

Enzymatic Mechanism and Energetics in the Optimized Biosynthesis of the Flavor and Fragrance Ester Geranyl Acetate



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Geranyl acetate, a significant natural fragrance compound, is typically available only in minute quantities from plant sources. This study focuses on the biosynthesis of this compound through enzymatic esterification of geraniol with acetic acid, utilizing the environmentally benign and generally safe solvent, acetone. The optimization process initially involved key synthesis variables, including acetic acid and geraniol concentrations, temperature, and enzyme loading. Subsequently, a thermodynamic investigation of the biosynthesis was conducted alongside kinetic elucidation, providing insights into the mechanism of the lipase-mediated esterification, which was revealed to follow a Ping-Pong Bi-Bi mechanism with no apparent inhibition by either substrate. The reaction is endothermic, with calculated changes in enthalpy (ΔH), and entropy, (ΔS) at 66 kJ mol^{-1} and $217 \text{ J mol}^{-1} \text{ K}^{-1}$, respectively. The activation energy, E_a , was determined to be 28 kJ mol^{-1} . Esterification was non-spontaneous below $30 \text{ }^\circ\text{C}$ but shifted to spontaneous at higher temperatures. These findings hold significance for the development of biocatalytic processes.

Keywords

geranyl acetate, esterification, *Candida antarctica*, kinetics, thermodynamic

Introduction

Global consumer demand for natural products largely includes the flavors and fragrances market segment. The demand is driven primarily by health concerns related to artificial ingredients and transparency issues in labeling when synthetic chemicals are involved. In the growing economic region of the world, expanding lifestyle and personal consumption expenditures encourage robust market growth for flavor- and fragrance commodities, such as packaged foods, cosmetics and toiletries, etc.¹ While North America and Western Europe continue to be vital markets for flavors and fragrances, the Asia/Pacific region will offer the most substantial market gains, fueled by strong socio-economic growth in the developing countries².

Natural flavors and fragrances, extracted from natural sources such as plants and animals, are

deemed unsustainable due to the limited supply and seasonal variations. Conversely, artificial flavors and fragrances are often synthesized without adhering to the green chemistry principles, posing potential health hazards. To be classified as a natural flavor/fragrance, a compound must either be a plant or animal extract, such as an essential oil, or be prepared by modifying a natural extract through enzyme- or microbial processes^{3,4}. Geranyl acetate (3,7-dimethyl-2,6-octadiene-1-ol acetate), derived from geraniol, is highly sought after as a flavor and fragrance compound in the food and cosmetics industries for its sweet fruity taste and floral scent, attributed to its neryl acetate content. The production of geranyl acetate reaches several hundred tons annually⁵. The parent molecule, geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol), is commonly found in various fruits, flowers, and berries, existing as an acyclic monoterpene alcohol with the chemical formula $\text{C}_{10}\text{H}_{18}\text{O}$; a mixture of two *cis-trans* isomers, namely geraniol (*trans*) and nerol (*cis*). Geraniol is found in 76 % of deodorants available on the European market, in 41 % of domestic and household

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products, and in 33 % of cosmetic formulations based on natural ingredients, with an annual production exceeding 1000 metric tons⁶. US and European legislations stipulate that, for a substance to qualify as a ‘natural’ flavor, it must be directly extracted from plant material through physical processes or be bioconverted from nature-derived precursors using enzymes or microbes⁷. However, natural flavors/fragrances are expensive due to the large amount of source materials required, often leading to unsustainable practices.

However, natural compounds such as flavor/fragrance esters synthesized enzymatically may qualify as “natural” and be labeled as such⁸. Utilizing enzymes as catalysts for ester production offers distinct advantages over traditional chemical methods due to the mild reaction conditions and minimal use of hazardous solvents. In this study, we employed immobilized *Candida antarctica* lipase B as a catalyst in the esterification reaction between a selected carboxylic acid and a monoterpene alcohol to synthesize a fragrance ester. Previous studies have demonstrated that *C. antarctica* lipase B (Cal B) is a robust enzyme for catalysis in organic synthesis, exhibiting high catalytic efficiency in esterification or transesterification reactions⁹. It also proves to be a suitable catalyst for the synthesis of short-chain flavor esters¹⁰. Exploring the diversity of biocatalytic routes for geranyl acetate, Bhavsar and Yadav¹¹ demonstrated its synthesis through the transesterification of geraniol with ethyl acetate. Notably, Lozano *et al.*⁹ and Couto *et al.*¹² reported a simpler approach using direct esterification with acetic acid. As reviewed by Liu *et al.*¹³, the performance of geranyl acetate synthesis is critically influenced by the origin and concentration of the biocatalyst, acyl donor properties (acid chain lengths, concentration, and purity), temperature, reaction media, and operational settings (temperature, pressure, reaction time, agitation). In their comprehensive review, the authors documented a remarkable range in conversion efficiency across various optimization studies, with values as low as 4 % and as high as 99.9 %. However, the results profoundly depend on the specific parameter ranges explored in their optimization study¹³.

In this study, we investigated the lipase-mediated esterification of geraniol with acetic acid in acetone as a reaction solvent to produce geranyl acetate. Acetone is an excellent solvent for such biosynthesis, exhibiting low acute and chronic toxicity when compared to other equally applicable solvents, such as *n*-hexane and toluene. Acetone is produced endogenously by the human body, and can be detected in exhaled breath, urine, blood, and breast milk. It is naturally eliminated within 1–3

days, particularly for the lower acetone levels prevalent in the general population. The health effects associated with acetone are mild. The U.S. Environmental Protection Agency (EPA) determined in 2003 that available data were insufficient for the assessment of acetone’s potential as a human carcinogen¹⁴.

In addition to determining the optimum conditions for the biosynthesis of geranyl acetate, this study explores its kinetics mechanism and thermodynamic parameters when acetone is used as a reaction solvent. An inclusive kinetic model, incorporating Michaelis-Menten parameters (K_m and V_{max}), is crucial for elucidating the individual contributions of these parameters to enzyme activity. The selection of an appropriate kinetic model, followed by rigorous validation, can provide valuable insights into how enzyme activity and substrate availability interact in enzymatic geranyl acetate synthesis. The thermodynamic parameters of Gibbs free energy unveil the spontaneity and energetic favorability of a reaction, guided by the equilibrium constant, enthalpy changes, and entropy. A comprehensive understanding of biocatalytic behavior is significant in developing a feasible upstream process for the production of geranyl acetate as a fragrance substance.

Materials and methods

Materials

Lipase from *Candida antarctica* immobilized on acrylic resin (CALB) (L4777), molecular sieve 4 Å (208604), and the acyl acceptor, geraniol (48798), were purchased from Sigma–Aldrich. Geranyl acetate (173495) was procured from Sigma-Aldrich. The acyl donor, acetic acid (695092), and acetone (270725) were supplied by Merck. *n*-octane (A13181.AP) was obtained from Acros, Germany. Analytical solvent and reagent were used as received.

