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Enzyme stability with essential oils and surfactants. Growing towards green disinfectant detergents

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ABSTRACT

This study investigates the stability of α-amylase and protease in disinfectant formulations incorporating essential oils (EOs), anionic surfactants (linear alkylbenzene sulfonate), non-ionic surfactants (alkylpolyglucosides and ethoxylated fatty alcohols), and mixtures of them, analysing the influence of the composition, temperature, and time using shelf-life assays. EOs reduce α-amylase activity showing first-order deactivation kinetics, and surfactants were found not to significantly affect its activity. However, protease activity increases with the presence of EOs, and it was also observed that the use of surfactants does not significantly affect protease activity either. This work represents a step forward in the development of environmentally friendly multifunctional detergents with high disinfectant and detersive capacity.

1. Introduction

Recent health crises have highlighted the need for thorough cleaning and disinfection of surfaces [1], especially in places such as hospitals or food production areas [2], leading to an increase in the production of disinfectants [3]. There is growing interest in developing eco-friendly alternatives to traditional disinfectants [4,5], and essential oils (EOs) are emerging as promising options due to their efficacy against pathogens [6] and their low environmental impact [7,8].

EOs are valued for their medicinal and antimicrobial properties. EOs come from renewable sources and are safer for humans and animals with lower environmental impact than traditional disinfectants [9]. Proper dosage, combined with other cleaning agents, makes EOs a promising green disinfectant alternatives. Thus, recent research reports their broad-spectrum antimicrobial effects against bacteria, fungi, and viruses [10]. Thyme, clove, and cinnamon essential oils enhance mango shelf life postharvest, reducing diseases and fruit firmness loss [11]. Oregano and thyme EOs exhibit antimicrobial properties against *Streptococcus*, *E. coli*, *Salmonella*, and *L. monocytogenes* [12]. Citrus EOs are effective against *E. coli*, *S. aureus*, *Salmonella*, *L. monocytogenes*, fungi, and biofilms [13]. Park et al. (2018) [6] found geranium essential oil combined with benzalkonium chloride more effective than sodium hypochlorite in disinfecting fresh-cut vegetables. Integration of cleaning is essential for effective disinfection, but traditional agents may not work well with surfactants or enzymes, so they need to be used separately. Enzymes improve detersive efficacy but are sensitive to harsh chemicals [14] and can be deactivated by disinfectants [15].

The use of enzymes and EOs as environmentally friendly cleaning and disinfection alternatives has been studied separately [6]. Their combination in a disinfectant detergent could increase their efficacy, despite the problems posed by their instability [16,17]. Combining EOs with surfactants and enzymes would generate detergents with cleaning and disinfectant capabilities, allowing effective dirt removal by surfactants and enzymes, while EOs would act as antimicrobials. This approach, which combines low-temperature cleaning and disinfection, would reduce the use of water and chemicals, saving costs and time. Recent research has explored the combination of enzymes and EOs in disinfectant and cleaning formulations. Moran-Martínez et al. (2018) [18] tested a formulation effective against *P. aeruginosa* biofilm, while Boels et al. (2007) [19] found antifungal activity and effective cleaning with a combination of enzymes, EOs, and a non-ionic surfactant. These works highlight the potential of combining EOs and enzymes in eco-friendly disinfectant and detergent formulations, and further research on their efficacy, safety and application in household and industrial

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products is needed. Further study of enzyme stability in formulations containing EOs and surfactants is essential due to possible enzyme inhibition or deactivation of enzymes.

Amylases and proteases are used to remove starchy and proteinaceous soils at low temperatures [20,21]. Amylase hydrolyses complex carbohydrates into smaller, soluble sugars [22,23], while protease breaks down protein stains and residues. Surfactants affect the stability of amylase and protease. Anionic surfactants, such as LAS, induce enzyme unfolding and deactivation [24], decreasing both amylase and protease activity [25]. However, non-ionic surfactants can preserve protease activity, suggesting a stabilising effect [26]. Mixtures of non-ionic surfactants, such as fatty alcohol ethoxylates and alkylpolyglucosides [27] or alkylpolyglucosides and amine oxides [28] stabilise proteases and amylases. Vicaria et al. (2022) [29] reported that non-ionic surfactants stabilise multi-enzyme systems containing amylase and protease. However, EOs may affect the stability of protease and amylase [30]. Interactions between surfactants, enzymes, and essential oils can modify the properties of detergents. Some studies show how nanoemulsions formed by a non-ionic surfactant and essential oils can enhance antibacterial properties [31,32]. In addition, cinnamon essential oil can inhibit the enzyme polyphenol oxidase [33]. However, these are preliminary studies in food science, and the stability of cleaning enzymes in complex matrices with surfactants and essential oils as potential disinfectants remains to be addressed.

