%)	S/R
1	1	5	6	1	59.23	14,39	1,11	7,73	35.4508
2	1	50	15	3	68.51	24,65	2,98	8,59	36.7151
3	1	95	24	5	82.21	36,45	3,48	5,23	38.2985
4	13	5	15	5	80.01	25,13	2,20	6,26	38.0629
5	13	50	24	1	65.91	24,70	1,68	6,79	36.3790
6	13	95	6	3	66.12	17,31	1,45	8,42	36.4067
7	25	5	24	3	76.25	21,52	7,36	14,12	37.6448
8	25	50	6	5	72.98	33,18	2,21	6,67	37.2641
9	25	95	15	1	55,26	26,76	1,60	4,33	34,5861

Source: Author (2023).

After analyzing the experiments carried out, the parameters that had the greatest influence on the reaction were identified, especially the percentage of biocatalysts and time. The percentage of biocatalysts was the most significant determining factor among all the analyzed parameters, as its variation had a direct impact on the obtained immobilization yields. Furthermore, it is important to point out that points 3, 4, and 7 of Table 5.2 were responsible for the three highest yields obtained, and at these points, the biocatalyst was applied at the

[9] Immobilization Yields
[10] Theoretical Activity
[11] Retrieved Activity
[12] Derivative Activity

highest levels, with points 3 and 4 at level 3 of biocatalyst and point 7 at level 2. The lowest yield was identified at level 1 of all parameters, showing that varying levels were efficient in optimizing the process.

In addition, as already mentioned, time was also fundamental in obtaining promising results in terms of immobilization yield, as can be seen in points 3 and 7 of the table, which show two of the three highest yield indices and were applied over time. of 24 hours, following this same logic, point 4 with a time of 15 hours is also among the best results. The experimental data obtained emphasize the importance of increasing the percentage of biocatalysts in the process.

Still based on the results of the experimental planning, it is possible to verify that the percentage of glutaraldehyde used in the activation of the support and the variation of the ionic strength, had little influence on the immobilization process. However, its importance in the process is not discarded, there were just more determining factors. Concerning the percentage of glutaraldehyde, at all analyzed levels, results were obtained that varied between higher and lower yield values, which leads to the conclusion that the variation of this parameter does not potentiate the process and that the use of higher concentrations would be material costs only. As for the ionic strength, the low influence may represent a good interaction between the enzyme and the activated support, requiring little buffer action to increase the binding, thus lower concentrations are sufficient for the process.

5.4.1.1 Analysis of the S/N ratio

To assess the importance of the analyzed parameters, the Taguchi method was used to calculate the S/N ratios. In this study, the "bigger is better" function was employed to identify these ratios based on fixed asset yield values, which are directly linked to the S/N ratio. Table 5.3 provides information on the average S/N of all levels of each factor, as well as the delta values, which are obtained by subtracting the factor with the highest S/N value and the lowest value. These results will be applied in the classification of factors and determine their importance for the process.

Table 5.3 — Response to mean S/N ratios and ordering of variables in enzymatic immobilization.

Factors/Levels	Glutaraldehyde (%)	Ionic Strength (mM)	Time (h)	Biocatalyst (%)
1	69.98	71.83	66.11	62.17
2	70.68	69.13	69.96	70.29
3	70.20	69.90	74.79	78.40
Delta	0.70	2.70	8.68	16.23
Ranking	4	3	2	1

Source: Author (2023).

When analyzing the ranking, it is possible to highlight the deltas of 16.23 and 8.68 which will make it possible to reaffirm that the percentage of biocatalyst and time were the most significant parameters, respectively. These results are consistent with the experimental data presented in Table 5.2, which also points to these parameters as the most important for the immobilization process. In the case of the percentage of biocatalyst, the S/N values increased significantly as the amount of biocatalyst in the reaction increased, with the variation from 1% (Level 1) to 5% (Level 3) being especially relevant, although already at Level 2 (3%), an increase in these factors would indicate good results. This level can receive more attention, because if its results are close to the results in Level 3, for the sake of cost reduction, it may turn out to be the most appropriate level to use.

5.4.1.2 Analysis of variance (ANOVA)

After carrying out the experimental planning, the Analysis of Variance (ANOVA) was performed based on statistical methods. The results of this analysis are presented in Table 5.4, with the p-value being the main highlight, since, according to the literature, it is this value that indicates the significance of the parameters in the immobilization process.

Table 5.4 — Results of analysis of variance (ANOVA) for parameters that affect enzyme immobilization.