Enzymatic synthesis of geranyl acetate

The lipase-catalyzed synthesis of geranyl acetate was conducted at various initial molarities of geraniol (0.1 to 0.3 mol L⁻¹) with different initial concentrations of acetic acid (0.1 to 0.3 mol L⁻¹) in acetone. The corresponding amount of acetone was added to reach a final volume of 3 mL in screw-capped tubes. The tubes were pre-incubated at a fixed reaction temperature (30 °C – 50 °C) for 10 minutes with horizontal shaking at 200 rpm speed. The reaction was initiated by adding immobilized *Candida antarctica* lipase B (CALB). The vials

Table 1 – Summary of the variable levels

Label	Variables	Unit	Level		
			Lowest	Middle	Highest
A	Acetic acid concentration	mol L ⁻¹	0.1	0.2	0.3
B	Geraniol concentration	mol L ⁻¹	0.1	0.2	0.3
C	Enzyme loading	mg	20	30	40
D	Temperature	°C	30	40	50

were incubated at a constant temperature (30 °C – 50 °C) with horizontal shaking at 200 rpm. Twenty μL aliquots were extracted at regular intervals. The aliquot volume was allowed to deplete *via* evaporation before the addition of 500 μL *n*-octane. The mixture was vortexed at room temperature for homogeneity before analysis using gas chromatography (GC). All experiments were conducted in triplicate.

To investigate the effects of key variables on the esterification process, the experiments were conducted according to the full factorial design (FFD) as devised in Minitab® 17 software. The effects of four selected variables: acetic acid concentration, A (mol L⁻¹), geraniol concentration, G (mol L⁻¹), enzyme loading, C (mg) and temperature, D (°C), were screened. Enzymatic synthesis of geranyl acetate was performed in random triplicates at the lowest, middle, and highest factor levels (Table 1). The effects of these variables were evaluated in terms of geranyl acetate concentration (mol L⁻¹) as the response variable.

Since there were four variables, each with three levels (minimum, middle, and maximum point values), a total of 34 experimental runs, including triplicates, were conducted. The design was completely randomized, along with the sampling.

$$v = \frac{V_{\max} [\text{Geraniol}][\text{Acetic acid}]}{K_m^{\text{Geraniol}} [\text{Acetic acid}] + K_m^{\text{Acetic acid}} [\text{Geraniol}] + [\text{Acetic acid}][\text{Geraniol}]} \quad (3)$$

where v is the initial reaction rate, V_{\max} is the maximum reaction rate (mol L⁻¹ h⁻¹), and $K_m^{\text{Acetic acid}}$ and K_m^{Geraniol} are the Michaelis constants for acetic acid and geraniol, respectively. The initial rates obtained at various acetic acid and geraniol concentrations were fitted to this model using non-linear regression (Polymath® software).

Analytical methods

Gas chromatography (GC)

The concentrations of geraniol and geranyl acetate were determined using a Thermo Scientific Trace GC Ultra equipped with an automated injector and a flame ionization detector. A TG-5MS column (30 m x 0.32 mm x 0.25 μm) was employed, with the following column oven temperature profile: program rate 10 °C min⁻¹, initial temperature 50 °C, final temperature 280 °C, and a holding period of 2 minutes. Helium served as the carrier gas at 35 mL min⁻¹. A splitless injection of 1 μL was performed. Standard concentrations of geraniol and geranyl acetate in *n*-octane were constructed separately to provide a standard calibration against peak area, as well as the reference retention time. The conversion was calculated according to equation (1):

$$\text{Conversion (\%)} = \frac{[\text{Geraniol}]_{\text{initial}} - [\text{Geraniol}]_{\text{residual}}}{[\text{Geraniol}]_{\text{initial}}} \cdot 100 \quad (1)$$

where $[\text{Geraniol}]_{\text{initial}}$ is the initial geraniol concentration, and $[\text{Geraniol}]_{\text{residual}}$ is the unconverted geraniol concentration in the reaction mixture. The conversion yield was validated through the assay of geranyl acetate concentration in the samples.

Thermo-kinetic parameters

Initial rate

The initial esterification rate was determined by a tangent drawn from the origin of the polynomial regression plot of product concentration against time. Thus, for geranyl acetate, the initial rate (v , mol L⁻¹ h⁻¹) was calculated using equation (2):

$$v = \frac{\Delta C_{[\text{GA}]}}{\Delta t} \quad (2)$$

where $\Delta C_{[\text{GA}]}$ (mol L⁻¹) are the changes in geranyl acetate concentration within Δt (h). Assuming no inhibition from both substrate and product, the initial rate can be represented using Ping-Pong Bi-Bi model:

In the esterification process, acetic acid (the acyl donor) initially forms an enzyme-acetate complex through a nucleophilic attack of the hydroxyl group of serine in the active site of the lipase. The first product is a water molecule, released simultaneously as the complex undergoes transformation into an enzyme-acyl intermediate. The second substrate, geraniol, then attacks the intermediate, leading to the formation of an enzyme-acyl-geraniol complex. Subsequently, this complex splits apart to release geranyl acetate, and the enzyme then recommences its catalytic activity in preparation for the next esterification cycle.

Activation energy

Activation energy (E_a) is defined as minimum energy required by reactant molecules to overcome energy barrier and pass through a transition state before progressing to the reaction product¹⁵. The activation energy (E_a , J mol⁻¹) of geraniol esterification was determined using the linearized Arrhenius equation:

$$\ln k_1' = \ln A_f - \frac{E_a}{RT} \quad (4)$$

where A_f is the frequency factor, R is the gas constant (8.3145 J mol⁻¹ K⁻¹), and T is the temperature (K). To ascertain the apparent first-order rate constant, k_1' , the reaction rate of geraniol esterification was determined at different initial concentrations of geraniol for each tested temperature (30 °C, 40 °C, 50 °C), while maintaining a constant initial acetic acid concentration of 0.3 mol L⁻¹. k_1' was determined from the slope of the resulting linear plot.

Thermodynamic parameters

The esterification reaction was considered to have reached equilibrium when no further conversion was observed over time at a specific temperature. The apparent equilibrium constant K_{eq} was calculated as follows:

$$K_{eq} = \frac{[\text{Geranyl acetate}]_{eq} [\text{Water}]_{eq}}{[\text{Geraniol}]_{eq} [\text{Acetic acid}]_{eq}} \quad (5)$$

where K_{eq} is the apparent equilibrium constant, $[\text{Geranyl acetate}]_{eq}$ and $[\text{Water}]_{eq}$ are the geranyl acetate (mol) and water concentration (mol) at equilibrium, respectively. $[\text{Geraniol}]_{eq}$ and $[\text{Acetic acid}]_{eq}$ are the respective concentrations of residual geraniol and acetic acid (mol) at equilibrium. $[\text{Water}]_{eq}$ was calculated from the stoichiometry of ester synthesis where 1 mol of water is produced per mol of ester synthesized. Similarly, 1 mol of acetic acid is converted per mole of geranyl acetate synthesized, therefore $[\text{Acetic acid}]_{eq}$ was calculated from the

initial acetic acid concentration minus the $[\text{Geranyl acetate}]_{eq}$ concentration.

K_{eq} was measured at different temperatures. According to the Van't Hoff equation, the temperature dependency of K_{eq} is represented as follows:

$$\ln K_{eq} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (6)$$

where ΔH is Van't Hoff enthalpy change (J mol⁻¹) and ΔS is entropy change (J mol⁻¹ K⁻¹).

