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Stability of clavulanic acid under variable pH, ionic strength and temperature conditions. A new kinetic approach

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ABSTRACT

Clavulanic acid (CA) is a β-lactam antibiotic that alone exhibits only weak antibacterial activity, but is a potent inhibitor of β-lactamases enzymes. For this reason it is used as a therapeutic in conjunction with penicillins and cephalosporins. However, it is a well-known fact that it is unstable not only during its production phase, but also during downstream processing. Therefore, the main objective of this study was the evaluation of CA long-term stability under different conditions of pH and temperature, in the presence of variable levels of different salts, so as to suggest the best conditions to perform its simultaneous production and recovery by two-phase polymer/salt liquid–liquid extractive fermentation. To this purpose, the CA stability was investigated at different values of pH (4.0–8.0) and temperature (20–45 °C), and the best conditions were met at a pH 6.0–7.2 and 20 °C. Its stability was also investigated at 30 °C in the presence of NaCl, Na_2SO_4, $CaCl_2$ and $MgSO_4$ at concentrations of 0.1 and 0.5 M in McIlvaine buffer (pH 6.5). All salts led to increased CA instability with respect to the buffer alone, and this effect decreased in following sequence: Na_2SO_4 > $MgSO_4$ > $CaCl_2$ > NaCl. Kinetic and thermodynamic parameters of CA degradation were calculated adopting a new model that took into consideration the equilibrium between the active and a reversibly inactivated form of CA after long-time degradation.

1. Introduction

Antibiotics represent industrially the most important group of secondary metabolites. They are encompassing a broad group of chemical compounds with a wide range of different molecular targets in fighting infectious diseases [1]. The β-lactam family is the biggest and most important class of clinical antibiotics [2]. Their sales are estimated at US$ 15 billion, so they represent the major biotechnology products with worldwide dosage sales at around 65% of the total market of antibiotics [3]. β-Lactams act by inhibiting bacterial cell wall peptidoglycan biosynthesis. They have a common four-membered β-lactam ring attached to different structures [2].

Currently, bacteria have shown resistance to β-lactam antibiotics by producing β-lactamases, which degrade and make them inactive. Despite this problem, these antibiotics are still important and effective in antimicrobial therapy in many situations, leading the pharmaceutical industry to develop strategies to contain the expansion of β-lactamases. One of the strategies to circumvent this problem is the use of inhibitors of β-lactamases that are structurally similar to β-lactam antiobiotics such as the clavulanic acid (CA) [4].

CA is a bicyclic β-lactam (condensed β-lactam and oxazolidine rings) that does not possess either the penicillin or the cephalosporin nucleus. It is a metabolite found in cultures of *Streptomyces clavuligerus* and was isolated during the early 1970s through a program of natural product screening designed to discover potential inhibitors of β-lactamases [5,6]. It has a powerful inhibitory activity against β-lactamases from a variety of Gram-positive and Gram-negative bacteria [7]. Potassium clavulanate is often used in conjunction with amoxycillin and prescribed clinically as co-amoxiclav (Augmentin™) or with ticarcillin as Timentin™ [8].

Like other β-lactam compounds, CA in its crude form is chemically unstable, due to susceptibly of the carbonyl group linked to the β-lactam ring to suffer an acidic (H^+)- or alkaline (OH^-)-catalyzed attack by water molecules [9,10]. Thus, in addition to the use of CA in the pharmaceutical industry, studies covering its stability in a wide range of pH, temperature, types and concentrations of salts are of particular concern. Several research-groups have conducted studies on the stability of CA from various sources in aqueous solutions under different conditions [7,11–14]. To quote only a few examples,

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Haginaka et al. [7], who investigated in different buffers the stability of CA at 35 °C and ionic strength of 0.5, observed, in the pH range 3.1–10.1, that the rate of its degradation followed pseudo-first-order kinetics, was strongly pH-dependent and increased with buffer concentration. Moreover, since the highest CA stability was obtained at 6.39, they concluded that either acidic or alkaline catalysis modulated by the buffering salts played a significant role in the hydrolysis.

Bersanetti et al. [14], investigating the stability of CA from several sources in the temperature range 10–45 °C at pH 6.2 and 7.0, observed that CA from fermentation was less stable than the commercial one employed for medical use and that its degradation followed first-order kinetics. So, they used the Arrhenius equation to relate the rate constant of CA degradation to temperature and to estimate its activation energy. Nevertheless, it is evident in the above studies that, under some conditions, this kinetics diverged from the classical first-order model, suggesting us that a preliminary equilibrium may precede the irreversible degradation.