Factors	SS	DF	IN	*F value*	*p-value*	Contribution (%)
Glutaraldehyde (%)	0.068	two	0.034	0.04	0.8547	0.01
Ionic Strength (mM)	5.607	two	2,304	3.13	0.1515	1.08
Time (h)	113.014	two	56,507	63.11	0.0014	22.02

Biocatalyst (%)	395,282	(two)	197,641	220.75	0.0001	76.89
Total	**513,971**	6	-	-	-	100

Source: Author (2023).

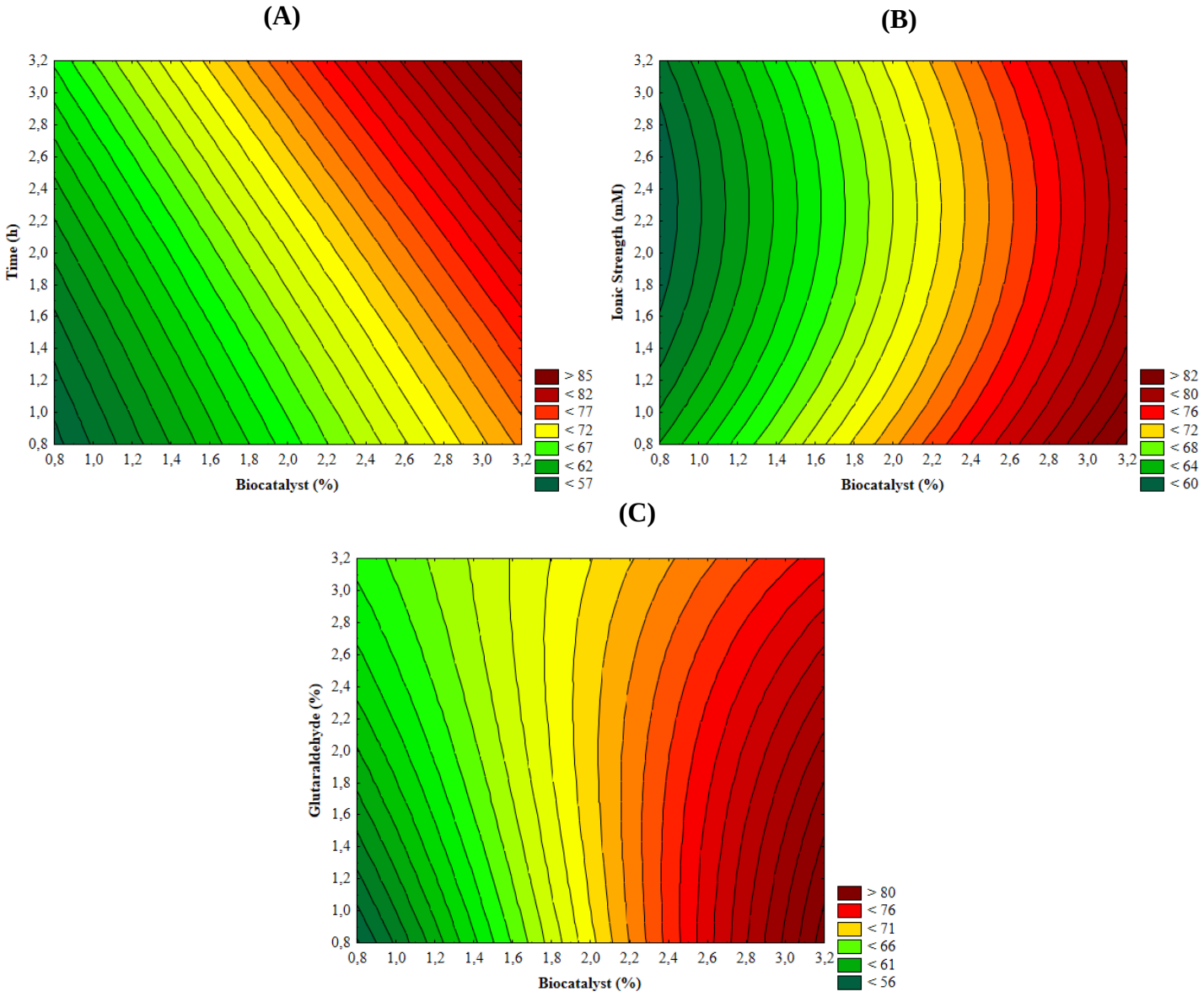
Based on the data obtained using the Taguchi method, it was possible to determine the significance of the parameters using the p-value, which can guarantee up to 95% confidence, provided it is less than 0.05. In this sense, the percentage of biocatalyst and time were responsible for significance values within the best reliability range, with a p-value equal to 0.0001 and 0.0014, respectively. However, the contribution of the percentage of biocatalysts was 76.89% overlapping the 22.02% contribution of time.