The Gibbs free energy change (ΔG , J mol⁻¹) at constant pressure and temperature for the reaction under non-standard conditions was determined using the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

Results and discussion

Enzymatic synthesis of geranyl acetate

A full factorial design (FFD) was employed to screen the effects of the selected esterification variables, namely acetic acid concentration (mol L⁻¹), geraniol concentration (mol L⁻¹), enzyme loading (mg), and temperature (°C). To evaluate the efficiency of the enzymatic esterification process, the range of variables was carefully selected drawing upon optimization studies conducted by previous researchers^{16–18}. A total of 34 experimental runs were conducted, with the analyzed response being the geranyl acetate concentration after an eight-hour reaction period (Table S1). The highest ester concentration, at 0.24 mol L⁻¹, was achieved with a variables combination of 0.3 mol L⁻¹ acetic acid, 0.3 mol L⁻¹ geraniol, 50 °C, and 40 mg enzyme. The lowest geranyl acetate concentration, (0.031 mol L⁻¹), was obtained with 0.1 mol L⁻¹ acetic acid, 0.1 mol L⁻¹ geraniol, 30 °C, and 20 mg enzyme.

Analysis of FFD experiments

ANOVA analysis was conducted to identify the significant variables utilizing Minitab® 17 software, as shown in Table 2. The effects of the main factors and their interactions were found to be significant at $p = 0.05$, corroborated by R^2 - and R^2 - adjusted values of 0.9897 and 0.9787, respectively, indicating a satisfactory fit of the model to the experimental data. All selected variables exhibited significant effects on the response ($p < 0.05$), except for the two-way interaction of Acetic acid*Geraniol (A*B). The F -values analysis in Table 2 revealed that temperature ($F = 897.59$) exerted the strongest influence on the response, followed by geraniol concentration ($F = 864.72$), the two-way interaction of temperature and geraniol concentration ($F = 494.43$), and the main

Table 2 – ANOVA of studied reaction variables showing their main effects and interactions

Source	DF	Adj SS ($\cdot 10^{-2}$)	Adj MS ($\cdot 10^{-3}$)	F-Value	P-Value
Model	16	10.85	6.78	198.42	0.00
Linear	4	7.11	17.78	520.37	0.00
Acetic acid (A)	1	0.28	2.77	80.92	0.00
Geraniol (B)	1	2.95	29.54	864.72	0.00
Enzyme (C)	1	0.81	8.14	238.26	0.00
Temperature (D)	1	3.07	30.67	897.59	0.00
2-Way Interactions	6	2.14	3.56	104.32	0.00
Acetic acid*Geraniol (A*B)	1	0.00	0.00	0.09	0.77
Acetic acid*Enzyme (A*C)	1	0.22	2.22	64.89	0.00
Acetic acid*Temperature (A*D)	1	0.08	0.78	22.76	0.00
Geraniol*Enzyme (B*C)	1	0.06	0.57	16.54	0.00
Geraniol*Temperature (B*D)	1	1.69	16.89	494.43	0.00
Enzyme*Temperature (C*D)	1	0.09	0.93	27.22	0.00
3-Way Interactions	4	0.97	2.42	70.73	0.00
Acetic acid*Geraniol*Enzyme (A*B*C)	1	0.24	2.44	71.38	0.00
Acetic acid*Geraniol*Temperature (A*B*D)	1	0.51	5.12	149.85	0.00
Acetic acid*Enzyme*Temperature (A*C*D)	1	0.12	1.16	33.99	0.00
Geraniol*Enzyme*Temperature (B*C*D)	1	0.09	0.95	27.70	0.00
4-Way Interactions	1	0.36	3.64	106.38	0.00
Acetic acid*Geraniol*Enzyme*Temperature (A*B*C*D)	1	0.36	3.64	106.38	0.00
Curvature	1	0.27	2.66	77.97	0.00
Error	17	0.06	0.03		
Total	33	10.90			

DF: Degree of freedom; ADJ SS: Adjusted sum of squares; Adj MS: Adjusted mean of square; F: F-statistics; P: p-statistics

effect of enzyme concentration ($F = 238.26$). The significant effects of two-, three- and four-way interactions indicated the presence of confounding effects among the selected variables.

It should be noted that a significant curvature effect was also found, suggesting the nonlinear relationship between the response and its variables.

The Pareto chart of the standardized effects plot analysis corroborates the findings of the variance analysis. In Fig. 1, the standardized effects are presented in bar charts, arranged from the largest effect on the right to the smallest effect on the left, with a reference line set at 2.11. The bars corresponding to the main factors D (temperature) and B (geraniol), followed by the BD interaction, extend farthest from the reference line (2.11). This positioning signifies that these effects are highly significant in the esterification process.

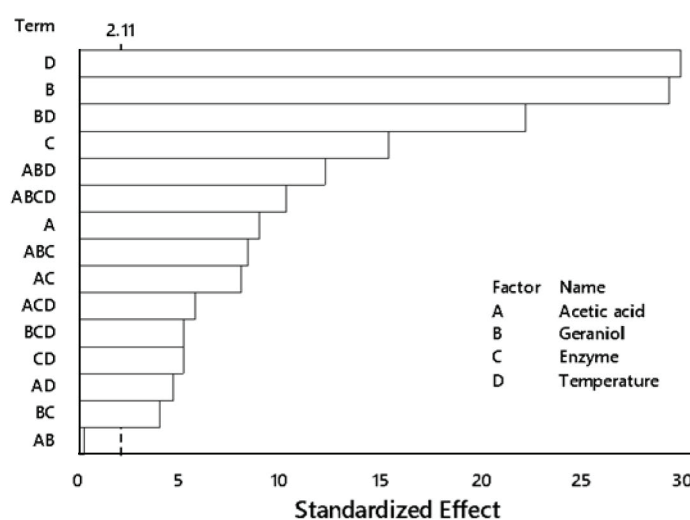


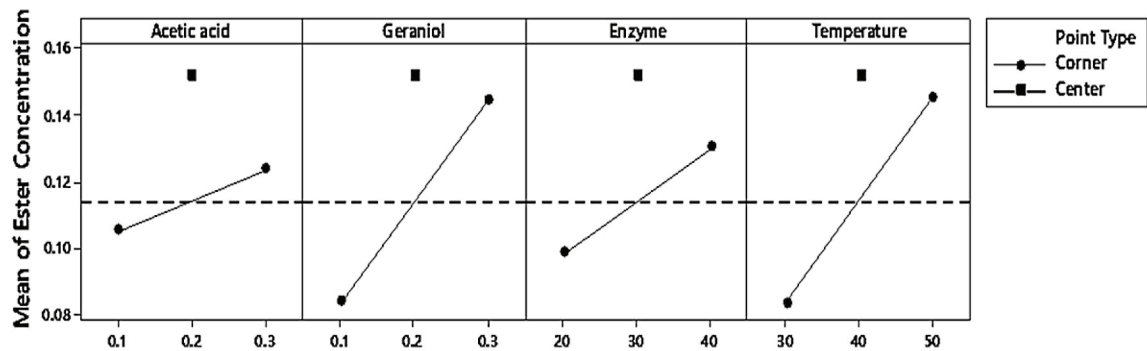
Fig. 1 – Pareto chart for the main effects and their interactions

Main effect plots

The response behavior in the main effect plots was based on the mean of the observed geranyl acetate production. The slope of a main effect plot serves as an indicator of the magnitude of response change concerning a variable change. A steeper line suggests a stronger effect that a variable exerts on the response. As illustrated in Fig. 2, both temperature and geraniol concentration clearly exhibited such strong effects. Conversely, acetic acid concentration and enzyme amount exhibited relatively weaker effects on the response. Nonetheless, for all the studied variables, the production of geranyl acetate improved as the magnitudes of the main effects increased from minimum to high level.

