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# OPTIMIZATION AND THEORETICAL ANALYSIS OF LIPASE-CATALYZED ENZYMATIC ESTERIFICATION OF GLYCEROL FOR EFFICIENT GLYCERIDES SYNTHESIS

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## ABSTRACT

*Eversa® Transform 2.0* is a lipase derived from *Thermomyces lanuginosus* produced by a genetically modified microorganism. It has a low production cost, high substrate specificity, and high catalytic activity in organic synthesis. The Taguchi method was used to evaluate the best conditions for the enzymatic esterification of glycerol with acetic acid. A yield of 84.8% was obtained, under the optimal conditions (temperature = 40 °C; molar ratio glycerol/acid = 1:1; biocatalyst = 15% w/w; time = 12.5 h). After the statistical analysis, the temperature was found to be the most significant parameter influencing the reaction conversion. Molecular docking, molecular dynamics, and QM/MM simulations were applied to understand the mechanism of esterification and to derive thermodynamic and kinetic data. The nucleophilic attack step was identified as the rate-limiting step for both acylation (13.1 kcal/mol) and deacylation (13.8 and 12.9 kcal/mol) reaction mechanisms. Although the enzyme can esterify all three alcohol groups of glycerol, the esterification of the primary alcohols is thermodynamically more favorable (5 kcal/mol), especially at higher temperatures, than their secondary counterpart.

Keywords: Glycerol. Eversa Enzyme. Taguchi Method. Molecular Docking. Molecular Dynamics.

## 1 INTRODUCTION

Esters derived from glycerol are essential in various industries, serving as lubricants, plasticizers, defoamers, and dispersing agents, among other applications requiring biological activity. These esters are usually sourced from natural materials like fatty acids and oils. However, current industrial methods yield low quantities, making the process economically unappealing. Traditional chemical synthesis routes using conventional catalysts require high temperatures and generate unwanted by-products[1]. Enzymes offer significant advantages over traditional inorganic catalysts. Enzymatic processes can greatly accelerate reaction rates and perform highly selective reactions, leading to higher product yields and purity under mild conditions. Additionally, enzymatic processes are environmentally sustainable as enzymes are biodegradable and can be sourced from microorganisms, animals, or plants. Biocatalysts are already used in producing high-value compounds from glycerol, such as monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG), which are raw materials in the pharmaceutical, fuel, materials, and food industries[2].

Lipases, enzymes in the hydrolases group, are crucial for their ability to hydrolyze lipids. These enzymes are used in a variety of biotechnological processes for chemical transformations. The mechanism of lipase action involves an acylation and deacylation cycle facilitated by a catalytic triad of serine (Ser), histidine (His), and aspartic acid (Asp) residues. The reaction begins with serine's hydroxyl group attacking the substrate's electrophilic carbon, initiating a proton transfer to histidine, forming an unstable gem-diol intermediate[3]. This intermediate collapses, releasing water and forming an acetylated enzyme intermediate, which then undergoes a nucleophilic attack by an alcohol, forming another gem-diol intermediate that collapses to release the ester and regenerate the enzyme. *Eversa® Transform 2.0* (ET2) is a significant lipase derived from *Thermomyces lanuginosus*, produced by a genetically modified strain of Aspergillus oryzae. ET2 is cost-effective and efficient, but its academic usage is limited despite being patented by Novozymes[4].

Optimizing enzyme-catalyzed processes requires understanding the variables controlling reaction rate, yield, and product selectivity. The Taguchi method, a statistical approach, helps optimize these processes by designing experiments to reduce errors, costs, and increase efficiency. Computational chemistry techniques like molecular docking, molecular dynamics, and quantum mechanics provide insights into enzyme-catalyzed reactions, aiding in the optimization of glyceride ester synthesis using ET2. Combining experimental and computational approaches, this study characterizes the enzymatic esterification of glycerol and acetic acid by ET2, confirming the formation of MAG, DAG, and TAG through NMR analysis. Theoretical and experimental data correlate well, highlighting the potential of enzymatic processes in industrial applications for their efficiency, sustainability, and economic viability[5].

## 2 MATERIAL & METHODS

For the experiment, conditions optimized by the Taguchi method were used for a higher conversion rate. 73.1 μL of glycerol (1 mmol) and 57.2 μL of acetic acid (1 mmol) were combined in a capped 1 mL Eppendorf tube to prevent evaporation. The reaction

was initiated with 19.5 μL of Eversa® Transform 2.0 enzyme (15% of the reaction medium), stirred at 250 rpm, and maintained at 40°C for 12.5 hours. A control reaction without the enzyme was conducted under the same conditions to serve as a standard.

