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Kinetic and thermodynamic investigation on clavulanic acid formation and degradation during glycerol fermentation by *Streptomyces* DAUFPE 3060

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ABSTRACT

Clavulanic acid (CA) is a potent inhibitor of β-lactamases, produced by some resistant pathogenic microorganisms, which allows efficient treatment of infectious diseases. The kinetic and thermodynamic parameters of CA production by a new isolate of *Streptomyces* DAUFPE 3060 and its degradation were evaluated. The effect of temperature on the system was investigated in the range 24–40 °C adopting an overall model accounting for (a) the Arrhenius-type formation of CA by fermentation, (b) the hypothetical reversible unfolding of the enzyme limiting the overall metabolism, and (c) the irreversible first-order degradation of CA. The higher rates of CA formation ($k_{CA} = 0.107\ h^{-1}$) and degradation ($k_d = 0.062\ h^{-1}$) were observed at 32 and 40 °C, respectively. The main thermodynamic parameters of the three above-hypothesized events were estimated. In particular, the activation parameters of degradation (activation energy = 39.0 kJ/mol; $\Delta H_d^* = 36.5$ kJ/mol; $\Delta S_d^* = -219.7$ J/(mol K); $\Delta G_d^* = 103.5$ kJ/mol) compare reasonably well with those reported in the literature for similar system without taking into account the other two events.

1. Introduction

β-Lactamase activity in bacteria is responsible for the inactivation of β-lactam antibiotics, and thus for a form of antibiotic resistance in a number of bacteria. Clavulanic acid (CA) inhibits β-lactamase activity in bacteria by inserting its fused bicyclic β-lactam ring into the active site of β-lactamase and rendering it inactive [1]. The reaction of CA with the enzyme is irreversible as the products of the reaction keep attached to the enzyme [2]. Its application in conjunction with penicillin has proven to be successful in the solution of the problem of bacterial resistance to β-lactam antibiotics and makes CA very important both clinically and economically. However, it is known that CA in its crude form is chemically unstable like other β-lactam compounds; therefore, it is degraded during the bacterial cultivation [3,4]. Loss of product during the cultivation process through decomposition is a significant problem for the pharmaceutical industry. Establishment of the destination of the decomposed products of the antibiotic may provide an indication of ways to prevent the losses and thus to reduce costs [2].

To date, only a few studies have been performed on CA degradation, with particular concern to the effect of temperature, which is the main factor modulating this phenomenon [5,6]. Some authors investigated the stability of CA in buffered aqueous solutions at various pHs and temperatures [4–6]; however, only one systematic study dealt with the thermodynamic parameters of CA thermostability [4].

On the contrary, no information is available on the simultaneous formation and degradation kinetics during CA fermentation, with particular concern to the mechanism of degradation and the related kinetic and thermodynamic parameters, which is the objective of this study. To this purpose a new kinetic model is proposed here that takes into account (a) the Arrhenius-type formation of CA, (b) the reversible unfolding of the enzyme limiting the metabolism, and (c) the irreversible first-order degradation of CA.

2. Theory

2.1. Phenomenological model

The model proposed in this study to describe the influence of temperature on the kinetics of clavulanic acid (CA) formation and degradation is based on the hypothesis that the formation of CA from glycerol (G) by *Streptomyces* DAUFPE 3060 fermentation may

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be modulated by the activity of one enzyme limiting the overall metabolism, which can be subject to a reversible unfolding:

$$E \underset{k_2}{\overset{k_1}{\leftrightarrow}} EI \quad (1)$$

where E and EI are the active and the unfolded, inactive form of the enzyme, respectively, and k_1 and k_2 (h^{-1}) are the kinetic constants of direct and reverse reactions of this equilibrium, respectively.

Such a general hypothesis was previously proposed with success for completely different biosystems [7–9]. Because the rate of a reaction catalyzed by one enzyme is modulated by both concentrations of the active enzyme and substrate, the rate of CA formation directly depends on this equilibrium. As stressed in previous studies made on different biological systems [7,8,10–13], the so-called "thermodynamic approach" proposes that the rate increase with temperature of every biosystem would be contrasted by the "reversible unfolding" described by Eq. (1), which is responsible for a net decrease of activity. The above kinetic constants can be estimated by semi-log plots of $\ln(CA/CA_o)$ versus time, under conditions where direct and reverse reactions do not interfere or interfere as less as possible each other, being CA and CA_o the CA concentrations after a certain time and at the beginning, respectively.

Once CA has been formed by fermentation, it is subject to irreversible degradation to a generic product (DP) as a consequence of CA instability, according to the temperature-dependent first-order reaction:

$$CA \xrightarrow{k_d} DP \quad (2)$$

where k_d (h^{-1}) is the rate constant of CA degradation.