Thus, with the analyzes carried out, the optimized reaction conditions were defined to guarantee greater immobilization yields. Thus, it was defined that the best time was presented at Level 3, with 24 h (L3), the best percentage of glutaraldehyde in the activation of the support was 12% (L2), the percentage of biocatalyst was defined as 5% (L3) and, finally, the ionic strength stood out in Level 1, at a concentration of 5 mM (L1). In theory, the yield presented at this optimized point would be 84.34%. However, after the experimental application of this point, the yield was 82.54%.

5.4.2 Statistical immobilization yields analysis

For the statistical analysis of conversion, contour surface graphs were prepared that show the responses of immobilization yields from the relationship between the percentage of biocatalyst, the main parameter of influence, with the other planning parameters. These graphs are shown in Figure 5.1.

Figure 5.1 — Contour surfaces for immobilization yields. (A) Time (h) versus Biocatalyst (%). (B) Ionic strength (mM) versus biocatalyst (%). (C) Glutaraldehyde (%) versus biocatalyst (%).



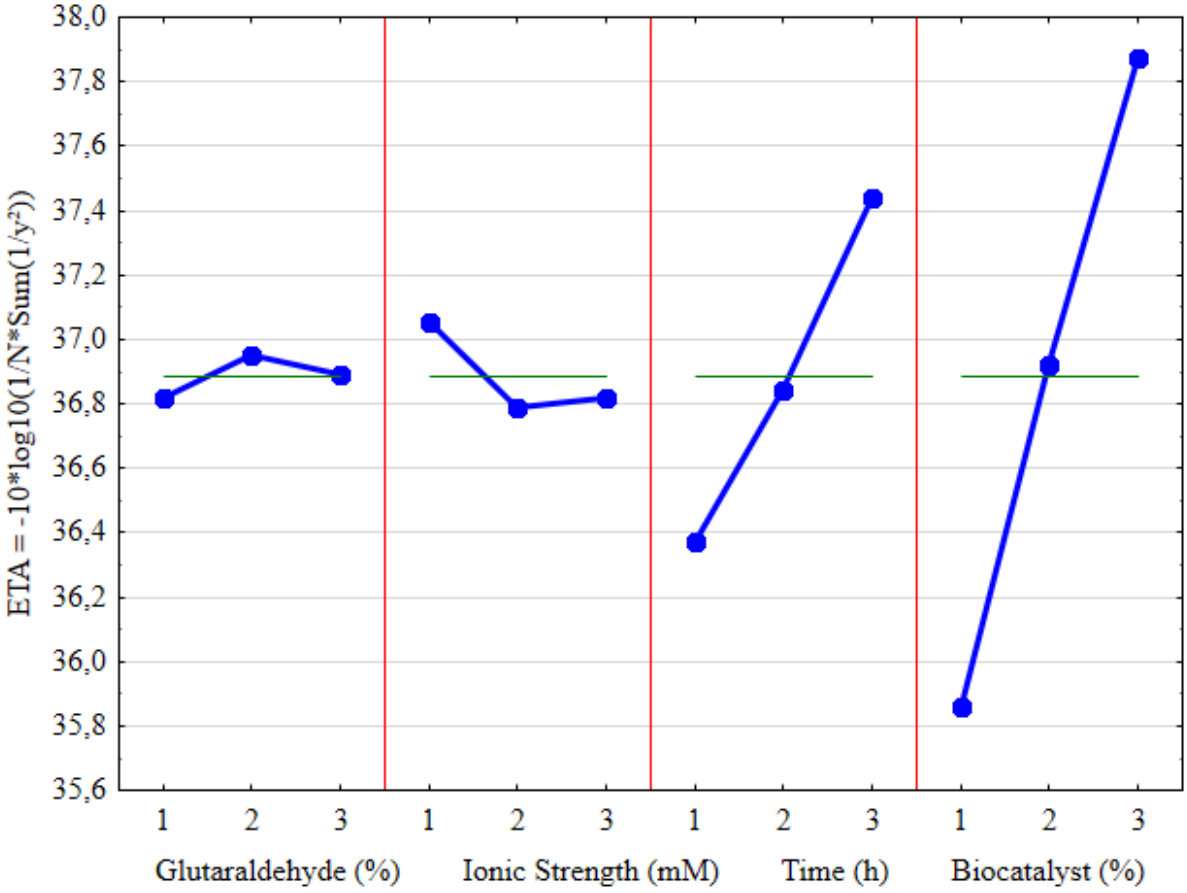
Source: Author (2023).