This study aimed at evaluating the stability of CA at different pH, temperature and salts conditions along the time. The kinetic and thermodynamic parameters of its degradation were then calculated adopting a new model that takes into consideration the equilibrium between the active form and a reversibly inactivated form of CA after long-time degradation.

2. Materials and methods

2.1. Materials

Potassium salt of clavulanic acid (CA) of pharmaceutical grade (54% purity) obtained from Galena (Campinas, São Paulo, Brazil) was used in the stability tests, while the one used to prepare standard solutions was purchased from Sigma–Aldrich (St. Louis, MO, USA) (99% purity). All the other reagents were of analytical grade and used as received. The solutions for the CA stability tests were prepared in McIlvaine buffer by adding disodium phosphate and citric acid at different molar concentrations depending on pH. In particular, the concentrations of disodium phosphate were 3.08, 5.05, 5.30, 17.4 and 7.78 mM and those of citric acid 2.46, 1.47, 1.38, 1.82 and 0.11 mM at pH 4.0, 6.0, 6.5, 7.2 and 8.0, respectively. Deionized water, obtained passing tap water through a Millipore Milli-Q ion-exchange system (Bedford, MA, USA), was always used.

2.2. Experimental procedure

All stability tests were performed in solutions containing an initial CA concentration of 30 mg/L. They were prepared by diluting a concentrated stock solution (300 mg/L) with McIlvaine buffer with the selected pH. The solutions were then homogenized in orbital shaker, model 400110 (Branstead/Thermolyne, Dubuque, IA, USA), at 8 rpm for 5 min at room temperature and kept in a bath, model 521/2DE (New Ethics, Vargem Grande Paulista, SP, Brazil), at the desired temperature. Aliquots of the solutions were withdrawn at different times, and the concentration of undegraded CA was determined as described later. All experiments were performed in triplicate, and the standard deviations and confidence intervals calculated. The limit of significance for the statistical analysis was resulting in a confidence interval of 95%.

A first set of experiments was performed where the CA stability was determined at pH 4.0, 6.0, 6.5, 7.2 and 8.0 and temperatures of 20, 25, 30 and 45 °C.

In a second set of experiments that lasted 6 h, the CA stability was determined at pH 6.5 and 30 °C but varying the ionic strength (μ) by addition of different salts, specifically NaCl, $CaCl_2$, $MgSO_4$ and Na_2SO_4, at concentrations of 0.1 or 0.5 M. To relate the CA degradation to the ionic strength, such a parameter was calculated by the equation [15]:

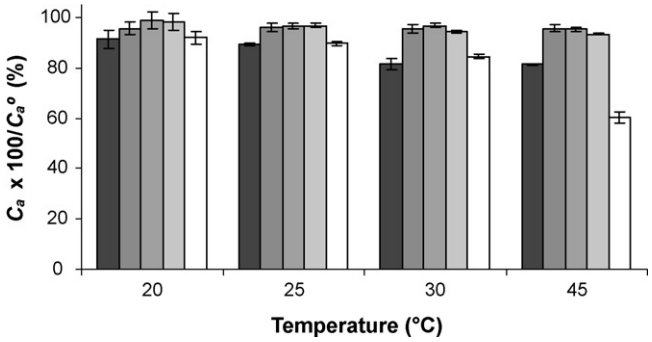

Fig. 1. Stability of CA in McIlvaine buffer at different values of temperature and pH after 3 h. pH: 4.0 (■), 6.0 (■), 6.5 (■), 7.2 (■), 8.0 (□).

$$\mu = 0.5 \times (C_1 Z_1^2 + C_2 Z_2^2 + \cdots + C_n Z_n^2) = 0.5 \times \sum_{i=1}^{i=n} C_i \times Z_i^2 \quad (1)$$

where C_i is the molar concentration of each ion, Z_i is its charge number, and the sum is taken over all ions in the solution. Thus, the ionic strength of a solution is a function of all ions present.

The final part of the study was devoted to the determination of CA degradation kinetics at different temperatures (15, 20, 30 and 45 °C) for 24 h as well as to the estimation of the related thermodynamic parameters making use of simple Arrhenius-type model.

