Electronic Journal of Biotechnology 18 (2015) 154–160

HOSTED BY

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

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

ARTICLE INFO

Article history:
Received 30 November 2014
Accepted 22 January 2015
Available online 31 March 2015

Keywords:
Adsorption
Clavulanic acid
Extractive fermentation
Integrated processing
Product inhibition

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).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.03.001

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

the difference between the mass of CA in the fermentation broth, before and immediately after the extraction step.

The cumulative CA mass in the culture broth was calculated using:

$$m_{CA-ci} = C_{CAi} \cdot V_{bi} + \sum_{1}^{i} m_{CA-desi} \cdot \quad \text{[Equation 5]}$$

The cumulative CA concentration was calculated using:

$$C_{CA-ci} = \frac{m_{CA-ci}}{V_{ai}} \quad \text{[Equation 6]}$$

where $m_{CA\text{-}ci}$: cumulative CA mass at time i (mg); $C_{CA\text{-}ci}$: cumulative CA concentration at time i (mg/L); V_{bi}: broth volume before extraction at time i (L); $\sum_{1}^{i} m_{CA\ -\ desi}$: sum of the mass of desorbed CA up to time i (mg); and V_{ai}: broth volume after extraction at time i (L).

In previous work, Costa and Badino [2] demonstrated that the average value of the degradation constant (k_{dCA}) at 20°C was 0.00173 ± 0.00046 h^{-1}. Substituting the values of k_{dCA} and C_{CA} in [Equation 7] enabled calculation of the profile of the CA degradation rate (r_{dCA}, mg/L·h). From the experimental CA accumulation data (dC_{CA}/dt, mg/L·h) and r_{dCA}, values of the CA production rate during the fermentation (r_{CA}, mg/L·h) could be obtained from the mass balance for CA [Equation 8]. The dC_{CA}/dt values were obtained as the simple derivatives of C_{CA} according to time.

$$r_{dCA} = k_{dCA}.C_{CA} \quad \text{[Equation 7]}$$

$$r_{CA} = \frac{dC_{CA}}{dt} + r_{dCA} \quad \text{[Equation 8]}$$

It was therefore possible to obtain the profiles of r_{dCA} and r_{CA} ([Equation 7] and [Equation 8]) during the course of the conventional and extractive fermentations, and evaluate the individual effects of CA extraction on r_{dCA} and r_{CA}.

2.5. Microorganism and culture media

The microorganism used in this work was *S. clavuligerus* ATCC 27064, stored as vegetative cells (5 g/L dry weight) at -70°C in 4 mL cryotubes containing glycerol (10%, v/v).

The seed medium proposed by Rosa et al. [32] had the following composition (g/L): glycerol (15.0); bactopeptone (10.0); malt extract (1.0); K_2HPO_4 (0.8); $MgSO_4 \cdot 7H_2O$ (0.75); $MnCl_2 \cdot 4H_2O$ (0.0001); $FeSO_4 \cdot 7H_2O$ (0.001); $ZnSO_4 \cdot 7H_2O$ (0.001); and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (21.0), at pH 6.8.

The composition of the inoculum medium (Teodoro et al. [9]) was (g/L): glycerol (15.0); soybean protein isolate (25.0); K_2HPO_4 (0.8); $MgSO_4 \cdot 7H_2O$ (0.75); $MnCl_2 \cdot 4H_2O$ (0.0001); $FeSO_4 \cdot 7H_2O$ (0.001); $ZnSO_4 \cdot 7H_2O$ (0.001); and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (21.0), at pH 6.8. The production media had the same composition as the inoculum medium, but with varying glycerol concentrations (B15 and EF15: 15.0 g/L; B30 and EF30: 30.0 g/L; EF15 + 15: 15.0 plus 15.0 g/L). All media were autoclaved at 121°C for 15 min.

2.6. Conventional and extractive batch fermentations

Vegetative cell suspensions were transferred from the cryotubes to 500 mL Erlenmeyer flasks containing 50 mL of seed medium and incubated in a rotary shaker at 250 rpm for 24 h at 30°C. Erlenmeyer flasks (500 mL) containing 45 mL of the inoculum medium were inoculated with 5 mL of cultivated seed broth and incubated at 250 rpm for 24 h at 30°C. For the production stage of the fermentation, the inoculum suspension was transferred to a 3 L flask containing the production medium, under agitation, using a proportion of 10% (v/v). Aliquots (60 mL) of the inoculated medium were transferred to individual 500 mL Erlenmeyer flasks and incubated at 250 rpm and 20°C.

Two conventional batch cultures without product removal were performed with initial glycerol concentrations of 15.0 g/L (B15, denoted the control culture) and 30.0 g/L (B30). Another two batch cultures with product removal (extractive fermentations, EF) were performed with initial glycerol concentrations of 15 g/L (EF15) and 30 g/L (EF30). An additional batch culture with product removal was performed with initial glycerol concentration of 15 g/L and one pulse of glycerol so as to obtain a final concentration of 15 g/L (EF15 + 15).