Residual analysis of FFD experiments

The residual analysis was employed to determine the correlation between experimental data and the predicted model (Fig. 3). Residual values were derived by subtracting the model fitted values from the experimental values (Residuals = Experimental values – Model fitted values). The normal probability plot, (Fig. 3a), indicated no potential outlier in the data; the residuals exhibited a normal distribution with respect to the standardized residual. Fitted values of the model showed a well-distributed, random scatter of residuals on both sides of standardized positive and negative values, falling within the acceptable range of -2 to $+2$ (Fig. 3b). Furthermore, the experimental responses displayed a normal dis-



All displayed terms are in the model.

Fig. 2 – Main effects plot for the studied reaction variables

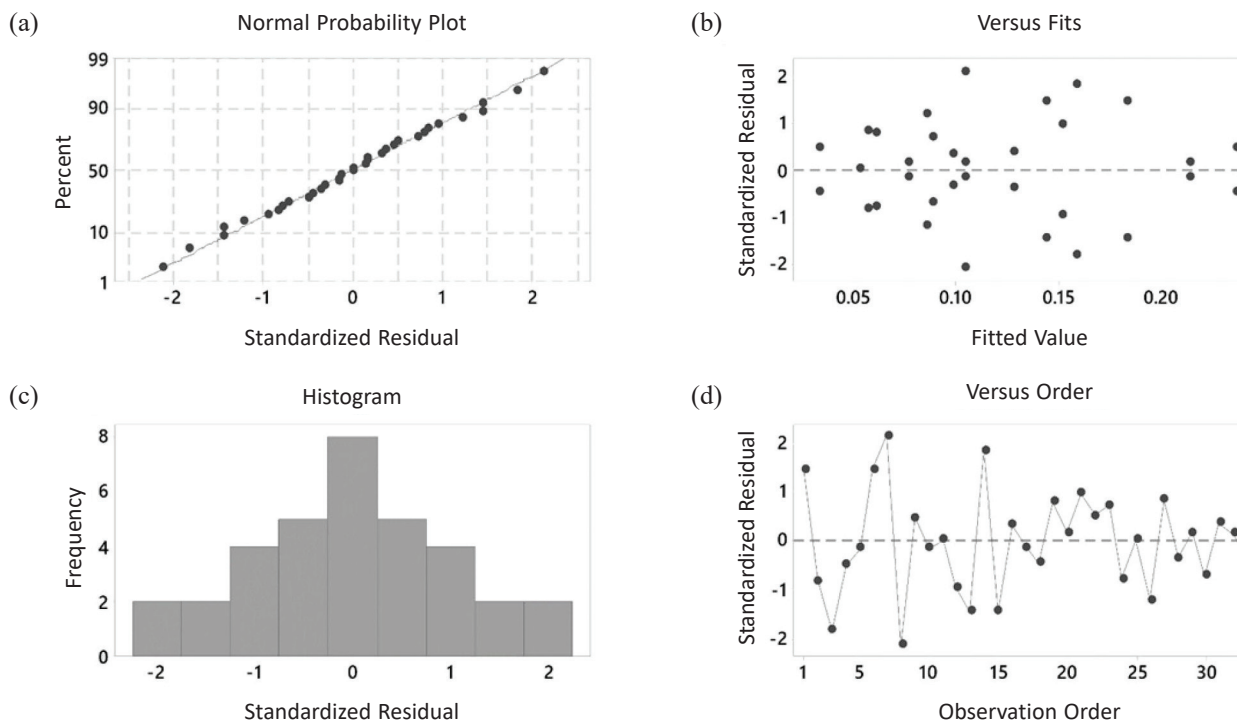


Fig. 3 – Residual plots for geranyl acetate concentration: (a) normal probability plot, (b) standardized residual vs fitted value, (c) histogram plot, and (d) standardized residual vs observation order

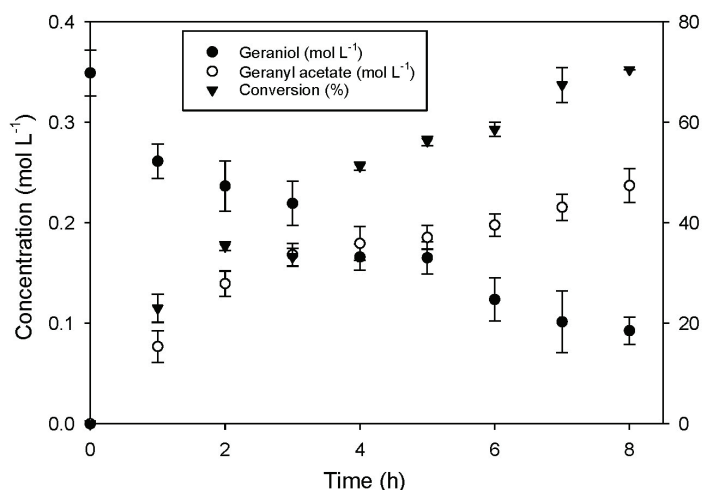


Fig. 4 – Plots of conversion (%), geraniol concentration (mol L^{-1}), geranyl acetate concentration (mol L^{-1}) against time (h). Reaction conditions: 50 °C; 40 mg of lipase; 200 rpm (Standard deviation of the triplicate measurements <15 %)

tribution, as shown by the approximate bell-shaped curve in the plotted histogram (Fig. 3c). The order of data collection had no influence on the response, as shown by the lack of a discernible pattern in the standardized residuals versus the observation order graph (Fig. 3d).

While Table S1 clearly indicates that the maximum response of 0.24 mol L^{-1} geranyl acetate was attained at 0.3 mol L^{-1} geraniol, 0.3 mol L^{-1} acetic acid, 40 mg lipase, and 50 °C, these conditions were validated using the Response Optimizer function in Minitab® 17. The optimization goal was to maximize the geranyl acetate concentration within the constraints of the empirical conditions presented in Table S1. Consequently, the optimal levels of variables for maximum response returned by the optimizer were identical, with a composite desirability of 0.9909. This suggested the possibility of achieving the predicted response in 99 out of 100 runs¹⁹.

