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Overproduction of clavulanic acid by extractive fermentation

Cecília Ladeira Lopes Costa *, Alberto Colli Badino

Chemical Engineering Graduate Program, Federal University of São Carlos, Cx. Postal 676, São Carlos, SP, Brazil

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ABSTRACT

Background: Clavulanic acid is an important beta-lactamase inhibitor produced as a secondary metabolite by the actinomycete *Streptomyces clavuligerus*. Clavulanic acid is chemically unstable; therefore, it is degraded during bacterial cultivation. In this work, the adsorbents clinoptilolite, activated carbon, calcined hydrotalcite, and Amberlite IRA 400 anionic exchange resin were studied in terms of their ability to adsorb clavulanic acid during extractive fermentation, in order to prevent product degradation and avoid product concentrations reaching inhibitory levels. Adsorption assays were used to investigate the effect of pH, and the decrease in the clavulanic acid concentration in the culture broth was measured for each adsorbent.
Results: IRA 400 was found to be most effective, with 78% adsorption of clavulanic acid. The maximum production of clavulanic acid in Erlenmeyer flask cultures increased 86% in terms of mass of CA, and 248% in cumulative CA concentration, with the use of Amberlite IRA 400 as adsorbent in extractive fermentation, compared to control fermentation performed without product removal.
Conclusions: The results indicated that extractive fermentation using a solid phase could be an important way of enhancing clavulanic acid titers. It was also possible to show that clavulanic acid acts as an inhibitor of its own synthesis.

1. Introduction

The continued clinical use of beta-lactam antibiotics has caused the emergence of a broad spectrum of bacterial resistance, one of the most important of which is related to the production of beta-lactamases. As a result, beta-lactamase inhibitors have emerged as a means of overcoming the problem of bacterial resistance to beta-lactam antibiotics. Clavulanic acid (CA), an inhibitor that has been successfully employed in clinical practice, is a secondary metabolite produced by the actinomycete *Streptomyces clavuligerus* [1], and is used in combination with beta-lactam antibiotics that are sensitive to attack by beta-lactamase enzymes. It acts by specifically inhibiting the active centers of these enzymes, hence avoiding loss of the beta-lactam antibiotic and restoring the antimicrobial action.

The clinical importance of CA has resulted in extensive research with a focus on increasing CA production using a variety of process control strategies, considering aspects such as temperature [2], agitation speed [3], fed-batch operation [4,5], and new microbial strains [6]. However, CA fermentation processes still present problems such as low CA concentrations [4,5,7,8], and it is essential to obtain higher production titers of this valuable product using more effective fermentation methods. In recent studies employing wild-type *S. clavuligerus*, a maximum CA concentration (C_{CAm}) of about 1.6 g/L was reported by Teodoro et al. [9] for fed-batch cultivations using bench-scale bioreactors with glycerol and ornithine feeding, and by Costa and Badino [2] for low temperature batch cultivations with glycerol pulses. Furthermore, Roubos et al. [10] showed that a high CA concentration (1.3 g/L) in the fermentation broth decreased the microbial growth rate. These limitations could be indicative of an inhibition effect of CA on microbial growth, as well as feedback inhibition of CA biosynthesis by CA itself.

Extractive fermentation is a technique that removes the product during fermentation. This process has been successfully employed in biotechnology as an effective approach for reducing feedback inhibition and increasing the product titer [11,12,13,14,15,16]. The advantages of extractive fermentation also include diminution of toxic effects of the product on microbial growth [17], and extended fermentation times [18]. Moreover, continuous product removal during the entire fermentation minimizes the degradation effects caused by temperature and pH [19], by reducing its exposure to such damaging conditions. This is particularly beneficial for labile products including CA.

Adsorbent resins have been successfully employed to improve the production of valuable biomolecules in extractive fermentations performed using different microorganisms. For example, Singh et al. [20] achieved a 100-fold increase in the production titer of

* Corresponding author.
E-mail address: cecilia.ladeira@gmail.com (C.L.L. Costa).
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cercosporamide, a self-toxic secondary metabolite, in fermentations carried out in culture media containing Diaion HP 20 resin. Lee et al. [18] demonstrated an improvement in teicoplanin production by reducing fungal self-toxicity. Jia et al. [21] obtained a 1.25-fold increase in pristinamycin production in batch fermentation by *Streptomyces pristinaespiralis* after the addition of JD-1 polymeric resin. Since the CA molecule is negatively charged at neutral pH, anionic exchangers such as Amberlite IRA 400 resin can be used in downstream CA processes [22,23]. These materials could be used as potential tools for extraction of CA from the culture broth in extractive fermentation processes. Zeolites and activated carbon have also been proposed for the immobilization of biomolecules including amino acids, antibiotics, and enzymes [24,25,26]. Their high surface areas, porous structures, and ion-exchange properties [24] enable these substances to be used as adsorbents for a diverse range of inorganic and organic anions. Similarly, hydrotalcites are a class of materials that can be described as positively charged planar layers consisting of divalent and trivalent cations that must be compensated by anion intercalation [27]. Therefore, hydrotalcites have applications that include adsorption of anionic species, such as CA. Despite the fact that these materials are inexpensive alternatives for the removal of CA from solution, only a few experimental studies have investigated their potential for the extraction of CA [28,29,30].