In order to extract CA during the cultivations, 30 mL of fermentation broth was centrifuged at 3725 × g and 4°C for 15 min. The cells were returned to shaker flasks containing the fermentation broth, and the supernatants were transferred to new flasks containing 3 g (10%, w/v) of Amberlite IRA 400 resin that had been treated as described above and washed in sterile deionized water. The flasks were incubated in a shaker at 25°C and 250 rpm for the time required for the attainment of equilibrium (as determined in the adsorption kinetics experiments). The resin was then separated from the broth by centrifugation at 3725 × g, and the broth was returned to the shaker flasks. This procedure was performed every time the CA concentration in the broth was in the range 500–700 mg/L. All the extractive fermentations were performed in triplicate.

2.7. Analytical methods

The CA concentration (C_{CA}) in the fermentation broth was determined by UV spectrophotometric analysis [31]. CA contained in the pharmaceutical product Clavulin® (Glaxo-SmithKline Farmacêutica, Rio de Janeiro, Brazil) was used as a standard.

The glycerol concentration (C_{Gly}) in the supernatant was determined by an enzymatic method using a triglycerides GPO-PAP test kit (Laborlab, Brazil).

The method proposed by Mayer and Deckwer [30] was used to determine the cell concentration in the presence of solid particles. The measured cell dry weight concentration (C_{Cell}) was correlated with the optical density at 600 nm (OD_{600}), using cultivations employing solid-free media, which generated a linear model describing the relationship between cell dry weight and OD_{600}. In the cultivations with media containing insoluble particles (soybean protein isolate, SPI), the broth was decanted for 45 s (the time necessary to precipitate the SPI) and the OD_{600} was measured. The value of C_{Cell} was obtained from the linear regression equation described above.

3. Results

3.1. Adsorption kinetics studies

In order to evaluate the effects of CA removal from the culture broth, the kinetics of adsorption of CA onto the activated carbon, clinoptilolite, calcined hydrotalcite, and Amberlite IRA 400 were studied using CA concentrations of 15% (w/v) in cell-free fermentation broth at 25°C and pH 7.0. The performance of the adsorbents was assessed based on the decrease of the CA concentration in the liquid phase, as well as pH, measured over a period of 120 min. The results are shown in Fig. 1.

In the assay performed with activated carbon, the pH reached 7.5 after approximately 10 min, and a low CA adsorption of 23% ($C_{CA}/C_{CA0} = 0.77$) was achieved after 120 min. About 65% of the CA ($C_{CA}/C_{CA0} = 0.35$) was adsorbed using calcined hydrotalcite. However, the pH showed a sudden increase after the addition of the adsorbent, with a value of 8.5 reached in 10 min. When clinoptilolite was used as the adsorbent, the pH remained practically unchanged at around pH 7.0, and a constant and very low CA adsorption of 6.5% ($C_{CA}/C_{CA0} = 0.935$)

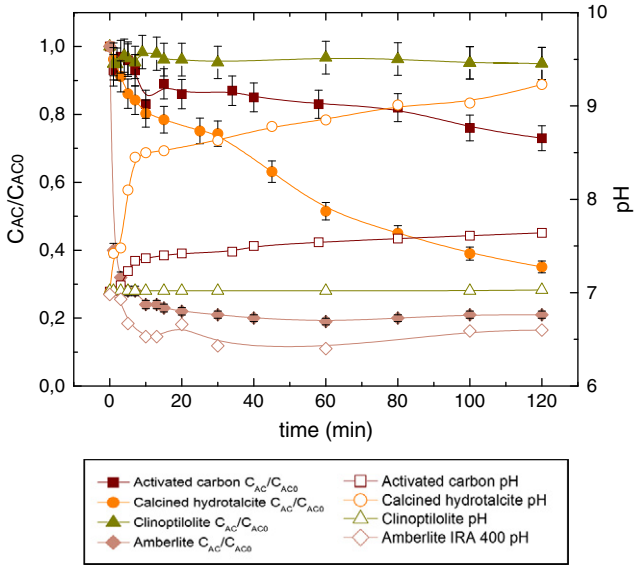

Fig. 1. pH profiles and adsorption kinetics using different adsorbents in cell-free fermentation broth containing 500 mg/L of CA.

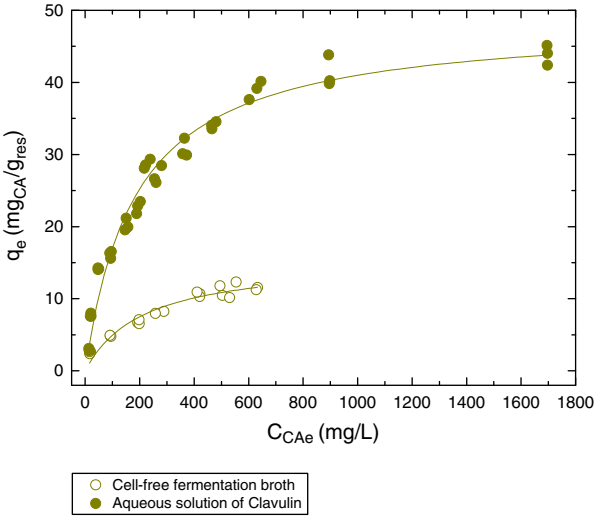

Fig. 2. Adsorption isotherms for the adsorption of CA on IRA 400 resin: Aqueous solution of Clavulin® and cell-free fermentation broth, at 25°C and pH 7.0.

was observed. In the adsorption assay with Amberlite IRA 400, the pH fell sharply during the first few minutes and then remained constant at around 6.5. This was the best value obtained for the different adsorbents, showing that this adsorbent was the most suitable for minimizing CA degradation. IRA 400 also showed the most promising results in terms of CA removal, since it was able to adsorb 78% of the CA ($C_{CA}/C_{CA0} = 0.22$) and was the fastest to reach equilibrium (in <20 min).