2.2. Kinetic modeling of CA formation

The above phenomenological model for CA formation can be kinetically described by a combination of the Arrhenius and Eyring equations (see Section 3.7), so as to estimate all the thermodynamic parameters of the system.

At temperature lower than the optimum ($T < T_{opt}$), it is reasonable to assume that the unfolding of the limiting enzyme is negligible; therefore, the rate constant of CA formation (k_{CA}) coincides with k_1, whose dependence on temperature can be described by the typical Arrhenius relationship [14]:

$$k_1 = Ae^{-E^*/RT} \quad (3)$$

where E^* is the activation energy of the enzyme unfolding (kJ/mol), A the frequency factor or Arrhenius constant (h^{-1}), R the ideal gas constant (8.3145 J/(mol K)) and T is the absolute temperature (K).

The inactivation equilibrium responsible for the transformation of the active form of the limiting enzyme (E) to its unfolded form becomes predominant at $T > T_{opt}$, and can be described by the equation:

$$E = \frac{E_o}{1 + K_i} \quad (4)$$

where E_o is the initial concentration of the enzyme and K_i is the unfolding equilibrium constant.

Applying the Gibb's equation to this equilibrium, we obtain

$$K_i = Be^{-\Delta H_i^\circ/RT} \quad (5)$$

where ΔH_i° is the standard enthalpy variation of the unfolding equilibrium and

$$B = e^{\Delta S_i^\circ/R} \quad (6)$$

an additional pre-exponential factor depending on the standard entropy variation of the same equilibrium (ΔS_i°).

Combining Eqs. (3) and (4)–(6) with the Michaelis–Menten equation adapted to enzyme unfolding, we obtain

$$k_1 = \frac{Ae^{-E^*/RT}}{1 + Be^{-\Delta H_i^\circ/RT}} \quad (7)$$

At low temperature, the second addend at the denominator of this equation becomes negligible, the denominator approaches unit, and Eq. (7) simplifies to the Arrhenius equation. On the contrary, at high temperatures, the contribution of the inactivated form of the enzyme becomes predominant owing to K_i increase, so the unit becomes negligible with respect to the second addend, and Eq. (7) simplifies to:

$$k_1 = \frac{A}{B}e^{(\Delta H_i^\circ - E^*)/RT} \quad (8)$$

2.3. Kinetic modeling of CA degradation

In contrast to CA formation, the kinetics of degradation of CA and similar compounds has widely been explored by several authors [2,5,6,15,16]. Parameters of the kinetic model for CA degradation owing to the irreversible reaction (Eq. (2)), include the reaction rate constant (k_d) and the related activation energy (E^*_d). According to other authors [4–6,16–18], the rate of CA degradation can be modeled as a first-order equation:

$$\frac{d\,CA}{dt} = -k_d CA \quad (9)$$

where t is the time (h).

On the hypothesis that, after maximum CA production, the progressive decrease of its concentration is the result of its degradation without any further formation, a linear regression analysis can be performed on the integrated semi-log form of this equation, utilizing only the experimental data collected after this occurrence:

$$\ln\frac{CA}{CA_o} = -k_d t \quad (10)$$

Likewise the direct reaction of the unfolding equilibrium described by Eq. (1), the temperature dependence of k_d can be modeled through the Arrhenius equation as follows:

$$k_d = A_d e^{-E^*/RT} \quad (11)$$

3. Materials and methods

3.1. Reagents

Potassium clavulanate from *Streptomyces clavuligerus* was used for the calibration curve needed to determinate the concentration of clavulanic acid (CA). The imidazole used in the CA determination was provided by Sigma–Aldrich (São Paulo, Brazil). The salts, glycerol, bacto-peptone, malt extract, and yeast extract used to prepare the media were analytical grade reagents.

3.2. Microorganism

The *Streptomyces* spp. DAUFPE 3060 strain, which is still under characterization, was kindly provided by the Microorganism Collection of the Department of Antibiotics of the Federal University of Pernambuco, Recife-PE, Brazil. The method suggested by Lawrence [19] was used to isolate this strain directly from soil samples. Spores obtained in Petri plates from colonies grown on solid culture medium were collected and grown in liquid culture medium for 96 h. After this cultivation, biomass was determined by dry weight (around 6–7 g/L), lyophilized and stored in cryotubes (glycerol 10%, v/v) at −70 °C and used throughout the present work.

3.3. Culture media

The seed medium had the following composition (in g/L distilled water): glycerol, 15; bacto-peptone, 10; malt extract, 10; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.75; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001. After adjusting the pH at 6.8 with NaOH 5.0 M, the medium was autoclaved at 121 °C for 15 min.

To prepare the inoculum, we used the medium proposed by Maranesi et al. [20] that had the following composition (in g/L distilled water): glycerol, 10; soybean