The relationship between the percentage of biocatalysts and time, shown in Figure 5.1A, shows that at higher levels of both parameters, immobilization occurs satisfactorily. Figure 5.1B, on the other hand, shows that the ionic strength variation does not influence the immobilization, which starts to occur more efficiently with the increase in temperature. Finally, Figure 5.1C highlights that at high concentrations of glutaraldehyde, immobilization tends to reduce its yield, even though this parameter is not a highlight in influencing the process.

From the analysis of the graph of averages shown in Figure 5.2, it is possible to observe which parameters have the greatest impact on lipase immobilization. Again, the

percentage of biocatalysts proved to be the most important factor, showing greater mean variations, more pronounced slopes, and more significant shifts between the graph points. On the other hand, the percentage of glutaraldehyde remains a low-impact parameter, as evidenced by the low slope and small average change in the graph, which does not differ much from the variation in ionic strength.

Figure 5.2 — Graph of the averages of the variations of the immobilization parameters.



Source: Author (2023).

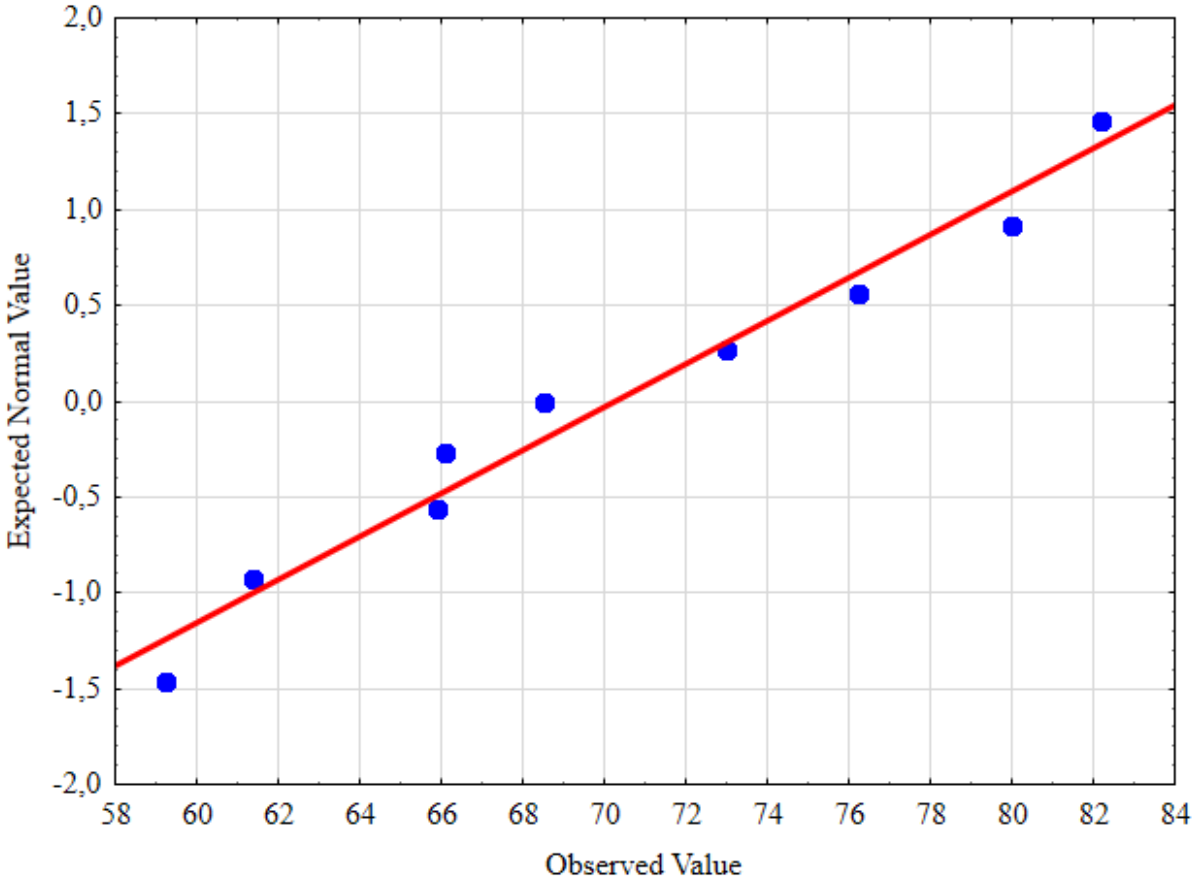
With the Taguchi method, it is possible to obtain data that allow the determination of a regression equation that considers four dependent variables and estimates that the conversion follows the presented parameters. Thus, Equation 5.5 represents this regression equation.

$$\boldsymbol{IY}\ (\%) = \mathbf{51,84} + \mathbf{0,0089}\,\boldsymbol{A} - \mathbf{0,0215}\,\boldsymbol{B} + \mathbf{0,4822}\,\boldsymbol{C} + \mathbf{4,058}\,\boldsymbol{C} \qquad \textbf{(5.5)}$$

Where *IY* is the immobilization yield and *A*, *B*, *C*, and *D* are the encoded values of percent glutaraldehyde, ionic strength, time, and percent biocatalyst, respectively.