This work investigates the stability and deactivation of amylase and protease with EOs from oregano and citrus extracts, together with different conventional surfactants: anionic surfactant (linear alkylbenzene sulfonate, LAS), non-ionic surfactants (alkylpolyglucosides and ethoxylated fatty alcohols), and mixtures of them. The kinetics of amylase and protease deactivation are also discussed using shelf-life assays. Our overall objective is to deepen the understanding of the interaction between enzymes, surfactants, and essential oils to facilitate the development of green disinfectant detergents that combine enzyme and EOs to effectively clean and disinfect at lower temperatures, while minimising the environmental impact of conventional disinfectants.

2. Materials and methods

2.1. Materials

The activity and stability of two enzymes acting together were analysed: **AMYL** (Termamyl 120®, Sigma-Aldrich, α-amylase from *B. licheniformis*, stable range 40–60 °C, pH = 7–9) and **PROT** (Everlase 16.0L®, Sigma-Aldrich, protease from *Bacillus sp.*, stable range 35–65 °C, pH = 7–9). Both enzymes maintained a constant activity during the experimental period. Mico E-PRO DMC® (Domca S.A.) was a food-grade product used as a disinfectant, composed of a mixture of natural extracts of orange and oregano (EOs), lactic acid, fatty acids, malic acid, and citric acid. This disinfectant showed its effective against *Penicillium* or *Aspergillus*, yeasts, and bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica*, *Clostridium perfringens*, or *E. coli* [13].

The influence of three surfactants, and a mixture of them, on the stability of enzymes with EO was studied. **APG** (Glucopon 600® 600 CS, Sigma-Aldrich) was an alkylpolyglucoside (non-ionic surfactant, humidity 47 %, CMC (25 °C) = 28.9 mg/L, alkyl chain length C_{12}-C_{14}, polymerization degree = 1.5) [34] derived from natural sources with a good environmental profile [35] that provided excellent detergency, wetting, dispersing, and of interfacial tension reduction properties. **EA** (Findet® 1214 N/23, Kao Chemicals, Spain) was one of the most widely used fatty ethoxylated alcohols (non-ionic surfactant, humidity 0.38 %) [36] CMC (25 °C) = 16.7 mg/L, alkyl chain length C_{12} (70 %)- C_{14} (30 %), ethoxylation degree = 9.9 and with low toxicity [37], good detergent effect, foaming power, wetting effect, and emulsifying power. **LAS** (Petrelas®, Petresa, Spain) was a linear alkylbenzene sulfonate (anionic surfactant, humidity 53.2 %), CMC (25 °C) = 25.5 mg/L [38], and alkyl chain length C_{10}-C_{13}. It was very effective in the removal and solubilisation of soils and was one of the most widely used anionic surfactants.

2.2. α-amylase test method

A modification of the Sigma Aldrich Protocol [39] was used to determine α-amylase activity in solutions. 1 mL of potato starch solution (PanReac, AppliChem) (1 % w/v) was kept at 40 °C for 3 min before adding 1 mL of the enzyme solution (all solutions were prepared in Sorenson's buffer). This mixture was stirred and thermostatised at 40 °C for 3 min. 1 mL of DNS solution was added and stirred, keeping the final solution at 100 °C for 15 min (AccuBlockTM Labnet Digital Dry Bath). The tube containing the solution was cooled with an ice bath and kept at room temperature for 10 min. Absorbance measurement was performed at 540 nm (THERMO® Helios Alpha with UNICAM UV–visible spectrophotometer).

DNS solution was prepared mixing 30 mL of Solution 1 (distilled water, 50–70 °C), 20 mL of Solution 2 (1.496 g/L of potassium sodium tartrate tetrahydrate (Sigma-Aldrich) dissolved in 2 M NaOH solution at 40-50° with stirring), and 50 mL of Solution 3 (219 g/L of 3,5-dinitrosalicylic acid (Sigma-Aldrich) dissolved in 100 ml of distilled water at 40–50 °C with stirring). The DNS solution was stored in an amber flask before use.

2.3. Protease test method

A modification of the Sigma Aldrich Protocol [40] was used to determine protease activity. 2.5 mL of aqueous haemoglobin solution (2 % w/v bovine blood solution, Sigma Aldrich) prepared in a 6 M urea solution; finally, the solution was adjusted to pH = 8 and was mixed and stirred with 0.5 mL of the protease solution. This mixture was kept at 40 °C for 20 min. Then, 5 mL of trichloroacetic acid aqueous solution (5 % w/v, AcrosOrganics) was added, and the tube was stirred. The solution was centrifuged (5 min, 8000 rpm, Hettich® Universal 320/320R). To 2.5 mL of the supernatant, 5 mL of NaOH solution (0.5 M) and 1.5 mL of 1 N Folin & Ciocalteu's phenol reagent solution (Sigma Aldrich) were added. This preparation was shaken and held for approximately 30 min before measuring the absorbance at 750 nm by spectrophotometer.