3.2. Desorption kinetics and isotherms

Evaluation of the desorption kinetics and isotherms was only conducted for the Amberlite IRA 400 anionic resin. After adsorption, the CA was desorbed from the resin employing NaCl (10%, w/v), and maximum recovery was achieved after 90 min. About 70% of the CA adsorbed by the resin was eluted under the conditions employed. Isotherms for the adsorption of CA by the IRA 400 anionic resin were used to describe the relationship between the mass of CA adsorbed by the adsorbent and the concentration of dissolved adsorbate in the solution at equilibrium. This provided an indication of the maximum adsorption capacity of the adsorbent, prior to its further use in extractive fermentation. Adsorption isotherms were obtained at pH 7.0 for batch systems with CA in aqueous solution and culture broth (Fig. 2). Values of the isotherm parameters were estimated by non-linear regression analysis.

The maximum capacity for adsorption (q_m) of CA from aqueous solution (48.5 ± 1.20 mg_{CA}/g_{res}) was greater than for adsorption from cell-free fermentation broth (14.9 ± 1.00 mg_{CA}/g_{res}). The Langmuir coefficient (k) values found for aqueous solution and cell-free fermentation broth were 87.34 ± 13.43 and 1095 ± 144.09 mg/L, respectively, showing the much higher affinity of the resin for CA present in aqueous solution.

3.3. Extractive CA fermentation

During the extractive fermentations, removal of CA was performed every time the CA concentration in the broth reached a value in the range 500–700 mg/L. An aliquot of fermentation broth (30 mL) was centrifuged, the cells were returned to the shaker flask, and the supernatant was placed in contact with 10% (w/v) IRA 400 resin for 20 min. The broth was then separated from the resin by centrifugation and returned to the shaker flask in order to continue the fermentation process. The results were compared to those obtained using conventional fermentation without product removal.

The quantity of IRA 400 required to extract a satisfactory amount of CA from the broth was chosen based on the results illustrated in Fig. 3. When the CA concentration reached values in the range 500–700 mg/L, an addition of 10% (w/v) of IRA 400 resulted in extraction of approximately 60% of the CA present in the broth.

Fig. 4, Fig. 5, and Fig. 6 show the time courses of glycerol consumption (C_{Gly}), cellular growth (C_{Cell}), and cumulative CA mass (m_{CA-c}) during the conventional and extractive fermentations. In comparison with conventional fermentation, the addition of IRA 400 to the cell-free fermentation broth in the extractive fermentation did not affect glycerol consumption (Fig. 4, Fig. 5). In the conventional and extractive fermentations with 15.0 g/L initial glycerol concentration (B15 and EF15), depletion of glycerol occurred after 142 h of cultivation, and the fermentation was continued until 238 h. In extractive fermentation with 15.0 g/L initial glycerol concentration and one pulse of glycerol (EF15 + 15), glycerol depletion occurred at 142 and 238 h, and the fermentation was continued until 300 h. In the conventional and extractive fermentations with initial glycerol

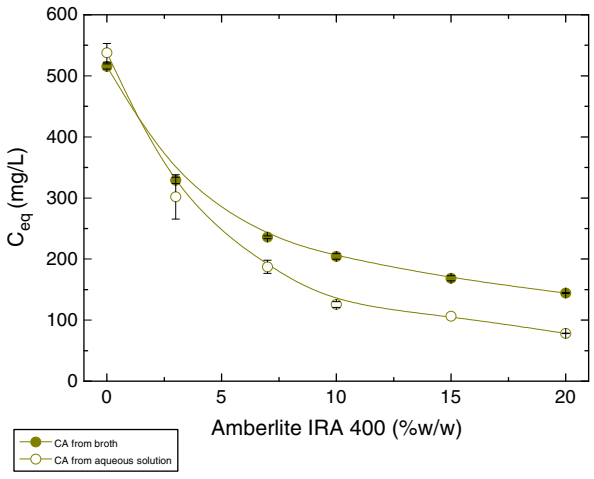

Fig. 3. CA concentrations at equilibrium (C_{CAe}) as a function of the quantity of IRA 400 used: CA from broth and CA from aqueous solution.

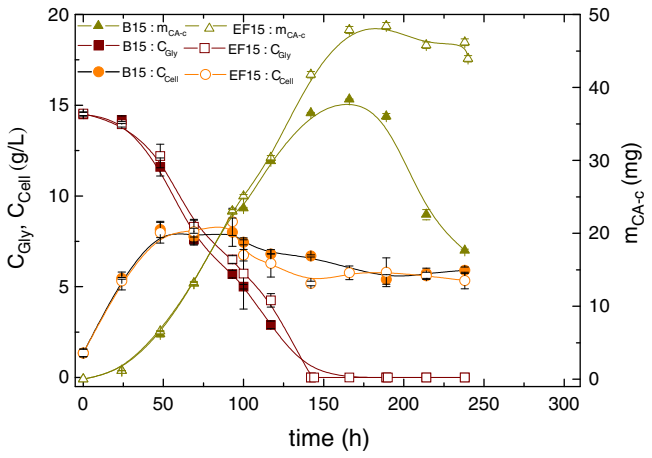

Fig. 4. Time courses of glycerol (C_{Gly}) and cellular (C_{Cell}) concentrations, and cumulative CA mass (m_{CA-c}), in the fermentation broth during conventional (B15: control cultivation) and extractive (EF15) fermentations at 20°C with 15 g/L initial glycerol concentration.