An aliquot of the reaction media at the optimal conversion point was analyzed using high-performance liquid chromatography (HPLC) with a Shimadzu LC-18 column and an ultraviolet detector. The isocratic method was employed with 70% methanol and 30% acetonitrile. The injection volume was 20 μL per minute for 5 minutes at 40°C, with the analysis wavelength set at 254 nm, where the spectrum showed higher absorptions. The reaction intermediates and products were further analyzed using one-dimensional Hydrogen (^{1}H NMR) and Carbon (^{13}C NMR) nuclear magnetic resonance spectroscopy. Spectra were recorded on a Bruker Advance DRX-300 spectrometer at the Northeast Center for Application and Use of Nuclear Magnetic Resonance, Federal University of Ceará (CENAUREMN-UFC). The hydrogen frequency was set at 300 MHz, and the carbon frequency at 75 MHz. Each sample was dissolved in deuterated chloroform ($CDCl_3$) and analyzed in 5 mm tubes.

The statistical Taguchi method was used to determine the number and variations of experiments, employing an L9 orthogonal matrix. The molar ratio of glycerol to acetic acid (GL: AA), the percentage of liquid enzyme in the reaction media (biocatalyst % w/w), temperature (°C), and reaction time (h) were the parameters chosen. A computational model of ET2 was built using homology modeling for classical and quantum simulations with acetic acid and glycerol as substrates. Their charges and electrostatic potentials (ESP) were calculated at the B3LYP/6-311G+ (d,p) level using Gaussian software. Forcefield parameters were developed with Antechamber in the AMBER package. Molecular docking with AutoDock Vina 1.5.6 generated 20 poses, with the three lowest-energy poses selected for all-atom molecular dynamics (MD) simulations. The 200 ns MD simulations in an aqueous solution used GROMACS and the AMBER forcefield, placing the enzyme-substrate complex in a 10×10×10 nm cubic box. Electrostatic interactions were computed with the particle mesh Ewald method and a 1.2 nm cutoff for Coulombic and van der Waals interactions. The TIP3P water model and $Na^+$ and $Cl^-$ ions neutralized the system, which was energy minimized for 3000 steps before each MD simulation. The MD simulations ran in an NPT ensemble with a 1 fs timestep, and cluster analysis identified the most representative structure[6].

## 3  RESULTS & DISCUSSION

Utilizing the Taguchi planning method with an L9 orthogonal matrix, we conducted statistical analysis to streamline and optimize experimental steps. This approach effectively elucidated the impact and interrelation of each parameter in our process. Temperature emerged as the pivotal factor influencing the conversion of glycerol to glyceride esters using ET2 as the biocatalyst, with optimal efficiency observed around 40°C. Beyond this temperature, enzymatic activity notably declined, potentially due to enzyme-substrate complex destabilization or enzyme denaturation.

In chromatographic analysis, peaks corresponded to mono-, di-, and tri-acetylated products, exhibiting maximum absorbance at 254 nm. Triacylglycerols predominated, with monoacylglycerols present in lesser amounts, as indicated by peak surface areas. The ^{1}H NMR spectrum of triacylglycerol illustrated characteristic signals at δ 2.11 and δ 2.09 ppm for methylene hydrogens adjacent to carbonyl groups, alongside multiplets near δ 4.3 – 3.3 ppm for internal hydrogens bonded to ester oxygens. Peaks around δ 3.70 and δ 3.55 ppm represented unreacted glycerol hydrogens, while peaks for mono- and di-acylated products were also discernible. The ^{13}C NMR spectrum highlighted the ester carbonyl carbon peak at δ 175 ppm, confirming alcohol conversion to ester forms.

After optimizing the structures of reactants and products to ensure they exhibited only positive frequencies, confirming they were not transition states, the lowest energy conformer was selected for docking with the enzyme. All of the structures were optimized and subjected to frequency calculations to verify their stability. Molecular Docking generated nine poses for each ligand, with the pose displaying the lowest energy and closest proximity to the enzyme's catalytic site chosen for further analysis using AutoDock Tools. The enzyme's active site consists of Ser153, His268, and Asp206, with Ser153 acting as a nucleophile crucial for substrate binding and catalysis.