Kinetics analysis

The course of esterification was profiled to determine the equilibrium of the reaction. Samples were collected regularly over an eight-hour period, revealing that geraniol conversion reached 70 % under the experimental conditions of 0.35 mol L^{-1} acetic acid, 0.35 mol L^{-1} geraniol, 40 mg CALB lipase, 50 °C (Fig. 4). An increase in geranyl acetate concentration was observed during the initial 4 hours, followed by a gradual slowdown. The profiles for geranyl acetate, geraniol, and conversion yield, indicated that equilibrium was achieved after eight hours of the reaction. Similar findings were observed for other temperatures investigated.

The profiles of conversion yield, geraniol and geranyl acetate concentrations at different temperatures with an initial geraniol concentration of

0.35 mol L^{-1} and initial acetic acid concentration of 0.35 mol L^{-1} are presented in Fig. 5(a)–(c), respectively. A substantial reduction in the conversion was observed from 6 hours onwards (Fig. 5a), comparably reflected in the profile of geraniol (Fig. 5b). Geranyl acetate production exhibited a negligible

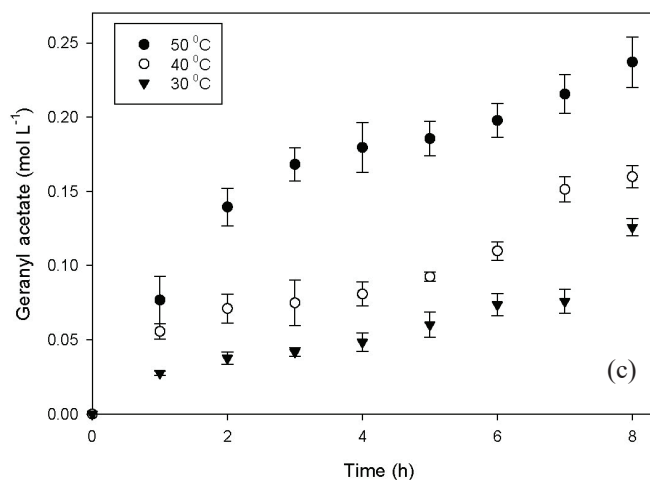
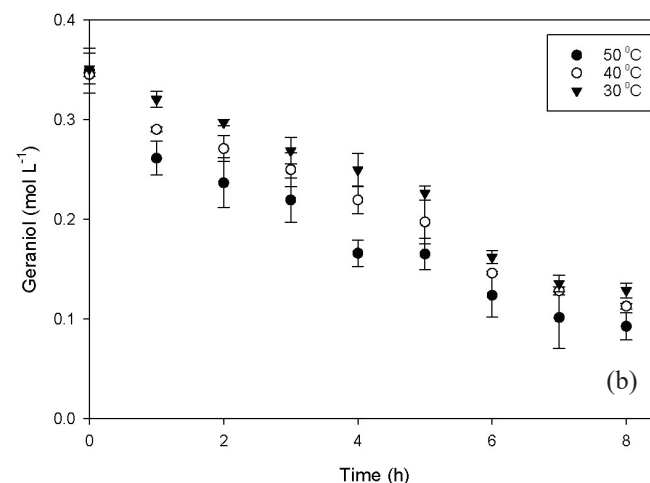
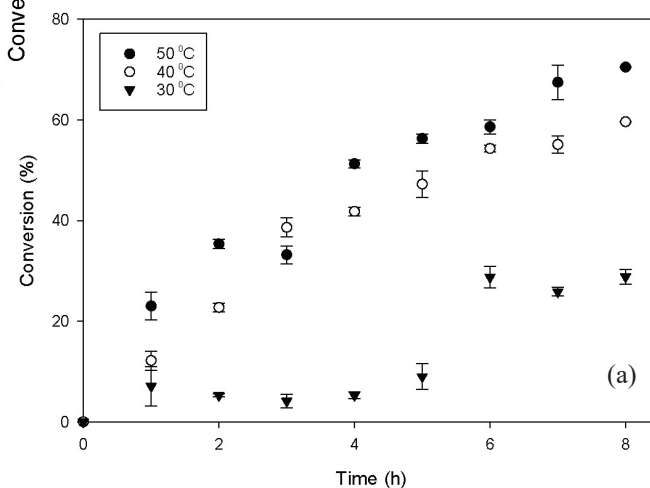


Fig. 5 – Profiles of (a) conversion yield (%), (b) geraniol concentration (mol L^{-1}), and (c) geranyl acetate (mol L^{-1}). Reaction conditions: 30 °C – 50 °C; 40 mg of lipase; 200 rpm (Standard deviation of the triplicate measurements <15 %)

increase after 7 hours onwards at 40 and 50 °C (Fig. 5c), while its production rate was consistently the slowest throughout the assay duration at 30 °C. Nevertheless, it is conceivable that equilibrium would be reached after 8 hours of reaction for the three temperatures studied. Reported reaction times for geranyl acetate production vary widely, from 0.25 hours to 120 hours¹³. In this study, the intrinsic properties of the chosen enzyme strongly suggest minimal deactivation under the specific conditions applied for 8 hours. The ability of CALB to withstand diverse solvents, temperatures up to 70 °C, and prolonged reaction times over 20 cycles makes it a reliable enzyme for a wide range of biocatalytic reactions. Studies by Salvi *et al.*²⁰ and Abd Razak *et al.*²¹ demonstrated CALB's ability to retain catalytic activity for 7 to 20 cycles in diverse esterification reactions.

Geraniol conversion reached a peak of 70 % at the highest temperature studied, maintaining a 1:1 molar ratio of geraniol and acetic acid. Across the temperature range of 30 °C to 50 °C, the initial rate of esterification and conversion yield at equilibrium were found to be highest at 50 °C. Similar results were reported by Claon and Akoh²².

Activation energy of esterification (E_a)

In Fig. 6a, the esterification kinetics exhibit first-order behavior for the initial geraniol concentrations used. The reaction rate of esterification increased with the corresponding increase in temperature and geraniol concentration, as evidenced by the rising slope of k_1' , indicating faster esterification.

A linear plot of $\ln k_1'$ against reciprocal temperature was obtained with a regression coefficient (R^2) of 0.9765 (Fig. 6b). From the slope, the E_a value was calculated at 28 kJ mol⁻¹, consistent with the activation energy of enzyme-catalyzed reactions, ranging from 25 to 83 kJ mol⁻¹²³. This activation energy in the current study is comparatively lower than literature values (83 kJ mol⁻¹, 80 kJ mol⁻¹, 57 kJ mol⁻¹) reported for similar reactions with different acyl donors (ethyl acetate¹¹, vinyl acetate^{25,26}). This lower energy implies a well-controlled enzymatic process and a more efficient reaction pathway.

Thermodynamics of esterification

The apparent enthalpy change (ΔH) and entropy change (ΔS) were determined from the slope and the y -abscissa intercept of the Van't Hoff plot (Fig. 7a), respectively.