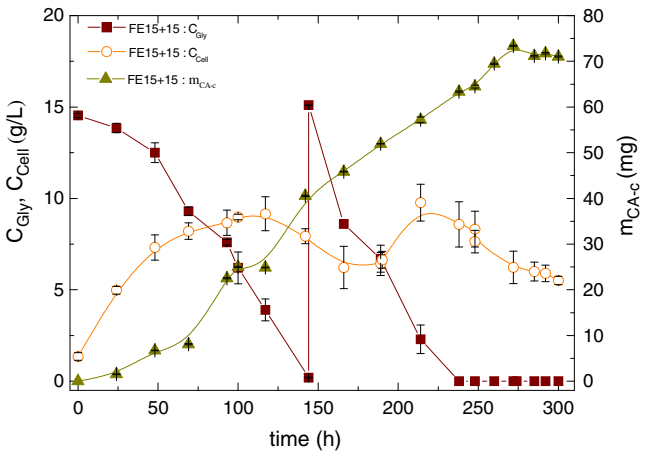

Fig. 6. Time courses of glycerol (C_{Gly}) and cellular (C_{Cell}) concentrations, and cumulative CA mass (m_{CA-c}), in the fermentation broth during extractive fermentation at 20°C with one glycerol pulse (EF15 + 15).

concentrations of 30.0 g/L (B30 and EF30), the carbon source was depleted at 214 h and the fermentation was extended until 300 h. After glycerol exhaustion, there were abrupt increases in pH, with maximum pH values of 7.5 and 7.7 for cultures B15 and EF15, respectively, and 7.1 for cultures B30 and EF30, at the end of the fermentation period (data not shown).

In the cultures with 15.0 g/L initial glycerol concentration (B15 and EF15), the cellular concentration (C_{Cell}) increased from soon after inoculation up to 50 h, when the exponential phase changed to the stationary phase (up to 93 h). The maximum cellular concentrations of 8.58 ± 0.75 g/L (B15) and 8.01 ± 0.77 g/L (EF15) were reached at about 93 h, before the time of the first CA extraction. Thereafter, approximately 35% decreases in cell concentrations were observed by the end of the cultivations (at 238 h). The time course of cellular concentration for the conventional fermentation with 30 g/L initial glycerol concentration and without product removal (B30) was similar to cultivations B15 and EF15, with a maximum value of 8.43 ± 0.11 g/L reached at 117 h. However, in cultivation B30, the cellular concentration showed different behavior, compared to the extractive fermentations with 15.0 g/L initial glycerol concentration and with one pulse of glycerol (EF15 + 15), and with 30.0 g/L initial glycerol concentration (EF30). After 117 h of fermentation, the cellular concentration of cultivation B30 decreased until 272 h, reaching a minimum value of 4.78 ± 0.68 g/L, while the cellular concentrations of cultivations EF30 and EF15 + 15 increased up to 214 and 238 h, with maximum values of 10.13 ± 0.57 and 9.07 ± 1.01 g/L, respectively. This was followed by decreases up to 300 h (5.63 ± 0.14 and 5.51 ± 0.23 g/L, respectively). In cultivations EF30 and EF15 + 15, the CA extractions were performed at 100, 142, 189, 238, and 272 h of fermentation.

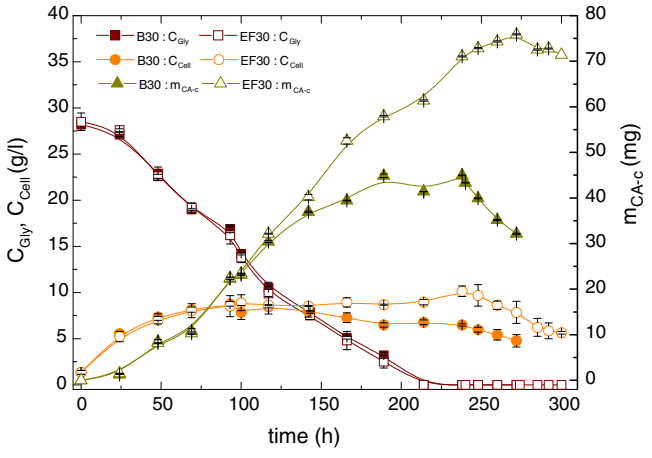

Fig. 5. Time courses of glycerol (C_{Gly}) and cellular (C_{Cell}) concentrations, and cumulative CA mass (m_{CA-c}), in the fermentation broth during conventional (B30) and extractive (EF30) fermentations at 20°C with 30 g/L initial glycerol concentration.