MD simulations were conducted to evaluate the interaction energies between ET2 and the ligands studied. Fig. 9 displays images generated from these simulations, illustrating the interaction of monoacylglycerol (MAG1 and MAG2) and triacylglycerol (TAG) ligands with ET2. MAG2 exhibited superior interaction energy and positioning within the enzyme's active site compared to MAG1. Triacylglycerol (TAG) demonstrated the most stable ligand-protein complex, displaying lower interaction energies and a favorable position in the catalytic pocket. Based on these findings, ligands with extreme energy values—high for monoacylglycerols and low for triacylglycerol—were selected for detailed Molecular Dynamics calculations to assess their energetic profiles and conformational dynamics. Triplicate MD simulations were performed for each ligand to ensure robust validation and consistency in results. RMSD analysis indicated minimal variation within triplicates, underscoring the stability of the ligand-protein complexes over the simulation period. Specifically, the TAG ligand showed the most stable variation in Cα enzyme backbone RMSD, suggesting a highly favorable interaction with ET2 compared to the monoacylglycerol ligands, which exhibited greater fluctuations during the simulation.

The active site of ET2, like other natural lipases, features a catalytic triad composed of Ser153, His268, and Asp206. Substrates must adopt suitable conformations to bind to these catalytic residues and undergo enzymatic catalysis. The catalytic mechanism involves two main phases: acylation and deacylation. During acylation, acetic acid is activated by the enzyme to form an acyl-enzyme intermediate. In deacylation, this intermediate transfers its acyl group to a free glycerol molecule, potentially forming mono-, di-, or tri-substituted products. In the acylation step, the nucleophilic attack by Ser153 on the electrophilic carbon of acetic acid occurs with a 13.1 kcal/mol energy barrier, a critical rate-limiting step. A proton transfer from Ser153 to His268 accompanies

this attack, facilitated by Asp206, enhancing His268's basicity. The subsequent gem-diolate intermediate (IA) is stabilized by Tyr29, albeit endergonically. Collapse of IA, catalyzed by protonated His268, yields the acylated enzyme (PA), exergonically.

For deacylation, glycerol's primary and secondary alcohols attack the acylated Ser153 carbon. These steps involve energy barriers of 13.8 kcal/mol and 12.9 kcal/mol, respectively, reflecting subtle differences in transition state energies due to substrate orientation in the enzyme's active site. His268 facilitates both nucleophilic attacks by acting as a base, influenced by Asp206. The resulting gem-diolate intermediates differ in stability by 3.5 kcal/mol, with the secondary intermediate being less stable and potentially prone to dissociation at higher temperatures.

The esterification of glycerol's primary alcohol yields a more stable thermodynamic product, favored under ET2's operational conditions (40°C). Conversely, the esterification of the secondary alcohol produces the kinetic product, energetically less favorable but potentially preferred under lower temperature conditions. These mechanistic insights underscore ET2's selectivity and efficiency in producing glyceride esters, crucial for various industrial applications.

## 4  CONCLUSION

Through the application of the statistical Taguchi method, significant parameters influencing the enzymatic conversion of glycerol to glycerides were identified. Among these, temperature emerged as the most critical factor impacting conversion efficiency within a fixed reaction time. Statistical analyses including S/N and ANOVA confirmed that parameters such as substrate molar ratio, biocatalyst load, and reaction duration exerted less influence compared to temperature. Optimal conditions derived from these analyses for efficient glyceride synthesis using ET2 were determined as 40°C, a glycerol to acetic acid ratio of 1:1, 15% w/w biocatalyst concentration, and a reaction time of 12.5 hours. In MD simulations, the interaction potential energy (IPE) values corroborated experimental findings. Triacylglycerol (TAG) exhibited the lowest average IPE (-35.9 kJ/mol), aligning with its high yield observed experimentally. Comparative analysis between MAG2 (-13.8 kJ/mol) and MAG1 (-10.9 kJ/mol) indicated MAG2's preference, reinforcing the favorable conditions for triacylglycerol production. Using homology modeling, a computational model of ET2 was constructed with 286 amino acids, validated by Ramachandran plot analysis showing minimal unfavorable regions (0.4%). QM/MM mechanistic studies elucidated the esterification process involving acylation and deacylation cycles. Activation energies of 13.1 kcal/mol and 13.8/12.9 kcal/mol for primary and secondary alcohol esterification, respectively, underscored the kinetic and thermodynamic preferences of the enzymatic process. These findings underscore the robustness of ET2 in catalyzing glyceride synthesis under optimized conditions identified through integrated statistical and computational approaches.

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