The values of ΔH and ΔS were calculated at 66 kJ mol⁻¹ and 217 J mol⁻¹ K⁻¹, respectively. The K_{eq} value increased at higher temperatures (Fig. 7a). The positive ΔH value indicates the endothermic

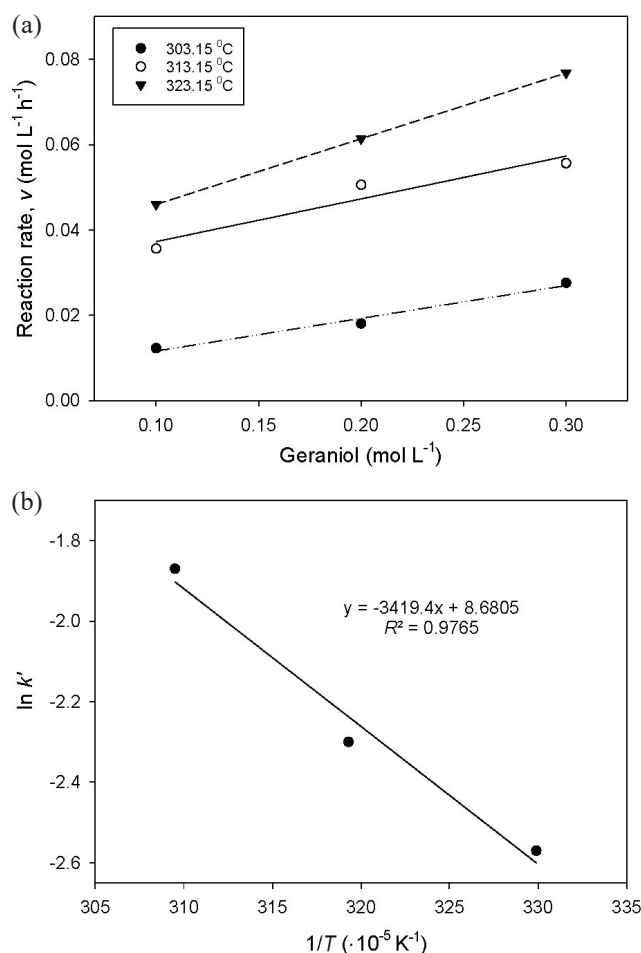


Fig. 6 – (a) Reaction rate of esterification (mol L⁻¹ h⁻¹) at different geraniol concentrations (mol L⁻¹) and temperatures, (b) Arrhenius plot. Reaction conditions: 0.3 mol L⁻¹ of acetic acid; 40 mg of lipase; and 200 rpm (Standard deviation of the triplicate measurements <15 %)

nature of the esterification process, while the positive ΔS value reflects increasing disorder as the temperature rises, attributed primarily to the dispersal of water molecules, a byproduct of the reaction. Given the endothermic nature of the reaction, an increase in temperature is expected to favor the direction of product formation¹⁵.

The ΔH and ΔS values were applied in the calculation of Gibbs free energy change, ΔG , using Eq. (7), indicating the spontaneity or lack thereof in the esterification process. As shown in Fig. 7b, esterification was non-spontaneous at 303 K, where the temperature dependence of ΔG is apparent. The plot also illustrates that $\Delta G = 0$ at approximately 308 K, indicating a spontaneous reaction, i.e., ΔG is negative above 308 K. The increase in temperature provides an energetically favorable path for geranyl acetate formation. These ΔG values were estimated under the assumption that the previously calculated ΔH and ΔS values remain constant within the narrow temperature range studied. Published data on

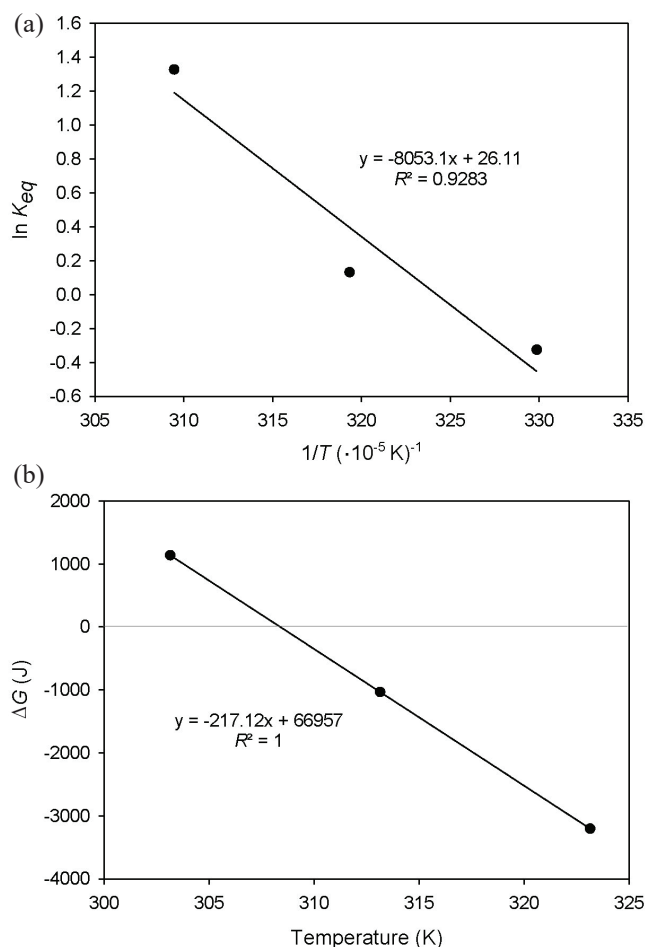


Fig. 7 – (a) Van't Hoff plot, (b) Gibbs free energy change for esterification as a function of temperature

the thermodynamics of lipase-catalyzed synthesis of geranyl acetate is rather limited. Converti *et al.* reported an apparent activation enthalpy of 35 kJ mol^{-1} for geraniol acetylation in *n*-heptane using lyophilized cells of *Aspergillus oryzae*²⁷.

Kinetic model

To determine the reaction rate of esterification, various initial concentrations of geraniol were reacted with different initial concentrations of acetic acid, keeping the quantities of acetone and enzyme constant. The concentration range of both substrates used in this experiment falls within the range studied by previous kinetics studies investigating similar reactions with non-identical substrates^{11,20,24}. Narrow ranges were employed to minimize competition, avoid inhibitory effects, and ensure efficient substrate utilization. Acetic acid functions as an inhibitor by binding to enzymes, thereby reducing their catalytic activity. This leads to a lower initial reaction rate and a subsequently reduced yield of geranyl acetate²⁰. Bhavsar and Yadav observed a declining reaction rate with an increase in ethyl acetate concentration while maintaining constant gera-

niol levels¹¹. Their findings suggested that, at higher concentrations, ethyl acetate forms a dead-end inhibition complex with lipase.

Profiles of geranyl acetate production (mol L^{-1}) as a function of time (h) at various initial geraniol and acetic acid concentrations are presented in Fig. S1. The initial rate was calculated from the initial slope of each graph using Eq. (2). Fig. 8(a)-(b) presents the initial rates as a function of geraniol and acetic acid concentrations.