2.4. Preparation of the formulations

Several formulations, containing a high concentration of surfactants, enzymes (α-amylase and protease), and EOs, were prepared to assess the activity and stability of the enzymes using shelf-life assays (see Table 1 for details). The formulations were prepared as follows: initially, the surfactants of each formulation (7.5 g/L, surfactant mass in dry weight) were added to 25 g of Mico E-PRO DMC®. For formulations **F1**, **F2**, and **F3**, the composition was 100 % LAS, 100 % EA, and 100 % APG, respectively. Formulation **F4** comprised a mixture of 50 % LAS, 25 % EA, and 25 % APG. Vicaria et al. (2022) [29] previously documented that these surfactant-enzyme ratios effectively preserved the activity of both amylase and protease. Formulation **F0**, designed as a control, did not contain any surfactant. Subsequently, 0.146 g of $NaH_2PO_4{\cdot}H_2O$ and 3.371 g of $Na_2HPO_4{\cdot}H_2O$ were added to each solution, and water was added up to 200 mL. The pH was adjusted to 8, serving as a pH-optimal buffer for both α-amylase and protease activity. 50 mL of these initial solutions (IS) were employed as blanks for the enzymatic activity assays of each formulation (**F0-F4**). Finally, 0.1125 g of AMYL and 0.1125 g of PROT were added to each remaining 150 mL, yielding formulations **F0** to **F4** (refer to Table 1). The concentration of Mico E-PRO DMC® in each formulation conferred disinfectant capacity to the final product. The formulations included a mixture of the three surfactants (**F4**), a common practice in the formulation of cleaning products due to potential synergistic effects in terms of efficiency [41], skin compatibility [42], or even their collective environmental impact [43].

Table 1
Composition of the formulations.

Chemical compounds	Concentration (g/L)	Composition	Formulations				
			F0	F1	F2	F3	F4
Surfactants concentration (in dry weight)	7.5	LAS (%)	0	100	0	0	50
		EA (%)	0	0	100	0	25
		APG (%)	0	0	0	100	25
Enzymes	1.5	AMYL (%)	50				
		PROT (%)	50				
Disinfectant (essential oils)	125	Mico E-PRO DMC® (%)	100				

2.5. Protocol to measure α-amylase and protease activity in formulations

To evaluate the stability of both enzymes in the formulations described in Table 1, shelf-life tests were carried out considering time and temperature. 50 mL of the initial solutions (IS) (as described in Section 2.4), together with 150 mL of each formulation (**F0** to **F4**), were kept at a constant temperature (35, 40, 45 and 50 °C), and their activities were assessed over a period of 0 to 7 days. Each formulation was renewed whenever the test temperature changed. The stability of α-amylase and protease was determined by evaluating the enzyme activity, as detailed in Sections 2.2 and 2.3, respectively. For these assays, the formulations were diluted with the initial solution (IS) at a ratio of