Figure 5. 3 presents a normal probability graph that allows the identification of the experimental behavior and defines how close the model is to reality. This graph makes a comparison between the theoretical and experimental methods.

Figure 5.3 — Normal probability plot of the enzymatic immobilization process.

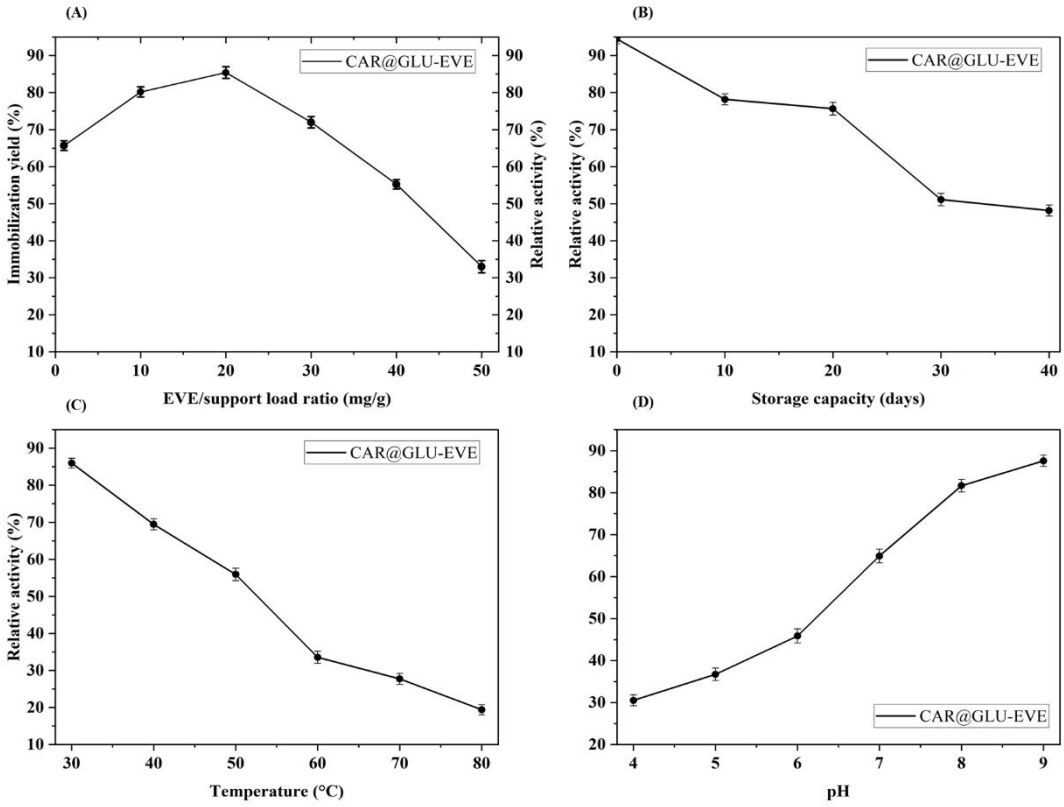


Source: Author (2023).

5.4.3 Analysis of the immobilization parameters of the biocatalyst CAR@GLU-EVE

Figure 4 presents the crucial parameters to understand the efficiency of the enzymatic immobilization protocol, the effects on the catalytic activity of the enzyme, as well as the method's effectiveness and the viability of the immobilization. All graphs related to the CAR@GLU-EVE biocatalyst are displayed.

Figure 5.4 — Enzyme immobilization parameters. A) Maximum enzyme load capacity (Maximum load test of CAR@GLU-EVE in time 24h, ionic load 5 mM, Glutaraldehyde 13% and protein load 1:1 - 1:50 mg/g); B) Storage capacity (Effect of storage time on the activity of the CAR@GLU-EVE biocatalyst over 24h, ionic load 5 mM, Glutaraldehyde 13% and protein load 1:5 mg/g); C) Thermal stability (at different temperatures 30 ◦C - 80 ◦C) of the soluble and immobilized EVE enzyme under optimal conditions; D) pH stability (at different pH 4 - 9) of soluble and immobilized EVE enzyme under optimal conditions.



Source: Author (2023).