The effects of substrate concentration on enzyme catalysis were investigated by varying the initial concentrations of geraniol and acetic acid. In a single-phase solution, these effects are consistent with Michaelis-Menten kinetics, assuming that mass transfer limitations of CALB are insignificant based on previous studies²⁸. The maximum initial rate of $0.08 \text{ mol L}^{-1} \text{ h}^{-1}$ was attained at 0.3 mol L^{-1} acetic acid and 0.3 mol L^{-1} geraniol (Fig. 8a-b),

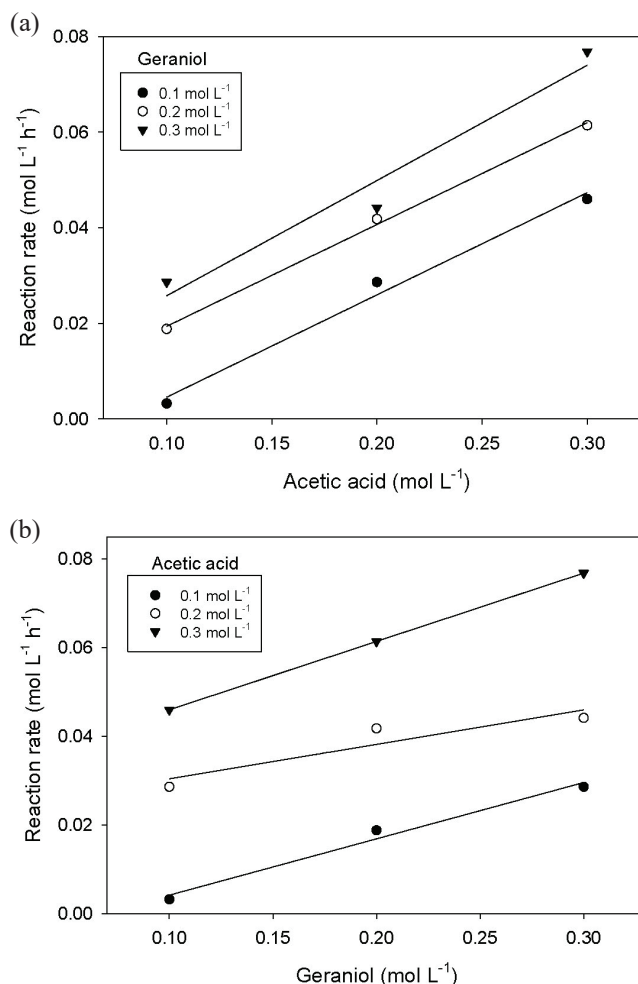


Fig. 8 – (a) Initial rate ($\text{mol L}^{-1} \text{ h}^{-1}$) as a function of initial acetic acid concentrations (mol L^{-1}) at different initial concentrations of geraniol (mol L^{-1}), (b) Initial rate ($\text{mol L}^{-1} \text{ h}^{-1}$) as a function of geraniol concentrations (mol L^{-1}) at different initial concentrations of acetic acid (mol L^{-1}). Reaction conditions: $50 \text{ }^\circ\text{C}$; 40 mg lipase; 200 rpm (Standard deviation of the triplicate measurements $< 15 \%$)

consistent with earlier findings and other studies regarding the optimal molar ratio of substrates for obtaining the best yield^{29–32}.

A double-reciprocal plot was employed to confirm the kinetic mechanism of the esterification. Double reciprocal plots of initial reaction rates (v^{-1}) versus acetic acid concentration ($[\text{Acetic acid}]^{-1}$) for several initial geraniol concentrations and *vice versa* are presented in Fig. 9 (a)-(b). The plotted lines substantiate that the reaction followed a Ping-Pong Bi-Bi mechanism. A similar mechanism has been proposed for other lipase-catalyzed esterifications^{33–37}.

In this study, no apparent enzyme inhibition by both geraniol and acetic acid was observed, as indicated by the absence of an upward curvature in both plots (Fig. 9 a-b). Huang and Chang reported that higher geraniol concentrations did not inhibit esterification, but observed an opposite effect for higher acetic acid concentrations, resulting in reduced yield of geranyl acetate³⁷.

The kinetic parameters of Eq. (3) were calculated using multiple regressions fitting of the experimental values. Therefore, the kinetic equation for the enzymatic synthesis of geranyl acetate using geraniol and acetic acid is given by:

$$v = \frac{0.471[\text{Acetic acid}][\text{Geraniol}]}{0.44[\text{Acetic acid}] + 1.51[\text{Geraniol}] + [\text{Acetic acid}][\text{Geraniol}]} \quad (8)$$

where $V_{\max} = 0.471 \text{ mol L}^{-1} \text{ h}^{-1}$, $K_m^{\text{Geraniol}} = 0.44 \text{ mol L}^{-1}$ and $K_m^{\text{Acetic acid}} = 1.51 \text{ mol L}^{-1}$. It was found that the experimental initial rates aligned very well with the calculated values, with an R^2 value of 0.86. The affinity of the enzyme towards geraniol was 3.4 times higher than for acetic acid, as evidenced by their respective K_m values.

Conclusions

While enzyme-mediated synthesis of high-value compounds in an environmentally- and health-benign solvent aligns with green chemistry principles, understanding quintessential reaction aspects is imperative for process development. This experiment employed response surface methodology, leading to optimal conditions for geranyl acetate production. Four critical factors, namely acetic acid, geraniol, enzyme, and temperature were investigated at three levels each using a full factorial design approach to optimize the process. A validation experiment was conducted using the predicted optimal values. The optimal conversion, reached at 70 %, resulted from the operating variables of 1:1 molar ratio of acetic acid:geraniol, 40 mg of enzyme loading, and a temperature of 50 °C. The lipase-catalyzed reaction was represented by a Ping-Pong Bi Bi model with no substrates inhibition, as reported herein for the biosynthesis of the valuable flavor and fragrance compound, geranyl acetate. The findings provide a baseline knowledge for designing a feasible upstream process.