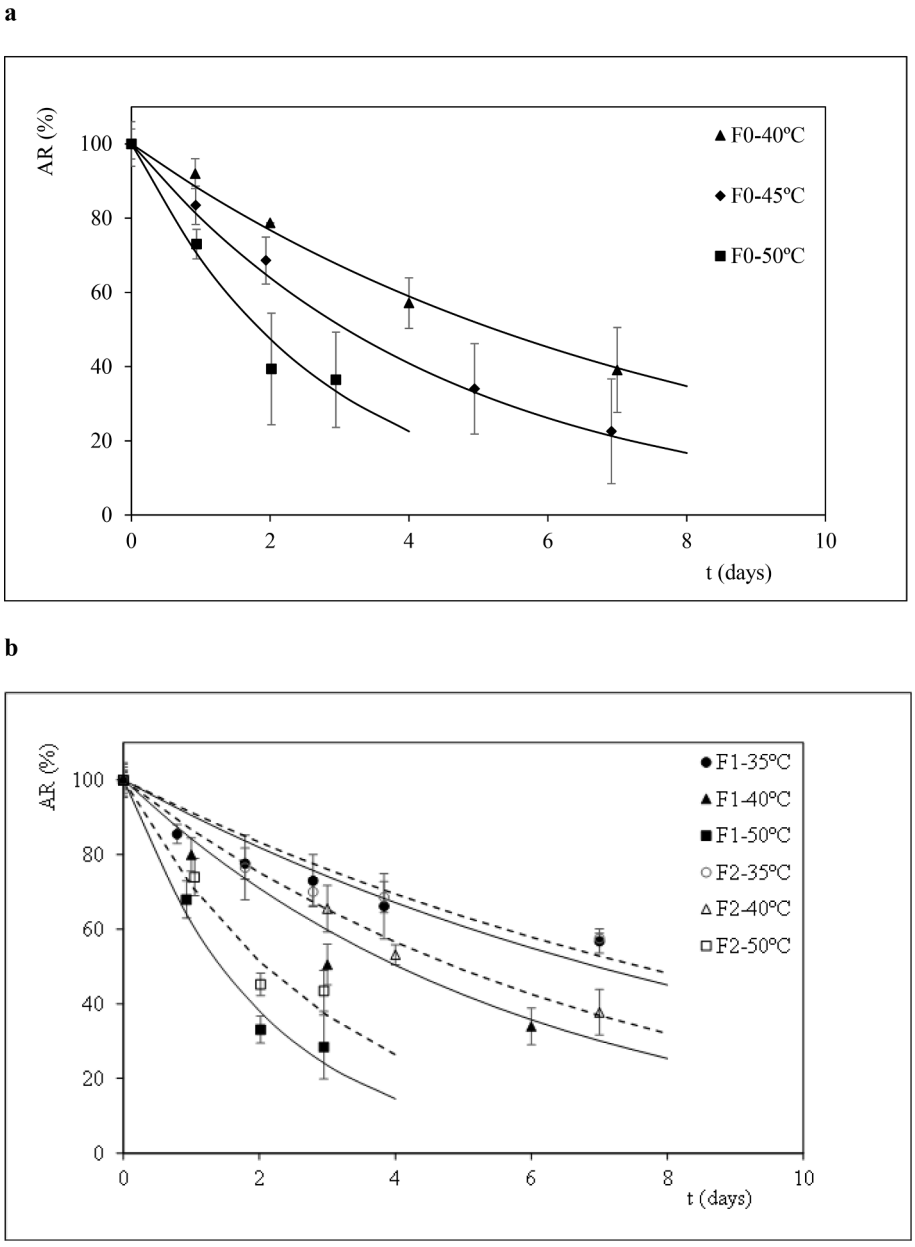

Fig. 1. α-amylase activity as a function of temperature, time, and type of formulation. The lines show the proposed deactivation model, and error bars represent the standard deviation of five assays. a) **F0**, b) **F1** (solid line) and **F2** (dashed line), c) **F3** (solid line) and **F4** (dashed line).

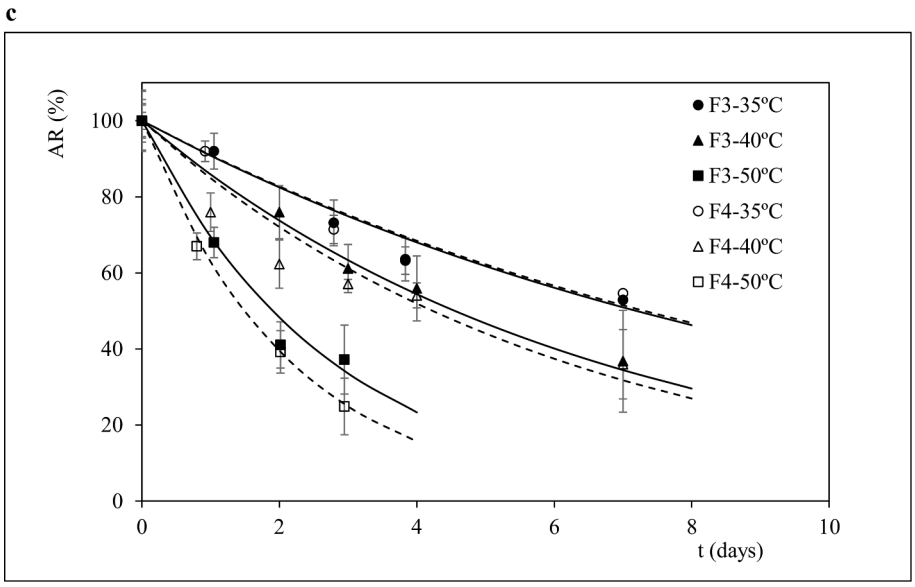

Fig. 1. (*continued*).

1:7.5 for both α-amylase and protease activity assessments. The relative activities of amylase and protease (AR, %) were calculated by dividing the activity at time 't' by the activity at t = 0. All tests were performed in quintuplicate.

3. Results and discussion

In the following sections, we present and discuss the results concerning the stability of α-amylase and protease in the presence of EOs, anionic, and non-ionic surfactants.

3.1. Enzymatic deactivation and essential oils: α-amylase stability in detergent formulations

Several studies explored the enzymatic deactivation of α-amylase using different kinetic models [44]. These models usually indicated deactivation of the enzyme in the presence of starch solutions. However, the complexity of this behaviour increased due to enzyme-surfactant interactions, particularly in the deactivation of α-amylase induced by anionic surfactants [27]. EOs were traditionally incorporated into detergent formulations as fragrances, but there is increasing interest in their use as sanitising agents due to their natural origin and antimicrobial properties [10]. There are no studies that have investigated the activity and stability of α-amylase in the presence of EOs that acted as disinfectant agents.

