# INFLUENCE OF GLYCEROL AND ORNITHINE FEEDING ON CLAVULANIC ACID PRODUCTION BY *Streptomyces clavuligerus*

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**Abstract** - The influence of glycerol and ornithine feeding on clavulanic acid (CA) production by *Streptomyces clavuligerus* was investigated. In batch experiments, CA maximum concentration ($Cp_{max}$) ranged randomly from 430 to 560 mg.L$^{-1}$, with a maximum increase of 10% in relation to the control run, without ornithine. However, the maximum volumetric productivity of CA ($Pp_{max}$) of 13.7 mg.L$^{-1}$.h$^{-1}$ was obtained with 0.66 g.L$^{-1}$ of ornithine, 44.2% higher than the $Pp_{max}$ in the control run. In fed-batch experiments, $Cp_{max}$ varied within the narrow range from 1.254 to 1.405 g.L$^{-1}$, 2.5 times higher than that obtained in the control run. The presence of ornithine increased the $Pp_{max}$, although it influenced only slightly the $Cp_{max}$. Concerning glycerol, the highest CA production of 1.6 g.L$^{-1}$ was obtained in the fed-batch with glycerol and ornithine (180 and 3.7 g.L$^{-1}$) in a 10-L bioreactor, showing a positive effect of ornithine and glycerol, in the proper proportion (48.6:1), on CA biosynthesis.
*Keywords:* Clavulanic acid; *Streptomyces clavuligerus*; Glycerol; Ornithine; Fed-batch.

## INTRODUCTION

One of the most important resistance mechanisms exhibited by a variety of gram-positive and gram-negative bacteria is their ability to produce β-lactamases, enzymes that deactivate penicillins and cephalosporins by hydrolyzing their β-lactam ring. Clavulanic acid (CA) is a potent inhibitor of β-lactamases, and its combination with amoxicillin is the most successful example of the use of a β-lactam antibiotic sensitive to β-lactamase together with an inhibitor of these enzymes (Mayer and Deckwer, 1996). The amoxicillin/clavulanate combination represents a broad-spectrum antibacterial agent which has been widely used for over 20 years (Buynak, 2006). CA is traditionally produced by *Streptomyces clavuligerus* using a complex culture media containing soybean derivatives and glycerol as

nitrogen, carbon, and energy sources, respectively (Mayer and Deckwer, 1996; Baptista-Neto et al., 2000; Chen et al., 2002; Chen et al., 2003; Baptista-Neto et al., 2005; Neto et al., 2005; Wang et al., 2005; Teodoro et al., 2006; Ortiz et al., 2007).
Glycerol plays an important role in CA production as the C3 precursor of the molecule (Baggaley et al., 1997). Amino acids in turn also have important roles in the regulation of secondary metabolism in *S. clavuligerus*, e.g. arginine and ornithine, which are the C5 precursors of the CA molecule (Townsend and Ho, 1985a). Ornithine is not a direct precursor of CA since this amino acid has to be converted to arginine before it can be incorporated into a CA molecule (Ives and Bushell, 1997). However, studies have demonstrated that exogenous ornithine, rather than arginine, effectively enhances CA production, provided that there is a

sufficient amount of C3 precursor (Chen et al., 2003; Bushell et al., 2007). Enhancements of CA production of 270% were observed in experiments with intermittent feeding of glycerol and ornithine, compared with the batch cultivation in shake flasks, and of 150% compared with cultures with glycerol and arginine feeding or when only glycerol was fed (Chen et al., 2003). These authors also observed that, in the absence of glycerol, feeding only ornithine or arginine could not enhance the CA production. Wang et al. (2005) obtained an increase of 50% in CA production in shake flasks batch cultivation under optimal concentrations of glycerol (15.0 $g.L^{-1}$) and ornithine (1.18 $g.L^{-1}$), compared to the non-optimized medium.