2.3. Analytical methods

According to Bird et al. [16], the CA concentration was determined by measuring the increase in the optical density at 312 nm due to the release of the product [1-(8-hydroxy-6-oxo-4-azooct-2-enol)-imidazole] of the reaction between CA and imidazole. The optical density was determined using a spectrophotometer, model UV-1650PC (Shimadzu, Kyoto, Japan). Standard solutions with different concentrations were used to get a calibration curve able to relate the optical density with the concentration.

3. Results and discussion

3.1. Study of CA stability at different temperatures and pHs

The stability of clavulanic acid (CA) in McIlvaine buffer at $4.0 \leq pH \leq 8.0$ and temperature ranging from 20 to 45 °C was determined after 3 and 6 h, and the related results are presented in Figs. 1 and 2, respectively. In order to make comparison of results possible, the above range of pH was selected on the basis of those

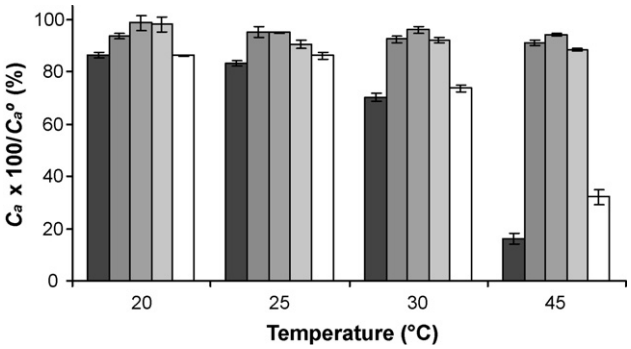

Fig. 2. Stability of CA in McIlvaine buffer at different values of temperature and pH after 6 h. pH: 4.0 (■), 6.0 (■), 6.5 (■), 7.2 (■), 8.0 (□).

reported in the literature as the most adequate for CA stability [7,14]. On the other hand, the range of temperature was selected, besides following this criterion, also taking into account previous finding [17,18] as well as the intention of utilizing these results in future CA extraction by aqueous two-phase systems with temperature-dependent binodal curves.

As expected, the longer the exposition time, the greater the stability loss under all conditions tested. Moreover, this loss of stability was more pronounced at extreme pHs (4 and 8) and the highest temperatures (30 and 45 °C). Although both these parameters exerted a significant influence on CA stability, the former showed a greater effect than the latter; significant stability variations were in fact already observed at different temperatures only at pH values quite different from the optimum stability pH reported for this drug (6.39) [7]. In particular, after 3–6 h at $6.0 \leq pH \leq 7.2$ no more than 5–10% of CA was degraded in the present study even at the highest temperature (45 °C), whereas, either under more acidic (4.0) or more alkaline (8.0) conditions, these loss percentages increased at 45 °C up to 60–83% and 40–67%, respectively.

The observed CA instability could have been the result either of acid- or alkali-catalyzed degradation. Taking into consideration that the alkaline conditions tested in this study (pH 8.0) are closer to the optimum stability range found in this study (pH 6.0–7.2) than the acidic ones (pH 4.0), it appears reasonable to conclude that the alkali-catalyzed degradation of CA was faster and stronger than the acidic one. These results are in good agreement with the literature. Haginaka et al. [7], studying the stability of CA in aqueous solution at 35 °C, ionic strength of 0.5 M in the pH range of 3.1–10.1, found that the rate of its degradation is highly pH-dependent and reached a minimum value at pH 6.39. This value is within the pH range of stability found in this study. Moreover, our results agree with those of Bersanetti et al. [14], who investigated the rate of degradation of this drug from various sources in the temperature range 10–40 °C and demonstrated that at the lowest temperature CA was more stable at pH 6.2 than 7.0, irrespective of its source.

The stronger influence of pH with respect to temperature evidenced in this work on CA stability may be due to the presence in its structure of the carbonyl group of the β-lactam ring, which is susceptible to attack by protons or hydroxide ions and water molecules, according to the conditions [9].

These results as a whole demonstrate that the best conditions for CA stability are in the pH range of 6.0–7.2. Therefore, in view of future adoption of two-aqueous phase extractive fermentation system, the subsequent investigation on the effects of different salts and concentrations has been performed just under these conditions.