The effects of product removal on the final production of CA by the cells, in terms of cumulative CA mass (m_{CA-c}), are shown in Fig. 4, Fig. 5, and Fig. 6. The maximum CA mass (m_{CAm-c}) obtained from cultivation EF15 was 46.2 mg at 238 h of fermentation, corresponding to a cumulative CA concentration (C_{CA-c}) of 1184 ± 17 mg/L. This represented a 20% increase in mass, compared to the conventional fermentation (B15) (m_{CAm-c} = 38.4 mg at 166 h; C_{CA-c} = 798 ± 17 mg/L). CA production was also improved by the increased glycerol concentration in the culture media. The maximum CA mass (m_{CAm-c}) obtained in conventional fermentation B30 was 45.0 mg at 238 h (1034 ± 5 mg/L), representing a 17% increase in mass compared to fermentation B15. The maximum cumulative CA mass (m_{CAm-c}) increased from 45.0 mg at 238 h (B30) to 71.4 mg at 300 h (EF30), corresponding to an increase of 58%. In terms of the cumulative CA concentration, the increases were equivalent to 1034 ± 5 mg/L (B30) and 2644 ± 14 mg/L (EF30), respectively. The results obtained in extractive fermentation EF30 were similar to the extractive fermentation with one pulse of glycerol (EF15 + 15), with a maximum CA mass (m_{CAm-c}) of 71.8 mg reached at 272 h of cultivation. This corresponded to increases of 86% and 59%, compared to conventional fermentations B15 and B30, respectively, and a cumulative CA concentration (C_{CA-c}) of 2841 ± 14 mg/L.

Fig. 7 illustrates the profiles of the rates of CA production and degradation. The CA degradation rates (r_{dCA}) during the extractive fermentations (EF15, EF30, and EF15 + 15) were lower than in the conventional fermentations without product removal (B15 and B30), due to lower CA concentrations (C_{CA}) in the broth. After the beginning of the extractions (at 100 h), the CA production rates (r_{CA}) were always greater for extractive fermentation than for conventional fermentation. The maximum CA production rates (r_{CAm}) obtained in conventional fermentations B15 and B30 were very similar (8.4 mg/L.h at 93 h and 8.6 mg/L.h at 166 h, respectively). With product removal, the values of r_{CAm} achieved were 10.9 mg/L·h at 166 h for cultivation EF15 (29% higher compared to B15), 12.9 mg/L·h at 166 h for cultivation EF30 (49% higher compared to B30), and 13.6 mg/L·h at 214 h for cultivation EF15 + 15 (62% higher compared to B15).

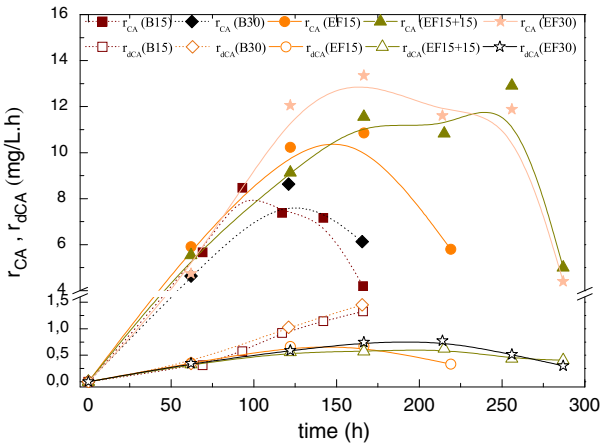

Fig. 7. Profiles of CA production rate (r_{CA}) and CA degradation rate (r_{dCA}) during conventional (B15 and B30) and extractive (EF15, EF30, and EF15 + 15) fermentations.

4. Discussion

In this work, extractive fermentation with a solid phase was successfully implemented as a way of improving CA production by *S. clavuligerus*. The aims were to evaluate the potential of different extractant materials, considering their recovery characteristics and costs, for reduction of the losses of CA during its production. This involved the removal of CA from the fermentation broth, followed by its recovery from the solid phase. The different stages of this integrated process are discussed below.

In order to evaluate the extractive fermentations performed with the proposed solid phases, it was necessary to measure pH changes and the times taken to reach equilibrium. It is well known that pH affects the degradation of CA. As reported by Bersanetti et al. [19], CA is most stable at pH 6.2 and is highly unstable at pH exceeding 8.0. During the kinetics experiments, pH increases were observed using activated carbon and calcined hydrotalcite as adsorbents. This behavior has been reported previously in other studies employing these materials in adsorption systems [28,34]. The increase in pH causes degradation of CA by basic hydrolysis, resulting in over estimation of the adsorption. This effect was supported by the fact that equilibrium was not attained after 120 min. An additional consideration is that an increase in pH is not desirable for extractive fermentation, due to the negative effect on cell growth.

Another important aspect to consider in the selection of an adsorbent for application in an extractive fermentation system is a short adsorption equilibrium time, which minimizes the contact time of the broth with the adsorbent during the extractive fermentation, hence reducing the effects of degradation losses caused by metabolites present in the culture broth.

Both of these characteristics (short time to reach equilibrium, and low pH variation) were shown by Amberlite IRA 400, which was therefore selected for further studies of CA extractive fermentation. To ensure that the resin was free of biological contaminants, the pretreated material was previously washed with sterilized deionized water in a sterilized glass column, using a ratio of 5 L of water to 20 g of resin.