5.4.3.1. Maximum enzyme load capacity

In Figure 4-A, illustrates the maximum load that the EVE can support for an efficient interaction with the CAR@GLU-EVE support. The graph represents the efficiency of immobilization and the relative activity of the biocatalyst. The analysis of these parameters allows a proper interpretation and a precise understanding of the maximum support capacity of the biocatalyst. This allows us to verify the adhesion of the enzyme to the support and determine if all enzymes stabilized on the support remain active or suffer inactivation due to structural, conformational modifications or the formation of enzymatic multilayers, resulting in a partial inactivation of the biocatalyst due to a spherical blockade.

The immobilization yields gradually increased until reaching the optimal point of 20 mg/g of enzyme on the support, reaching a growth of up to 85%. From that point on, the yield began to decline in percentage terms. The activity of the derivative reached its maximum

at 20 mg/g, which indicates that the support is capable of immobilizing 20 mg/g of enzyme more than the optimal point (5 mg/g) before reaching surface saturation and starting to have their activity reduced. Excess enzyme in solution can lead to interactions between enzyme molecules, resulting in the formation of conglomerates that inhibit the elasticity of the enzyme conformation, leading to spherical inactivation and activity deficiency.

5.4.3.2. Storage stability

Figure 4B shows the storage stability of the biocatalyst produced over a period of 40 days at a temperature of 4 °C. The storage capacity of the immobilized enzyme is extremely important in the production of biocatalysts. In general, the enzyme in its soluble form is not stable during storage, with its activity gradually decreasing over time. After 40 days, the relative activity of this biocatalyst was 48%.

The heterofunctional immobilization of the scaffold provided a favorable activity rate, reducing the influence of autolysis and possibly stabilizing the active free form of lipase. This immobilization strategy allowed greater preservation of the biocatalyst activity during the storage period, demonstrating its viability and efficiency for practical applications in industry.

5.4.3.3. Thermal stability

The Figure 4C examines the thermal deactivation of soluble and immobilized EVE (enzyme) in response to temperature variations. It is generally observed that immobilized enzymes have a higher thermal resistance than the free form, due to restriction of enzyme mobility and increased rigidity. In this study, it was possible to verify that the highest activities were observed at temperatures of 30°C and 40°C. This indicates that the immobilized biocatalyst has significant thermal stability in these temperature ranges, which makes it promising for applications involving higher reaction conditions.

5.4.3.4. pH stability

The analysis of the effect of pH on enzyme performance is shown in Figure 4D. In this study, a range of pH values from 4 to 9 was investigated and it was observed that the surface charges of the transporter have an influence on the ideal pH for enzymatic activity. Supports with a positive charge changed the ideal pH to a lower value, while supports with a negative charge had the opposite effect. In the context of this work, it was verified that the maximum hydrolytic activity of the biocatalyst occurred between pH 7 and 9. The stability of immobilized enzymes at different pHs may be related to the interaction between the support and the enzyme, which acts in stabilizing the structure of the site active and reduced enzymatic mobility after immobilization.

5.4.4 Application of the biocatalyst

The application of the biocatalyst in the synthesis of aroma esters, carried out under the optimized conditions in Chapter 4, resulted in 91.68±0.07% conversion of butyric acid plus geraniol into Geranyl Butyrate. The expected response for this reaction would be 95.98 %. Conversion using free lipase was 85.17 ± 0.06%. Thus, the use of CAR@GLU-EVE increased the conversion of this reaction, although it did not reach the value expected by the planning.

5.5 Conclusions

By comparing the most and least significant parameters, it can be concluded that the support was able to support all levels of the amount of biocatalyst based on the mass percentage of the support. The increase in immobilization time contributed to greater interaction between activated support (CAR@GLU) and the biocatalyst (EVE), thus ensuring a more efficient immobilization. Variations in glutaraldehyde and ionic strength were a few determinants of the process. The application of the new biocatalyst (CAR@GLU-EVE) presented the expected results, based on theoretical studies carried out and its effectiveness was superior to the use of lipase Eversa® in its free form since the yield increased by approximately 85% to 91%. Regarding the biomass used, as a result of the experimental planning, it proved to be suitable for use as an enzymatic support, thus opening up another possibility for the application of biomass extracted from carnauba.

CHAPTER 6

CHAPTER 6 – FINAL CONSIDERATIONS

6.1 Final considerations

In summary, the application of lignocellulosic biomass as a support for enzyme immobilization is an alternative to be considered, as it constitutes a sustainable methodology and addresses problems related to the storage and management of these residues. Thus, immobilization in lignocellulosic materials has been highlighted in scientific research in several areas, such as in the chemical, pharmaceutical, biotechnological, food, and environmental industries. Therefore, it is possible to determine that biocatalysts on lignocellulosic supports represent an ecologically correct method and aim to make bioprocesses in this area more sustainable.