In this section, we analyse the stability of α-amylase in the presence of EOs and protease (**F0**). Furthermore, anionic surfactant (LAS, **F1**), non-ionic surfactants (fatty ethoxylated alcohol, **F2**; alkylpolyglucoside, **F3**), and a mixture of anionic and non-ionic surfactants (**F4**) were incorporated into the formulations (Table 1). As detailed in section 2.5, α-amylase activity was assessed at different temperatures (35–50 °C) and time (0–7 days) to perform shelf-life assays and evaluate α-amylase deactivation (Fig. 1).

Different kinetic models were tested to model the enzymatic activity of α-amylase as a function of time and temperature, such as first-order kinetic model, kinetic model with partially unfolded and reversibly denatured, kinetic model considering a partially unfolded and reversibly denatured, and kinetic model with irreversible deactivation of the enzyme, among others. None of them modelled the data coherently except the first-order kinetic model. α-amylase activity over time and temperature was fitted to a first-order kinetic model ($AR = \exp(-k_d \cdot t)$), where k_d represents the deactivation kinetic constant. Non-linear regression was used to evaluate the deactivation kinetic constants (k_d) for each formulation at different temperatures, yielding correlation coefficients (r^2) ranging from 0.905 to 0.999, showing a good fit (Table 2). The Arrhenius equation was applied to the kinetic constants (k_d) as a function of temperature ($k_d = k_{d0} \exp(-E_a/RT)$), allowing the pre-exponential value (k_{d0}) and the activation energy (E_a) to be calculated for each formulation (Table 2). The analysis showed strong correlation (r^2 between 0.987 and 0.999). Increasing temperature reduced α-amylase activity in all experiments (Fig. 1a-c, Table 2). As temperature increased, α-amylase exhibited reduced rigidity and a loss of the α-helical structure, causing its denaturation and decreased activity [45]. Fig. 2 illustrates the relationship between the deactivation constant and temperature following the Arrhenius equation. The proposed model provided an acceptable modelling of the results (Fig. 1a-c).

For formulation **F0** (Fig. 1**.a**), α-amylase activity decreased with EOs, time, and temperature in shelf-life tests. Previous work [29] observed a constant amylase activity at 40 °C over time without the addition of EOs. Therefore, it appeared that the addition of EOs was a contributing factor to the decrease in amylase activity in the **F0** formulation. Some studies in the medical and botanical fields demonstrated the ability of EOs to inhibit α-amylases [30,46,47], which could be applicable to the proposed formulations. This study revealed a reduction of activity of up to 39.1 % at 40 °C after 7 days for **F0**, while the activity remained almost

Table 2
Deactivation kinetic constant (k_d) and activation energy (E_a) in α-amylase shelf-life tests with formulations **F0-F4**.

Formulation	T (°C)	k_d (day^{-1})	r^2	k_{d0} (day^{-1})	Ea (Kcal/mol)	r^2
F0	40	0.135	0.997	$4.71 \cdot 10^{+13}$	20.8	0.995
	45	0.215	0.999			
	50	0.381	0.977			
F1	35	0.0930	0.936	$5.66 \cdot 10^{+13}$	20.8	0.987
	40	0.190	0.987			
	50	0.465	0.987			
F2	35	0.0910	0.905	$1.22 \cdot 10^{+11}$	17.1	0.999
	40	0.143	0.995			
	50	0.333	0.923			
F3	35	0.0990	0.923	$2.60 \cdot 10^{+11}$	17.5	0.997
	40	0.146	0.994			
	50	0.369	0.980			
F4	35	0.0970	0.954	$6.66 \cdot 10^{+13}$	20.9	0.999
	40	0.158	0.946			
	50	0.469	0.999			