The maximum capacity for adsorption of CA (q_m) from aqueous solution obtained here using Amberlite IRA 400 was considerably greater than the value reported previously by Barboza et al. [23] ($q_m = 24.6\ mg_{CA}/g_{res}$ at 23°C). This difference could be related to the different conditions of pH and temperature employed in the two studies. The competition between CA and other anions present in the fermentation broth for binding sites on the ionic resin could explain the higher CA q_m value obtained using the aqueous solution, compared to the cell-free fermentation broth [33]. In relation to the Langmuir coefficient (k), the values found for aqueous solutions were similar to that reported by Barboza et al. [23] (72.2 mg/L). The values obtained for cell-free fermentation broth were higher, compared to aqueous solution, which could be attributed to the affinity of the resin binding sites for other compounds present in the medium.

The process of desorption of CA from the Amberlite IRA 400 was slower than adsorption, possibly because of strong ionic interaction between the CA molecules and the active sites of the resin [23]. In the desorption kinetics experiments, the pH of the eluting solution increased rapidly and reached values of 8.6 after 90 min of elution (data not shown). The degradation of CA by alkaline hydrolysis could contribute to losses. According to Mayer et al. [33], this occurs due to irreversible interactions with the resin matrix, causing degradation of the CA at the ion-exchange phase and consequently low recovery. It is likely that a higher recovery, in a shorter time, could be achieved under optimized conditions of pH, temperature, and eluent concentration.

The finding by Roubos et al. [10] that extractive fermentation employing a solid phase improved the production of biomolecules was supported by the decrease in the microbial growth rate under high CA concentrations. Comparison was therefore made between CA production using conventional fermentation and extractive fermentation with IRA400 resin. The cell concentration profile for EF15 indicated that at the times of CA extraction (100, 142, and 189 h), cell concentrations were slightly lower than those obtained using conventional fermentation (B15). This was probably related to biomass losses during the CA extraction procedure, but did not affect the performance of the process. The frequent removal of product in cultivation EF30 resulted in higher cell concentrations, compared to cultivation B30, suggesting that an excess of CA in the fermentation broth was associated with cytotoxicity.

Even at high carbon source concentrations, the control of glycerol consumption using low temperatures [2] enables better incorporation of the carbon source in the production of secondary metabolites such as CA. As expected, extractive fermentation with 30 g/L initial glycerol concentration (EF30) or with a pulse of glycerol (EF15 + 15) resulted in greater production of CA, which could be recovered using IRA 400. Higher cumulative CA concentrations were therefore obtained. The exhaustion of glycerol is followed by increased consumption of proteins (amino acids) as carbon source, and the consequent release of ammonia provides an explanation for the increase in pH. Therefore, the excess of carbon source in the batch cultures with 30 g/L glycerol and with one glycerol pulse (15 + 15 g/L) controlled the pH, preventing the effects of CA degradation by basic hydrolysis and enhancing cellular viability.

The use of extractive fermentation for CA production by *S. clavuligerus* significantly improved the CA production rate (r_{CA}), compared to fermentation without product removal (Fig. 7), indicating that the cellular biomass remained viable for longer, in agreement with the results obtained for the cell concentrations. Lynch and Yang [35] evaluated the effect of addition of clavulanic acid degradation products in *S. clavuligerus* cultures. It was found that the degradation rate (r_{dCA}) was equivalent to the clavulanic acid production rate (r_{CA}), indicating that clavulanic acid was both produced and degraded in the cultures of *S. clavuligerus*, and that the products of degradation were used by the organism, resulting in further production of the antibiotic. In the present work, the r_{CA} values were much higher than the r_{dCA} values, showing that the fermentation conditions employed here, such as low temperature, favored the accumulation of CA. Furthermore, the higher r_{CA} values obtained for extractive fermentation, compared to the conventional process, showed that CA acted as an inhibitor of its own biosynthesis. Similar findings have been reported for application of a solid phase for in situ removal of lactic acid. Gao et al. [26] demonstrated that product removal successfully decreased the inhibitory effects of lactic acid, resulting in significant increases in productivity and yield.

The use of IRA 400 adsorbent resin to remove CA during the fermentation process resulted in an increase of 86% in the mass of CA recovered, and a 248% increase in the cumulative CA concentration, compared to the control fermentation performed without product removal. The values found in the present study were much greater than the highest CA concentration (1.6 g/L) reported in the literature for cultures utilizing a wild type strain of *S. clavuligerus* [2,9]. Marques et al. [36] recently evaluated extractive CA fermentation using a two-phase aqueous PEG/phosphate salt system in a bench-scale bioreactor, and obtained a yield of about 691 mg/L. The results obtained in the present work indicate that the use of an anionic exchange resin as the extractor in extractive CA fermentation is more effective than an aqueous two-phase system.

The findings showed that high concentrations of CA in the culture broth reduced the CA production rate, and that removal of CA by Amberlite IRA 400 increased the cellular concentration and the product yield. An additional advantage of extractive fermentation with resin is that it provides partial purification of the product, reducing both the number of steps and the loss of product in downstream processes. An integrated fermentation-separation process is a promising technique for increasing CA production, and could be a new strategy suitable for use with other important secondary metabolites.