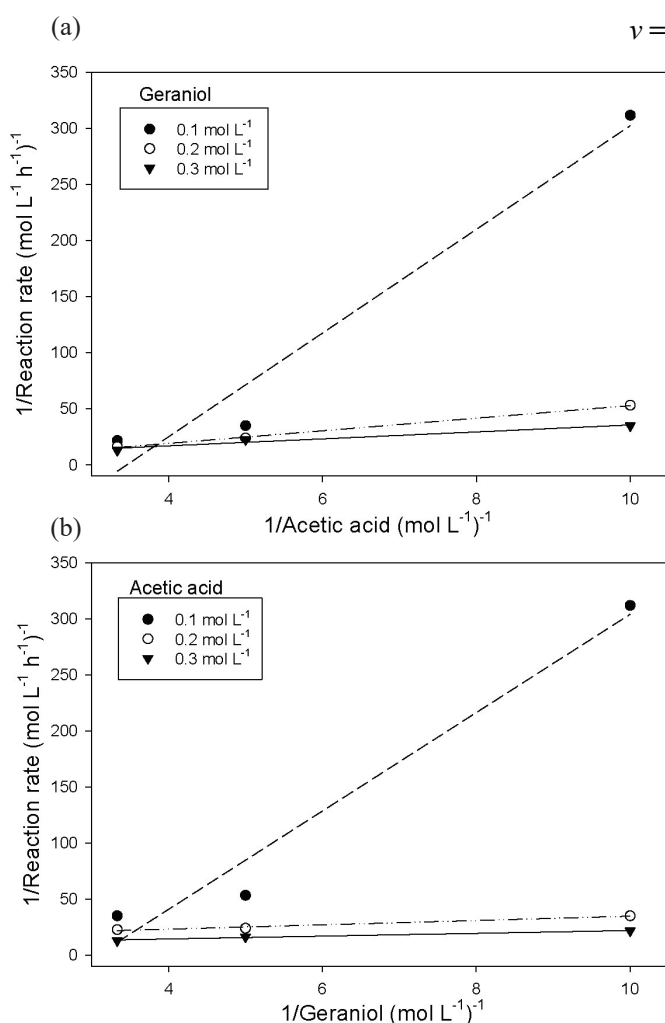
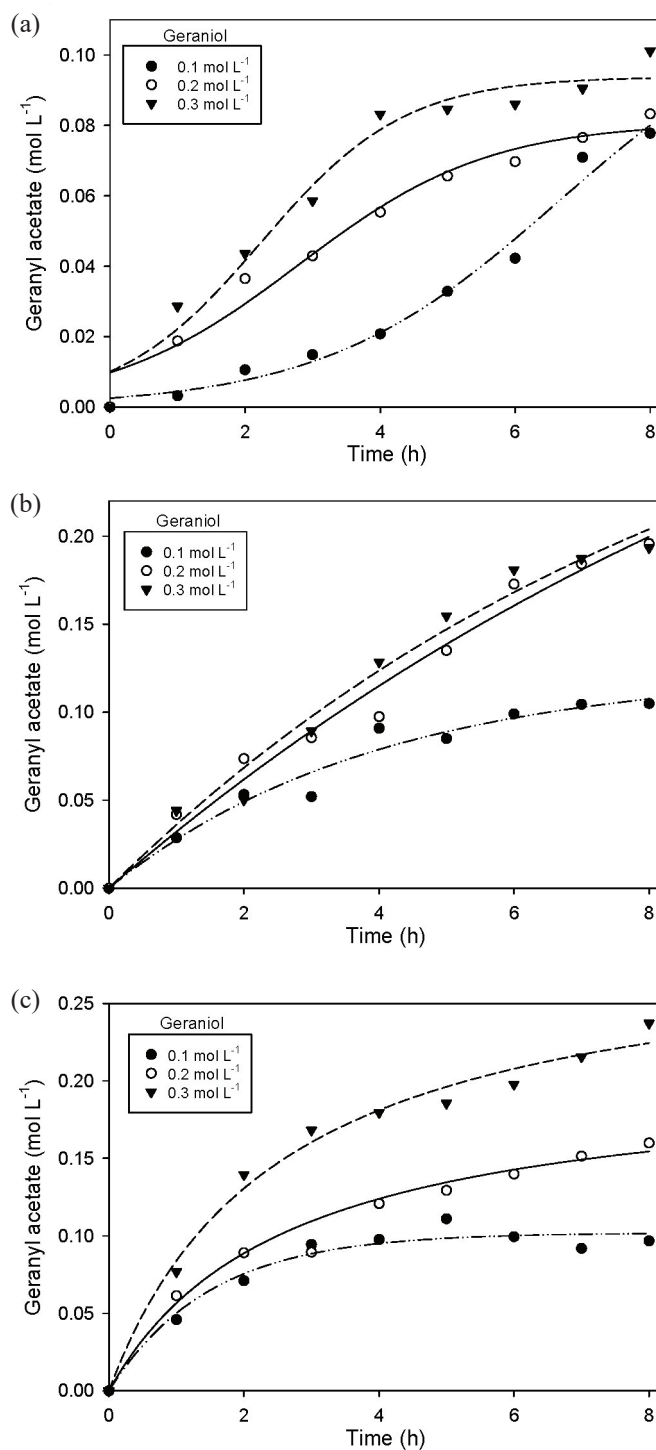


Fig. 9 – (a) Double-reciprocal plot of initial rates ($\text{mol L}^{-1} \text{ h}^{-1}$)⁻¹ against acetic acid (mol L^{-1})⁻¹ concentrations at fixed geraniol concentrations (mol L^{-1}), (b) Double-reciprocal plot of initial rates ($\text{mol L}^{-1} \text{ h}^{-1}$)⁻¹ against geraniol concentrations (mol L^{-1})⁻¹ at fixed acetic acid concentrations (mol L^{-1}). Reaction conditions: 40 mg lipase; 50 °C; 200 rpm (Standard deviation of the triplicate measurements <15 %)

SUPPORTING INFORMATION

Table S1 – FFD of selected esterification variables with geranyl acetate concentration as the response

Run	Acetic acid (mol L ⁻¹)	Geraniol (mol L ⁻¹)	Enzyme (mg)	Temperature (°C)	Geranyl acetate concentration (mol L ⁻¹)
1	0.1	0.3	40	30	0.150
2	0.3	0.1	20	30	0.053
3	0.1	0.3	20	50	0.152
4	0.1	0.1	20	30	0.031
5	0.3	0.3	20	50	0.214
6	0.1	0.3	40	50	0.189
7	0.3	0.3	20	30	0.113
8	0.3	0.3	20	30	0.096
9	0.3	0.3	40	50	0.239
10	0.1	0.1	20	50	0.076
11	0.1	0.3	20	30	0.053
12	0.2	0.2	30	40	0.148
13	0.1	0.3	40	50	0.177
14	0.1	0.3	20	50	0.167
15	0.1	0.3	40	30	0.138
16	0.3	0.1	40	50	0.099
17	0.1	0.1	40	50	0.103
18	0.3	0.3	40	50	0.235
19	0.3	0.3	40	30	0.064
20	0.1	0.1	20	50	0.078
21	0.2	0.2	30	40	0.156
22	0.1	0.1	20	30	0.035
23	0.3	0.1	20	50	0.091
24	0.3	0.3	40	30	0.057
25	0.1	0.3	20	30	0.053
26	0.1	0.1	40	30	0.080
27	0.3	0.1	20	30	0.060
28	0.3	0.1	40	30	0.127
29	0.3	0.3	20	50	0.215
30	0.3	0.1	20	50	0.085
31	0.3	0.1	40	30	0.130
32	0.1	0.1	40	50	0.105
33	0.3	0.1	40	50	0.097
34	0.1	0.1	40	30	0.090

Fig. S1 – Profiles of geranyl acetate production (mol L⁻¹) at various acetic acid concentrations (mol L⁻¹) (a) 0.1 (b) 0.2 (c) 0.3. Reaction conditions: 50 °C; 40 mg lipase; 200 rpm (Standard deviation of the triplicate measurements <15 %)

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STATEMENTS AND DECLARATIONS

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

List of symbols

E_a	– Activation energy, J mol ⁻¹
K_{eq}	– Equilibrium constant, –
K_m	– Michaelis constant, mol L ⁻¹
R	– Gas constant, 8.3145 J mol K ⁻¹
T	– Absolute temperature, K
v	– Initial rate, mol L ⁻¹ h ⁻¹
V_{max}	– Maximum velocity, mol L ⁻¹ h ⁻¹
ΔC	– Change in concentration, mol L ⁻¹
ΔG	– Gibbs free energy change, J mol ⁻¹
ΔH	– Enthalpy change, J mol ⁻¹
Δt	– Change in time, h
ΔS	– Entropy change, J mol ⁻¹ K ⁻¹

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