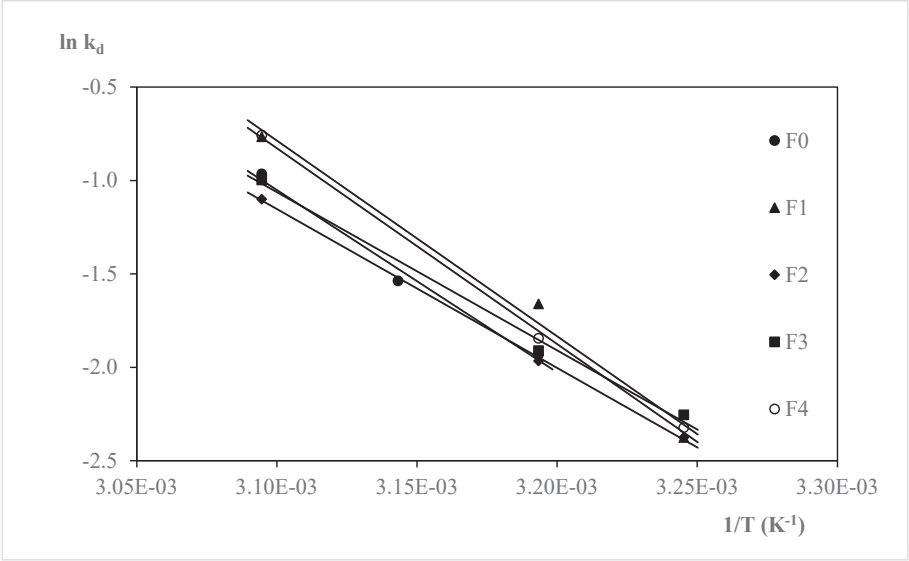

Fig. 2. Deactivation kinetic constant as a function of temperature for formulations **F0**, **F1**, **F2**, **F3** and **F4**.

constant without EOs. Similar relative activity values were observed at 45 and 50 °C after 5 and 3 days, respectively. Vicaria et al. (2002) [29] reported a significant reduction in α-amylase activity after 8 days, up to 30 % at 50 °C. Consequently, the addition of EOs to the formulation containing only protease and amylase without surfactants (**F0**) reduced the amylase activity. Several authors have explored the behaviour of amylases with EOs from citrus and oregano extracts. Koochi et al. (2022) [48] and Oboh et al. (2017) [49] reported the anti-amylase properties of sweet and bitter orange essential oils, which reduce the amylase activity more than 50 % in a short time. Being higher than the values reported in this study even with a lower EOs concentration. Radünz et al. (2021) [50] also studied the inhibition of α-amylase using oregano EO, and the inhibition percentage reached 81.4 %, which is above the percentages found in this study for the mixture of orange and oregano EOs.

Amylase activity also decreased with temperature and time in other formulations containing surfactants (**F1**-**F4**, Fig. 1.**b** and **1.c**). Fitting experimental results to a first-order deactivation model allowed the evaluation of the deactivation energy (E_a) of α-amylase (Table 2). The use of LAS in different proportions (formulations **F1** and **F4**) did not significantly affect the behaviour of α-amylase, as demonstrated by the similar pre-exponential and E_a values obtained for **F0**, **F1**, and **F4** ($4.71{\cdot}10^{+13}$/$5.66{\cdot}10^{+13}$/$6.66{\cdot}10^{+13}$ day^{-1} and 20.8/20.8/20.9 Kcal/mol, respectively). Therefore, it appeared that the addition of LAS did not significantly affect α-amylase activity in the presence of EOs. These results contradicted some works in the scientific literature where the addition of LAS to an enzyme system composed of α-amylase and protease resulted in a marked loss of enzymatic activity [29]. However, Lappas (1996) [51] found that linear alkylbenzene sulphonate stabilised and/or improved amylase performance, allowing a reduction in surfactant/amylase levels while maintaining the same detergency performance. This suggests that the reduction in activity is mainly caused by EOs, since LAS does not to alter amylase stability. The presence of fatty ethoxylated alcohol (EA) or alkylpolyglucoside (APG) in the EOs and enzyme formulations (**F2** and **F3**) also did not significantly affect the behaviour of α-amylase with time and temperature, exhibiting a stability similar to the formulations that included LAS, reaching pre-exponential and E_a values of $1.22{\cdot}10^{+11}$/$2.60{\cdot}10^{+11}$ day^{-1} and 17.1/17.5 Kcal/mol with **F2** (AGE 100 %, $r^2 = 0.999$) and **F3** (APG 100 %, $r^2 = 0.997$), respectively. So, shelf-life assays indicated that α-amylase activity was similar without and with surfactants. Therefore, this work suggests that the surfactants and surfactant mixtures with EOs, partially stabilise α-amylase.

3.2. Enzymatic deactivation and essential oils: Protease stability in detergent formulations

In this section, we explore the stability of protease in the presence of EOs and α-amylase (**F0**), and surfactants (**F1**-**F4**) (Table 1). Protease activity was evaluated at different temperatures (35–50 °C) and time intervals (1–7 days) in shelf-life assays to analyse protease deactivation (Fig. 3) within the surfactant formulations (Table 1). Unfortunately, a kinetic model could not be proposed for this data.