Financial support

Financial support for this work was provided by the Brazilian agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Proc. 2011/23807-1) and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of interest

None.

Acknowledgments

The authors would like to thank Prof. José M. Assaf (DEQ/UFSCar) and Dr. Marcus B. S. Forte (FEA/UNICAMP) for the donation of the hydrotalcite.

References

[1] Baggaley KH, Brown AG, Schofield CJ. Chemistry and biosynthesis of clavulanic acid and other clavams. Nat Prod Rep 1997;14:309–33. http://dx.doi.org/10.1039/NP9971400309.
[2] Costa CLL, Badino AC. Production of clavulanic acid by *Streptomyces clavuligerus* in batch cultures without and with glycerol pulses under different temperature conditions. Biochem Eng J 2012;69:1–7. http://dx.doi.org/10.1016/j.bej.2012.08.005.
[3] Large KP, Ison AP, Williams DJ. The effect of agitation rate on lipid utilisation and clavulanic acid production in *Streptomyces clavuligerus*. J Biotechnol 1998;63: 111–9. http://dx.doi.org/10.1016/S0168-1656(98)00082-0.
[4] Domingues LCG, Teodoro JC, Hokka CO, Badino AC, Araujo MLGC. Optimisation of the glycerol-to-ornithine molar ratio in the feed medium for the continuous production of clavulanic acid by *Streptomyces clavuligerus*. Biochem Eng J 2010;53:7–11. http://dx.doi.org/10.1016/j.bej.2009.05.006.
[5] Saudagar PS, Singhal RS. Optimization of nutritional requirements and feeding strategies for clavulanic acid production by *Streptomyces clavuligerus*. Bioresour Technol 2007;98:2010–7. http://dx.doi.org/10.1016/j.biortech.2006.08.003.
[6] Li R, Townsend CA. Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus*. Metab Eng 2006;8:240–52. http://dx.doi.org/10.1016/j.ymben.2006.01.003.
[7] Ortiz SCA, Hokka CO, Badino AC. Utilization of soybean derivatives on clavulanic acid production by *Streptomyces clavuligerus*. Enzyme Microb Technol 2007;40:1071–7. http://dx.doi.org/10.1016/j.enzmictec.2006.08.009.
[8] Wang YH, Yang B, Ren J, Dong ML, Liang D, Xu AL. Optimization of medium composition for the production of clavulanic acid by *Streptomyces clavuligerus*. Process Biochem 2005;40:1161–6. http://dx.doi.org/10.1016/j.procbio.2004.04.010.
[9] Teodoro JC, Baptista-Neto A, Araújo MLGC, Hokka CO, Badino AC. Influence of glycerol and ornithine feeding on clavulanic acid production by *Streptomyces clavuligerus*. Braz J Chem Eng 2010;27:499–506. http://dx.doi.org/10.1590/S0104-66322010000400001.
[10] Roubos JA, Krabben P, De Laar WTAM, Babuskaa R, Heijen JJ. Clavulanic acid degradation in *Streptomyces clavuligerus* fed-batch cultivations. Biotechnol Prog 2002;18:451–7. http://dx.doi.org/10.1021/bp020294n.
[11] Gao MT, Shimamura T, Ishida N, Takahashi H. Extractive lactic acid fermentation with tri-n-decylamine as the extractant. Enzyme Microb Technol 2009;44:350–4. http://dx.doi.org/10.1016/j.enzmictec.2008.12.001.
[12] Gao MT, Shimamura T, Ishida N, Takahashi H. Application of metabolically engineered *Saccharomyces cerevisiae* to extractive lactic acid fermentation. Biochem Eng J 2009;44:251–5. http://dx.doi.org/10.1016/j.bej.2009.01.001.
[13] Hollmann D, Switalski J, Geipel S, Onken U. Extractive fermentation of gibberellic acid by *Gibberella fujikuroi*. J Ferment Bioeng 1995;79:594–600. http://dx.doi.org/10.1016/0922-338X(95)94754-F.
[14] Jeon BS, Moon C, Kim B-C, Kim H, Um Y, Sang B-I. In situ extractive fermentation for the production of hexanoic acid from galactitol by *Clostridium* sp. BS-1. Enzyme Microb Technol 2013;53:143–51. http://dx.doi.org/10.1016/j.enzmictec.2013.02.008.
[15] Lazarova Z, Syska B, Schügerl K. Application of large-scale hollow fiber membrane contactors for simultaneous extractive removal and stripping of penicillin. J Membr Sci 2002;202:151–64. http://dx.doi.org/10.1016/S0376-7388(01)00748-7.
[16] Show PL, Tan CP, Shamsul Anuar M, Ariff A, Yusof YA, Chen SK, et al. Extractive fermentation for improved production and recovery of lipase derived from *Burkholderia cepacia* using a thermoseparating polymer in aqueous two-phase systems. Bioresour Technol 2012;116:226–33. http://dx.doi.org/10.1016/j.biortech.2011.09.131.