The results of the production of aromatic esters indicate that the methods used in this work have the potential for further scientific development, as optimization can increase the production of studies in this area. Statistical work has proven to be effective in reducing the time and cost of optimized stages of the process, combined with theoretical studies that guarantee the confirmation of experimental analyzes and allow adjustments aimed at greater efficiency in obtaining the target products. Both studies ensure that the use of enzymes as biological catalysts is an important tool for industrial use and has the potential for large-scale application.

Finally, the application of the new biocatalyst (CAR@GLU-EVE) presented the expected results, based on the theoretical studies carried out, and its efficacy proved to be superior to the use of lipase Eversa® in free form. It was also verified that the use of carnauba biomass as an enzymatic support is possible and thus guarantees one more possibility of application of its by-products.

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APPENDIX A – PRODUCTION DURING THE MASTER'S DEGREE

PRODUCTION 1/4: http://dx.doi.org/10.1016/j.procbio.2022.05.020

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Process Biochemistry

Volume 120, September 2022, Pages 1-14

Chemical modification of clay nanocomposites for the improvement of the catalytic properties of Lipase A from *Candida antarctica*

Antônio Luthierre Gama Cavalcante [a], Anderson Valério Chaves [a], Pierre Basílio Almeida Fechine [a], Jeferson Yves Nunes Holanda Alexandre [b], Tiago Melo Freire [a], Dalila Maria Barbosa Davi [c], Francisco Simão Neto [d], Isamayra Germano de Sousa [d], Katerine da Silva Moreira [b], André Luiz Barros de Oliveira [b], Marcos Carlos de Mattos [c], Maria Conceição Ferreira Oliveira [c], Maria Vieira de Brito [c, e], Stéphanie Ballereau [e], Vania Bernardes-Génisson [f, h], Aluísio Marques da Fonseca [g], José C.S. dos Santos [b, d]

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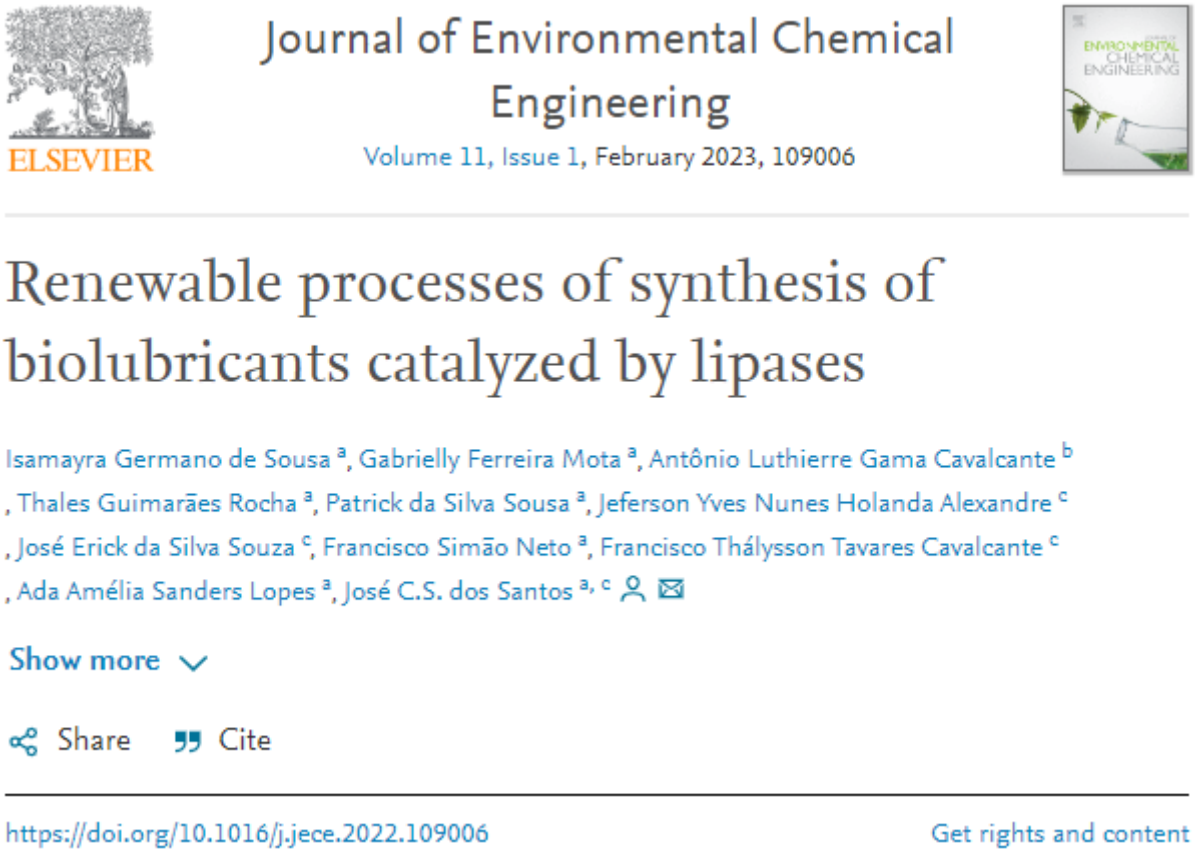