An increase in protease activity was observed over time at 35 °C, reaching a maximum value and then remaining constant. Formulations **F0** (173.1 %), **F3** (171.2 %), and **F2** (170.6 %) exhibited greater activity, with a slightly lower increase observed when LAS was used (159.2 % for **F1** and 146.5 % for **F4**). At 45 °C, the initial protease activity was maintained for all formulations during for the first 4 days, followed by a decrease after 7 days ranging from 65.4 % to 55.9 % for formulations **F0**-**F3**, and a decrease to 27.2 % for **F4**. At 50 °C, protease activity was reduced by up to 80 % after 3 days with formulations **F3** and **F4**. Vicaria et al. (2022) [29] reported a significant decrease in protease stability with time and temperature in formulations containing protease, α-amylase, and surfactants (LAS, EA, and APG). However, this work obtained a lower deactivation, which suggested that surfactants and surfactant mixtures can act as solubilisers of EOs, preventing possible denaturation of the protease and preserving its activity.

Contrary to our findings, Lund et al. (2012) [25] and Zhang and Zhang (2016) [14] reported a reduction in the stability of protease with anionic surfactants. Regarding the influence of non-ionic surfactants on protease, Zhang and Zhang (2016) [14] suggested that alkylpolyglucosides had minimal inhibitory effects on protease activity. They also reported that ethoxylated alcohols had a lesser impact on protease activity, proposing that non-ionic surfactants, due to their inability to ionise into anions, interacted with the protease through hydrogen bonds, exerting a weaker influence on protease conformation and active site compared to ionic surfactants.

Like amylases, only a few papers investigated the interaction between EOs and proteases. For instance, Fekry et al. (2022) [52] found that caraway EO at subinhibitory concentrations significantly inhibited protease activity. EO sulphur compounds from *Allium sativum* reacted with cysteine, inhibiting thiol-containing enzymes (proteases and alcohol dehydrogenase) [53]. Gogoi et al. (2023) [54] also reported that *A. malaccensis* EO acted as a protease inhibitor.