The goals of this work were to produce CA using *S. clavuligerus* in an extractive fermentation system with a solid phase, comparing the results obtained for batch fermentations performed with and without glycerol pulses, and to evaluate the effects of product removal on cumulative CA production in an extractive fermentation system. The materials investigated for extraction of CA from the fermentation broth supernatant were alternative adsorbents (calcined hydrotalcite, clinoptilolite, and activated carbon) and a conventional resin (Amberlite IRA 400). Significant improvement of CA production by *S. clavuligerus* ATCC 27064 under extractive fermentation was achieved by inclusion of IRA 400 resin in order to reduce feedback inhibition effects.

2. Materials and methods

2.1. Treatment of adsorbents and adsorption kinetics

Four different adsorbents were used for the adsorption of CA present in aqueous solution and in cell-free fermentation broth. Activated carbon (Synth) and clinoptilolite (kindly provided by Celta Brasil) were repeatedly washed with deionized water to remove impurities, and then dried at 70°C for 48 h. Hydrotalcite (Mg–Al–CO_3, Sasol GmbH, Germany) was calcined at 500°C for 4 h. Anionic exchange resin (Amberlite IRA 400, Sigma-Aldrich) was pretreated with 10% (w/v) NaOH, then washed several times with deionized water and regenerated with 10% (w/v) NaCl.

The kinetics of adsorption of CA was determined using batch experiments. The supernatant of fermentation broth containing 500 $mg \cdot L^{-1}$ of CA was separated by centrifuging at 3725 g for 15 min at 4°C, and was then used in the adsorption assays. The initial pH of the solution was adjusted to 7.0.

The kinetics experiments were performed in a stirred jacketed glass reactor containing 100 mL of cell-free fermentation broth and 15 g of adsorbent. The temperature was controlled at 25°C. Samples (500 μL) were periodically withdrawn and centrifuged at 3725 g and 4°C for 2 min, and CA concentrations were determined as described by Bird et al. [31].

These experiments were used to identify the most suitable adsorbent to employ in further tests. The criteria used were the shortest time to reach equilibrium in the adsorption kinetics experiments, as well as the pH conditions, since the CA molecule is most stable at pH near 6.2 [19]. All the adsorption kinetics assays were performed in triplicate.

The mass of CA adsorbed was obtained by mass balance in the reactor, using:

$$m_{CA-ads} = (C_{CA-b} - C_{CA-a}) \cdot V \quad \text{[Equation 1]}$$

where $m_{CA\text{-}ads}$: mass of CA adsorbed (mg); $C_{CA\text{-}b}$: CA concentration before extraction (mg/L); $C_{CA\text{-}a}$: CA concentration after extraction (mg/L); and V: volume of broth (L).

2.2. Adsorption isotherms

CA adsorption isotherms were only determined using the Amberlite IRA 400 resin, since this material provided the best results in terms of the adsorption kinetics and pH. Aqueous solutions of commercial potassium clavulanate (Clavulin®) and cell-free fermentation broth were employed, with CA concentrations of 100–5000 and 100–1000 mg/L, respectively. The experiments were conducted in shaker flasks at 150 rpm, 25°C, and initial pH of 7.0, with 0.2 g (wet mass) of IRA 400 added to 4.8 mL of the solution containing CA. After attainment of equilibrium, the CA concentration in the mixture was determined. All the adsorption isotherm assays were performed in triplicate.

The quantity of CA adsorbed per unit wet mass of adsorbent was calculated by mass balance as the difference between the initial (C_{CA0}) and final (C_{CAe}) concentrations of CA in solution, divided by the mass of the adsorbent:

$$q_e = (C_{CA0} - C_{CAe}) \cdot \frac{V_{sol}}{m_{ads}} \quad \text{[Equation 2]}$$

where q_e: amount of CA adsorbed at equilibrium (mg/g); C_{CA0}: initial CA concentration (mg/L); C_{CAe}: CA concentration at equilibrium (mg/L); V_{sol}: volume of solution (L); and m_{ads}: wet mass of adsorbent (g).

The experimental q_e and C_{CAe} data were treated using the Langmuir model and the values of the parameters q_m and k were determined by non-linear regression:

$$q_e = q_m \cdot \frac{C_{CAe}}{k \cdot + C_{CAe}} \quad \text{[Equation 3]}$$

where q_m: maximum CA adsorption capacity of the resin (mg/g); and k: Langmuir coefficient (mg/L).

2.3. Desorption of CA

The desorption characteristics were only determined for the best adsorbent (Amberlite IRA 400), selected based on its capacity to adsorb CA. The resin with adsorbed CA was eluted with 100 mL of NaCl (10%, w/v) at 25°C for 2 h. Aliquots (500 μL) were periodically withdrawn for determination of the CA concentration.

The desorption kinetics results were used to calculate the mass of CA desorbed ($m_{CA\text{-}des}$, in mg), using:

$$m_{CA-des} = \frac{p_{CA-des}\ (\%)}{100} \cdot m_{CA-ads} \quad \text{[Equation 4]}$$

where $p_{CA\text{-}des}$: amount of CA desorbed from the resin (%).

2.4. Calculation of cumulative CA mass, and rates of production and degradation

The effect of product removal on the final production of CA by the cells was evaluated by comparing the results in terms of cumulative CA mass ($m_{CA\text{-}c}$). The calculations considered the mass of CA extracted from the broth and recovered from the resin after desorption in each extraction step. The mass of CA removed from the broth was given by