3.2. Stability of clavulanic acid in different types of salts and concentrations

The second step of this study dealt with the effects of different salts and concentrations on the CA stability at an intermediate value (6.5) of the above optimum pH range. To this purpose, in order to take into consideration the highest variations as possible, the highest temperature (30 °C) of the optimum range previously selected (20–30 °C) was chosen for comparison. The salts utilized in this part of the study (NaCl, Na_2SO_4, $CaCl_2$ and $MgSO_4$) were selected so as to investigate a large range of ionic strength (0.02–3.02 M) [19] as well as to follow literature suggestions on polymeric [20] and micellar [21] aqueous two-phase systems. The maximum salt concentration investigated in this study (0.5 M) was selected on the basis of previous literature findings on the degradation of different β-lactam compounds [7,22,23]. On the other hand, the lowest concentration (0.1 M) was chosen taking into account that the partition systems to be used in future work only need a low concentration of salts [24].

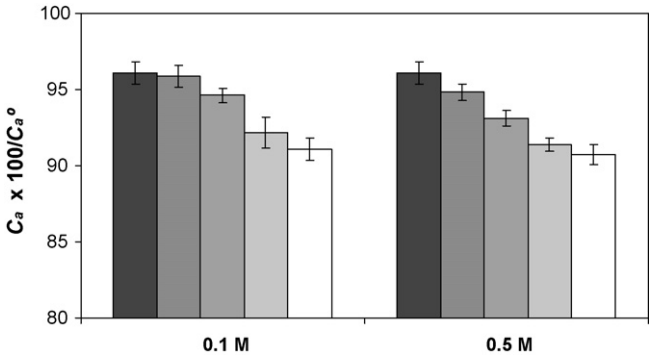

Fig. 3. Stability at 30 °C of CA in McIlvaine buffer (pH 6.5) supplemented with different salts at concentrations of 0.1 and 0.5 M after 6 h. Salt: None (■), NaCl (■), $CaCl_2$ (■), $MgSO_4$ (■), Na_2SO_4 (□).

Fig. 3 illustrates the results of these tests after 6 h using the above salts at concentrations of 0.1 and 0.5 M, which are compared with those obtained in the McIlvaine buffer alone. These results clearly show that the supplementation of different salts either at high (0.5 M) or low (0.1 M) salt level affected the stability of CA in the McIlvaine buffer. In particular, all salts led to an increased CA degradability with respect to the buffer alone (by about 4%), and this effect decreased in following sequence: $Na_2SO_4 > MgSO_4 > CaCl_2 > NaCl$. The error bars with 95% confidence interval suggest that the losses induced by an increase in the concentrations of the first two salts in McIlvaine buffer are practically the same. Thus, it seemed that the degradability of CA could be a function of the ionic strength.

This hypothesis is confirmed in Fig. 4, where the stability of CA is plotted versus the ionic strength for all the salts tested at the two selected levels. Although the percentage of undegraded CA always kept higher than 90% after 6 h, it is evident that this parameter linearly decreased with increasing the ionic strength and was strongly influenced by the kind of salt. But the most interesting finding is that two different straight lines were obtained at the two concentrations under investigation. The straight line obtained at the lower salt level had a slope about 5-fold of that referring to the higher one.

The above results suggest that the CA stability was not a simple linear function of the ionic strength, but, for a given value of this parameter, it is also influenced by other factors. For all the salts, the loss of CA stability consequent to the rise in concentration from 0.1

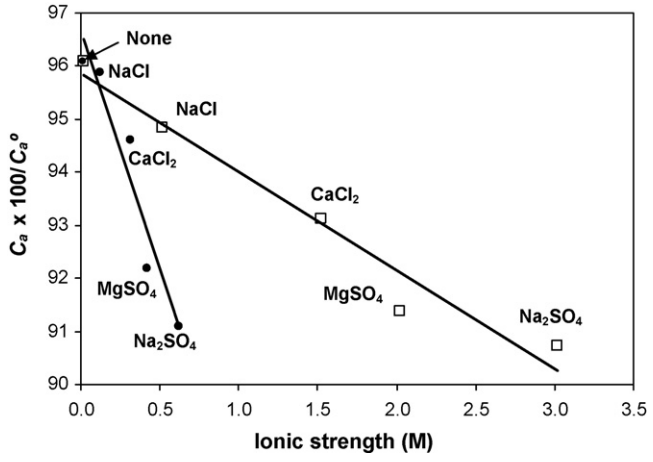

Fig. 4. Influence of the ionic strength on the stability at 30 °C of CA in McIlvaine buffer (pH 6.5) supplemented with different salts at concentrations of 0.1 M (●) and 0.5 M (□) after 6 h.