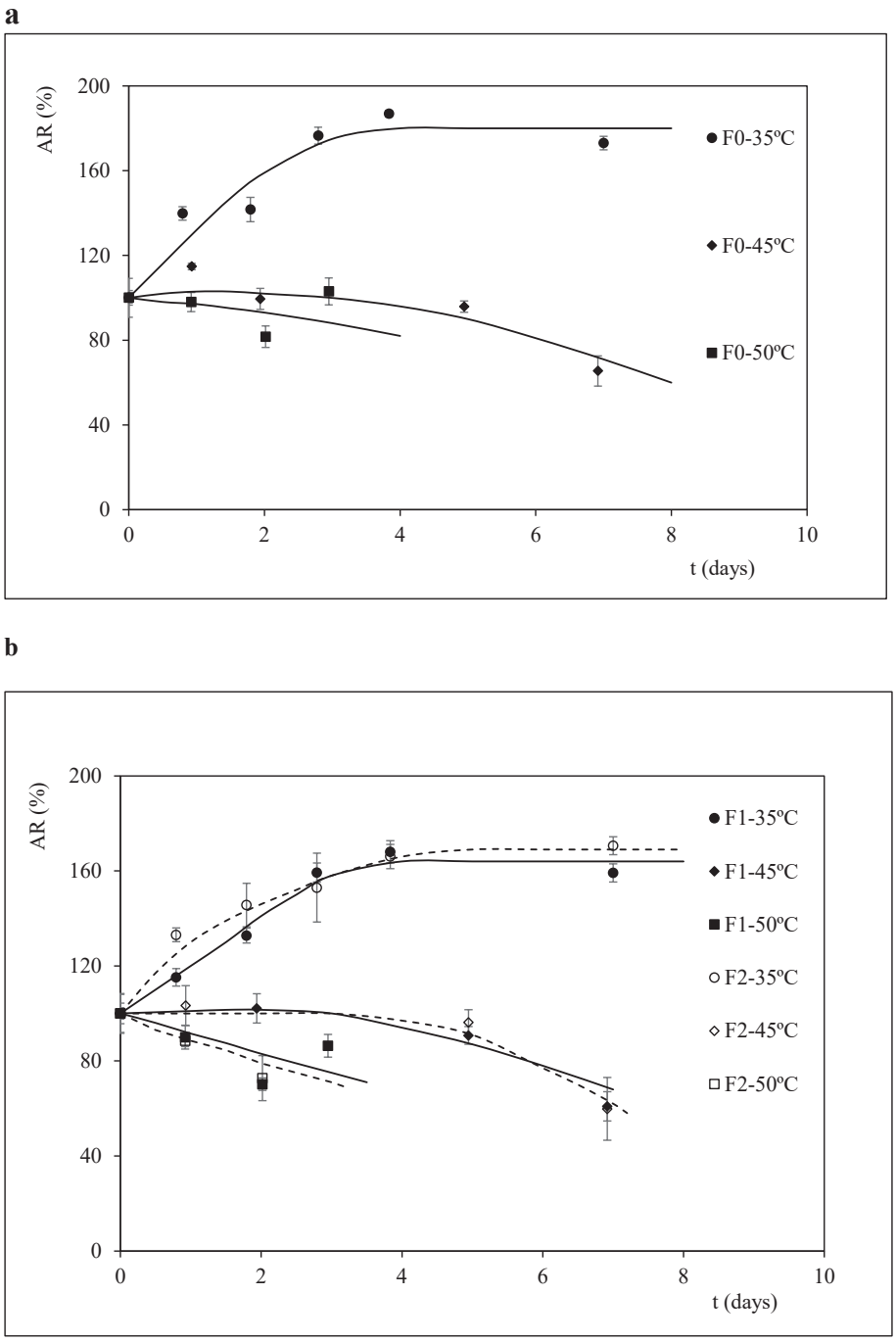

Fig. 3. Protease activity as a function of temperature, time, and type of formulation. The lines provide a visual aid, and error bars indicate the standard deviation of five assays. **(a) F0**, **(b) F1** (solid line) and **F2** (dashed line), (**c) F3** (solid line) and **F4** (dashed line).

4. Conclusions

This work reveals that essential oils (EOs) reduce the stability of α-amylase, but its activity was not significantly affected by the presence of LAS, EA, APG, or a mixture of them. α-amylase showed a decrease in activity over time, with a greater reduction observed at higher temperatures and time. α-amylase showed first-order deactivation kinetics, decreasing its activity with EOs (formulation **F0**). In contrast, the addition of ionic and non-ionic slightly affected α-amylase activity. Conversely, the EOs stabilised the activity of protease and the surfactants prevented the protease destabilisation (with the exception of LAS), maintaining its enzymatic activity. Therefore, EOs reduced α-amylase activity, while surfactants do not significantly affect its stability in the presence of EOs. Conversely, EOs stabilised the protease, and its activity was not affected by the surfactants, except the LAS.

These results allow to advance in the knowledge of the interactions between surfactants, enzymes, and EOs, offering formulations that optimise the stability of enzyme. These formulations can integrate the characteristics of these chemical compounds, providing a multifunctional product with high detergency, reduced temperature in cleaning processes, and disinfectant power.

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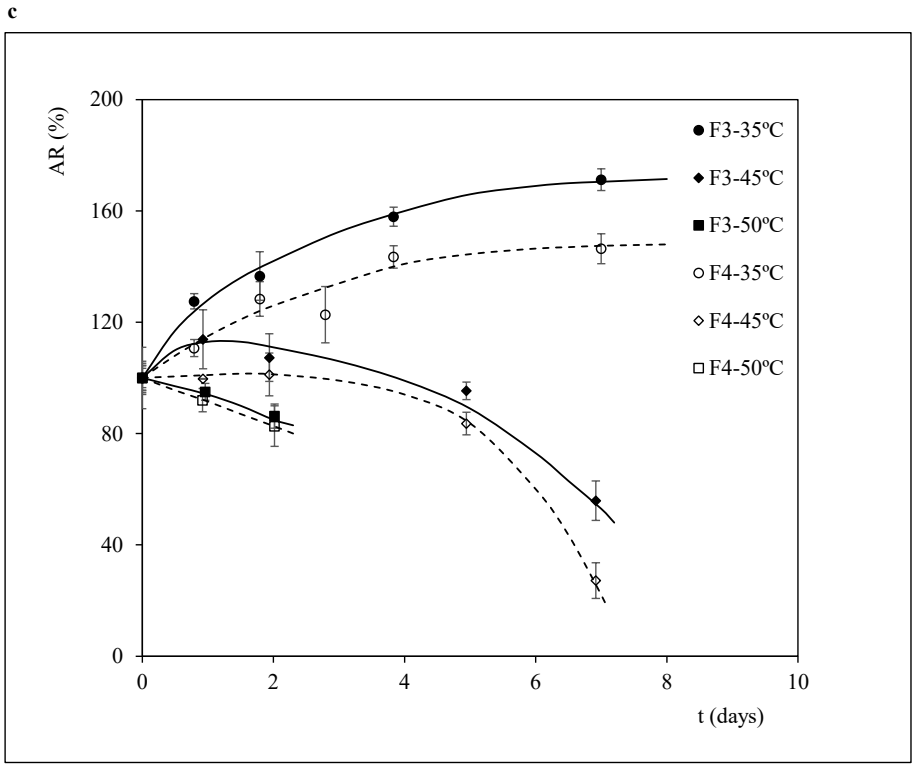

Fig. 3. (*continued*).

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CRediT authorship contribution statement

Eléa Sizaire: Investigation, Conceptualization, Methodology, Writing – original draft. **Sabrina Di Scipio:** Investigation, Conceptualization, Methodology, Writing – original draft. **José María Vicaria:** Investigation, Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Ana Isabel García-López:** Formal analysis, Writing – original draft, Writing – review & editing. **Francisco Ríos:** Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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