[17] Lu C, Dong J, Yang ST. Butanol production from wood pulping hydrolysate in an integrated fermentation-gas stripping process. Bioresour Technol 2013;143:467–75. http://dx.doi.org/http://dx.doi.org/10.1016/j.biortech.2013.06.012.
[18] Lee JC, Park HR, Park DJ, Lee HB, Kim YB, Kim CJ. Improved production of teicoplanin using adsorbent resin in fermentations. Lett Appl Microbiol 2003;37:196–200. http://dx.doi.org/10.1046/j.1472-765X.2003.01374.x.
[19] Bersanetti PA, Almeida RMRG, Barboza M, Araújo MLGC, Hokka CO. Kinetic studies on clavulanic acid degradation. Biochem Eng J 2005;23:31–6. http://dx.doi.org/10.1016/j.bej.2004.10.007.
[20] Singh MP, Leighton MM, Barbieri LR, Roll DM, Urbance SE, Hoshan L, et al. Fermentative production of self-toxic fungal secondary metabolites. J Ind Microbiol Biotechnol 2010;37:335–40. http://dx.doi.org/10.1007/s10295-009-0678-9.
[21] Jia B, Jin ZH, Lei YL, Mei LH, Li NH. Improved production of pristinamycin coupled with an adsorbent resin in fermentation by *Streptomyces pristinaespiralis*. Biotechnol Lett 2006;28:1811–5. http://dx.doi.org/10.1007/s10529-006-9157-9.
[22] Kang SH, Chang YK. Recovery of potassium clavulanate from fermentation broth by Ion exchange chromatography and desalting electrodialysis. Biotechnol Bioprocess Eng 2009;14:803–10. http://dx.doi.org/10.1007/s12257-008-0161-x.
[23] Barboza M, Almeida RMRG, Hokka CO. Influence of temperature on the kinetics of adsorption and desorption of clavulanic acid by ionic exchange. Biochem Eng J 2003;14:19–26. http://dx.doi.org/10.1016/S1369-703X(02)00103-1.
[24] Carabineiro SAC, Thavorn-Amornsri T, Pereira MFR, Figueiredo JL. Adsorption of ciprofloxacin on surface-modified carbon materials. Water Res 2011;45:4583–91. http://dx.doi.org/10.1016/j.watres.2011.06.008.
[25] Chang YK, Huang RZ, Lin SY, Chiu SJ, Tsai JC. Equilibrium study of immobilized lysozyme on the extrudate-shaped NaY zeolite. Biochem Eng J 2006;28:1–9. http://dx.doi.org/10.1016/j.bej.2005.08.029.
[26] Gao M-T, Shimamura T, Ishida N, Takahashi H. pH-uncontrolled lactic acid fermentation with activated carbon as an adsorbent. Enzyme Microb Technol 2011;48:526–30. http://dx.doi.org/10.1016/j.enzmictec.2010.07.015.
[27] Filho JFN, Barros Valim J. Temperature effect on the sorption of cholate anions on calcined LDH. J Phys Chem Solids 2010;71:620–3. http://dx.doi.org/10.1016/j.jpcs.2009.12.051.
[28] Forte MBS, Elias ECL, Pastore HO, Filho FM, Rodrigues MI. Evaluation of clavulanic acid adsorption in MgAl-layered double hydroxides: Kinetic, equilibrium and thermodynamic studies. Adsorpt Sci Technol 2012;30:65–80. http://dx.doi.org/10.1260/0263-6174.30.1.65.
[29] Forte MB, Costa F, Maugeri F, Rodrigues MI. Clavulanic acid separation by molecular sieving from fermentation broth of *Streptomyces clavuligerus* using experimental design. N Biotechnol 2009;25:S182. http://dx.doi.org/10.1016/j.nbt.2009.06.727.
[30] Mayer AF, Deckwer WD. Simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations. Appl Microbiol Biotechnol 1996; 45:41–6. http://dx.doi.org/10.1007/s002530050646.
[31] Bird AE, Bellis JM, Gasson BC. Spectrophotometric assay of clavulanic acid by reaction with imidazole. Analyst 1982;107:1241–5. http://dx.doi.org/10.1039/AN9820701241.
[32] Rosa JC, Baptista-Neto A, Hokka CO, Badino AC. Influence of dissolved oxygen and shear conditions on clavulanic acid production by *Streptomyces clavuligerus*. Bioprocess Biosyst Eng 2005;27:99–104. http://dx.doi.org/10.1007/s00449-004-0386-9.
[33] Mayer AF, Hartmann R, Deckwer WD. Diffusivities of clavulanic acid in porous sorption systems with ion pairing. Chem Eng Sci 1997;52:4561–8. http://dx.doi.org/10.1016/S0009-2509(97)00299-6.
[34] Seida Y, Nakano Y, Nakamura Y. Rapid removal of dilute lead from water by pyroaurite-like compound. Water Res 2001;35:2341–6. http://dx.doi.org/10.1016/S0043-1354(00)00523-6.
[35] Lynch HC, Yang Y. Degradation products of clavulanic acid promote clavulanic acid production in cultures of *Streptomyces clavuligerus*. Enzyme Microb Technol 2004; 34(1):48–54. http://dx.doi.org/10.1016/j.enzmictec.2003.08.003.
[36] Marques DAV, Lima-Filho JL, Converti A, Perego P, Porto ALF, Pessoa-júnior A. Extractive fermentation of clavulanic acid by *Streptomyces* DAUFPE 3060 Using aqueous two-phase system. Biotechnol Prog 2011;27:96–103. http://dx.doi.org/10.1002/btpr.526.