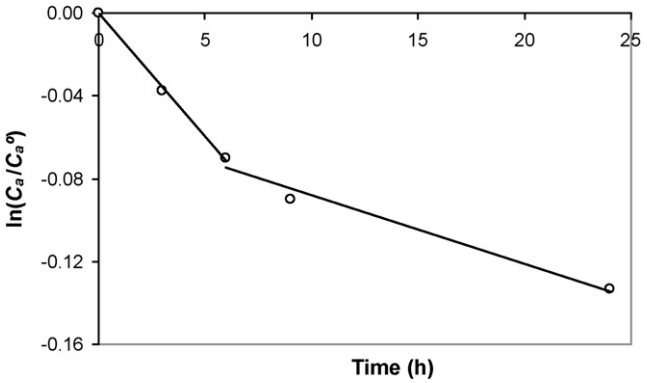

Fig. 5. Semi-log plot of the ratio of the residual to the initial CA concentrations (C_a/C_a^o) versus time. Tests performed in McIlvaine buffer at pH 6.5, 45 °C and 0.1 M NaCl.

to 0.5 M was significant but not so large; the fraction of degraded CA did in fact only increase from about 4 to 5% with NaCl, from 5 to 7% with $CaCl_2$, from 7 to 8% with $MgSO_4$ and from 8 to 9% with Na_2SO_4, respectively. This result partially agrees with those of Laidler [19], who stressed that the ionic strength, for a given salt, has no relevant influence on the rate of hydrolysis reactions.

No comparison with literature data is possible because, as earlier mentioned, most of the work in this field was done keeping constant the ionic strength at a value of 0.5 M [7,22,23]. Nevertheless, an indirect comparison can be made with the results reported by Haginaka et al. [7], who observed a rate of degradation increasing with the concentration of different buffers, for a given value of the ionic strength of the medium (0.5 M).

3.3. Kinetics of clavulanic acid degradation at different temperatures

The final part of the study has been devoted to the kinetics of CA degradation at the best conditions previously determined (pH 6.5 and 0.1 M NaCl) but varying temperature between 15 and 45 °C.

To this purpose, a pseudo-first-order model was previously proposed and successfully employed to describe the degradation of CA [7] and other β-lactam compounds [22] versus time. However, the results of Fig. 5, taken as an example, show that this model is able to effectively describe the kinetics of the system only at the beginning of the exposition at the selected temperature (45 °C), whereas a deceleration of the degradation took place in a second phase. Similar trends were also observed at the other temperatures.

This behavior, which can be recognized also under some of the conditions utilized in the work of Bersanetti et al. [14], is typical of equilibrium reactions or simultaneous opposite reactions [25] rather than of first-order irreversible reaction. Therefore, the kinetic approach proposed by the above authors has been implemented, under the conditions selected for this work, hypothesizing the preliminary occurrence of an equilibrium between the stable CA configuration (C_a) and a configuration more sensitive to degradation (C_a^*), as the likely result of the acid- or alkali-catalyzed complexation of CA, followed by a first-order reaction leading to the formation of the products of CA degradation (C_d). To this respect, it was demonstrated that different degradation products are obtained under acidic or alkaline conditions [7], but this is not relevant for the kinetic purposes of this study. The proposed phenomenological situation can be described by the reaction sequence:

$$C_a \overset{K}{\rightleftarrows} C_a^* \xrightarrow{k_d} C_d \tag{2}$$

where K is the constant of the complexation–decomplexation equilibrium and k_d the pseudo-first-order constant of the irreversible degradation reaction.

Table 1
Values of the constants of CA complexation–decomplexation equilibrium (K) and of the pseudo-first-order constants of the irreversible CA degradation (k_d) estimated at different temperatures.

Temperature (°C)	15	20	30	45
K	0.0248	0.0434	0.0636	0.1284
k_d (h^{-1})	0.0046	0.0068	0.0079	0.0119

At the start of the exposition at a given temperature, when the C_a^* concentration is almost negligible, the former equilibrium can be regarded as totally shifted towards C_a^* formation; therefore, the traditional first-order model approach can be applied successfully, because the degradation of C_a^* to C_d becomes the limiting stage. So, the slopes of the straight lines as that depicted in Fig. 5 up to 6 h give the values of k_d for each temperature, which are listed in Table 1. The value of k_d observed in this study at 30 °C is only 9% higher than that previously reported at the same temperature and pH 6.2 [14].