Journal of Environmental Chemical Engineering

Volume 11, Issue 1, February 2023, 109006

Renewable processes of synthesis of biolubricants catalyzed by lipases

Isamayra Germano de Sousa [a], Gabrielly Ferreira Mota [a], Antônio Luthierre Gama Cavalcante [b], Thales Guimarães Rocha [a], Patrick da Silva Sousa [a], Jeferson Yves Nunes Holanda Alexandre [c], José Erick da Silva Souza [c], Francisco Simão Neto [a], Francisco Thálysson Tavares Cavalcante [c], Ada Amélia Sanders Lopes [a], José C.S. dos Santos [a, c]

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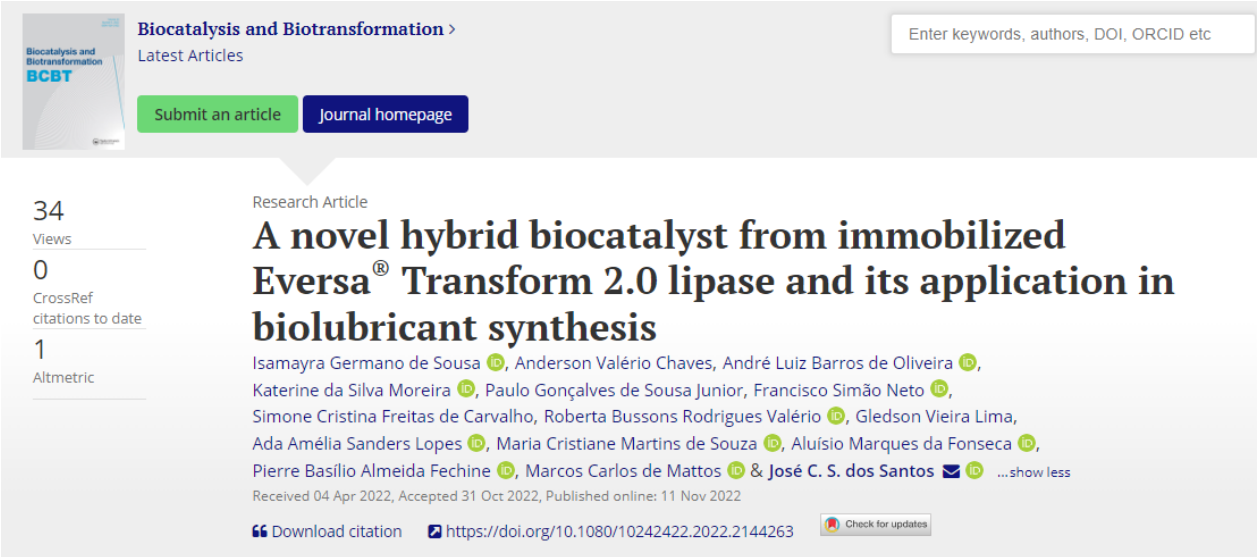

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Isamayra Germano de Sousa, Anderson Valério Chaves, André Luiz Barros de Oliveira, Katerine da Silva Moreira, Paulo Gonçalves de Sousa Junior, Francisco Simão Neto, Simone Cristina Freitas de Carvalho, Roberta Bussons Rodrigues Valério, Gledson Vieira Lima, Ada Amélia Sanders Lopes, Maria Cristiane Martins de Souza, Aluísio Marques da Fonseca, Pierre Basílio Almeida Fechine, Marcos Carlos de Mattos & José C. S. dos Santos ...show less

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Biocatalyst Immobilization

Foundations and Applications

Foundations and Frontiers in Enzymology

2023, Pages 87-114

Chapter 5 - Support-free immobilization

Roberta Bussons Rodrigues Valério [a], Isamayra Germano de Sousa [b], Antônio Luthierre Gama Cavalcante [a], José Erick da Silva Souza [b], Thales Guimarães Rocha [b], Francisco Simão Neto [b], Maria Cristiane Martins de Souza [b], José Cleiton Sousa dos Santos [b, c]

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