On the other hand, after this time, the concentration of C_a^* cannot be neglected anymore, and the preliminary equilibrium reaches a condition where C_a^* is shifted to C_a formation; therefore, it becomes the limiting step of the degradation, and the first-order reaction can be neglected. According to this last hypothesis, the results of Fig. 5 after $t > 6$ h, only depend on the equilibrium and can be used to estimate its kinetic and thermodynamic parameters.

After the achievement of the equilibrium we can write:

$$K = \frac{C_a^*}{C_a} \tag{3}$$

where:

$$C_a^* = C_a^o - C_a \tag{4}$$

being C_a^o the concentration of C_a at the start of each run.

Therefore:

$$\frac{C_a}{C_a^o} = \frac{1}{K+1} \tag{5}$$

The slopes of the straight lines as that in Fig. 5 for $t > 6$ h allowed estimating the values of K listed in Table 1 for the different temperatures selected for this study. It is noteworthy that the values of both parameters increased with temperature, which suggested Arrhenius-type behaviors.

Plotting these data in semi-log plots versus the reciprocal absolute temperature, the straight lines illustrated in Fig. 6 were obtained, which describe typical Arrhenius behaviors with good correlation in both cases ($r^2 = 0.972$ for K and 0.939 for

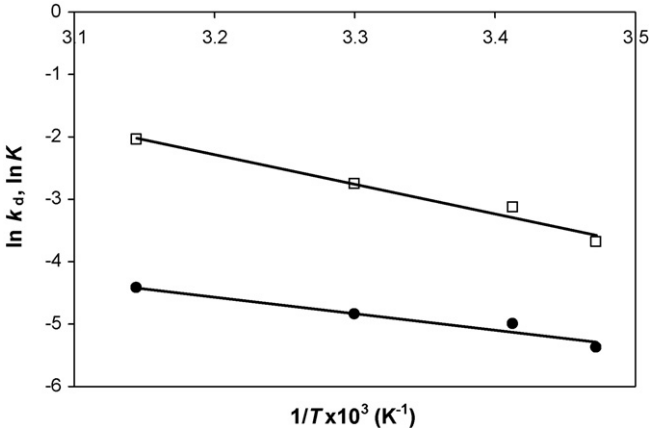

Fig. 6. Semi-log plots of the constant of CA complexation–decomplexation equilibrium (K) and of the pseudo-first-order constant of the irreversible CA degradation (k_d) versus the reciprocal absolute temperature. Tests performed in McIlvaine buffer at pH 6.5 and 0.1 M NaCl.

k_d, respectively). The slopes of these straight lines allowed estimating both the activation energy of the irreversible CA degradation (E_a = 22.0 kJ/mol) and the standard free energy variation of the preliminary complexation–decomplexation equilibrium (ΔG° = 39.3 kJ/mol). Whereas the latter values cannot be compared with literature because of the new approach proposed in this study, the former one is close to the activation energy reported at pH 6.2 for CA obtained by fermentation (24.0 kJ/mol), but lower than that obtained with commercial CA (46.1 kJ/mol) [14]. The higher values of activation energy previously reported (61.5–79.5 kJ/mol) [7] should be associated to the much quicker degradation obtained under different pH conditions because of a higher temperature range (35–65°).

4. Conclusions

In view of future development of a two-phase polymer/salt liquid–liquid extractive fermentation for simultaneous clavulanic acid production and recovery, the long-term stability of this inhibitor of β-lactamases has been investigated under different conditions of pH and temperature, in the presence of variable levels of different salts. The longer the exposition time, the greater its instability under all the conditions tested, especially at extreme values of pH and high temperatures. The effect of pH was stronger than that of temperature, especially under alkaline conditions. The degradation of CA appeared to be also a function of the ionic strength, since it increased in the present of all tested salts compared to the buffer alone, and this effect decreased in following sequence: $Na_2SO_4 > MgSO_4 > CaCl_2 > NaCl$.

Finally, a new kinetic model has been proposed for CA degradation, which hypothesizes equilibrium between the active form and a reversibly inactivated form of CA and subsequent first-order irreversible degradation. The resulting kinetic results have been utilized to estimate the thermodynamic parameters of both hypothesized phenomena.

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