Many authors have observed that a glycerol concentration above 15 $g.L^{-1}$ inhibited the biosynthesis of CA in batch cultivations (Romero et al., 1984; Chen et al. 2002). The fed-batch strategies have been considered as means of overcoming substrate inhibition and metabolite repression, besides controlling the growth rate and prolonging the stationary phase. Teodoro et al. (2006) investigated CA production in fed-batch cultivations and the best experimental conditions found for the volumetric flow rate and glycerol concentration in the complex feed medium were 0.01 $L.h^{-1}$ and 120 $g.L^{-1}$, respectively. Under these conditions, the maximum CA production was practically two-fold higher than that obtained in batch cultivation.

The literature reports work emphasizing the important role of glycerol and ornithine feeding on the CA production process either in batch or fed-batch cultures. However, these studies were performed in shake flasks in which oxygen limitation is unavoidable. So far, there are no reports in the literature dealing with the effect of ornithine and glycerol on CA production in bioreactors under proper oxygen supply. In this context, the aim of the present study was to evaluate the simultaneous influence of glycerol and ornithine feeding conditions on CA production in a submerged culture of *S. clavuligerus* in both batch and fed-batch cultivations in a bench scale bioreactor under conditions of no oxygen limitation. Furthermore, the consumption of amino acids was monitored during the cultivations in order to evaluate its relation to CA production.

## MATERIALS AND METHODS

### Microorganism

*Streptomyces clavuligerus* ATCC 27064, used throughout this work, was stored as vegetative cells

(5.0 $g.L^{-1}$ dry weight) at −70°C in cryotubes utilizing glycerol 20% w/v.

### Culture Media and Experimental Conditions

The seed medium proposed by Rosa et al. (2005) had the following composition (in $g.L^{-1}$ distilled water): glycerol, 15.0; bacto peptone, 10.0; malt extract, 10.0; yeast extract, 1.0; $K_2HPO_4$, 2.5; $MgSO_4.7H_2O$, 0.75; $MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, 21 (100 mM), and pH 6.8.

The inoculum medium proposed by Gouveia et al. (1999) had the following composition (in $g.L^{-1}$ distilled water): glycerol, 15.0; soybean protein isolate (containing about 15% w/v of total nitrogen), 20.0; $K_2HPO_4$, 0.8; $MgSO_4.7H_2O$, 0.75; salt solution 1.0 $g.L^{-1}$; $MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, 21 (100 mM), and pH 6.8. Under the medium preparation conditions, the soybean protein isolate was not completely soluble.

The production medium had almost the same composition as the inoculum medium except that no MOPS buffer was used and silicone antifoam (0.1 $mL.L^{-1}$) and soybean oil (1.0 $g.L^{-1}$) were added.

The experimental conditions and purposes of the batch (B) and fed-batch cultivations (FB) are presented in Table 1. In the first stage of the present work, the influence of ornithine on CA production was evaluated in four batch cultivations (B1-B4). The run performed without ornithine (B1) was considered the control. The effect of ornithine feeding on CA production was evaluated in four fed-batch experiments (FB1-FB4) with different ornithine concentrations. In the second stage, the influence of the global composition of the feed medium on CA production was evaluated by comparing the results obtained in two simultaneous fed-batch cultivations (FB5 and FB6). In the FB5 cultivation, the feed medium had the same composition as the production medium used in the fed-batch FB4. In FB6, the feed medium contained only glycerol, ornithine, and distilled water. In third stage, the influence of glycerol feeding was investigated in four fed-batch experiments (FB6-FB9) with the feeding media containing distilled water, ornithine, and four different glycerol concentrations. The best results in terms of CA production obtained on a 5-L scale were validated in two fed-batch experiments (FB10 and FB11) on a 10-L scale.

**Table 1: Experimental conditions utilized in batch and fed-batch experiments**

| Run | $C_{S\text{-}0}$ (g.L$^{-1}$) | $C_{orn\text{-}0}$ (g.L$^{-1}$) | $C_{S\text{-}F}$ (g.L$^{-1}$) | $C_{orn\text{-}F}$ (g.L$^{-1}$) | Main |
|---|---|---|---|---|---|
| B1 | 15.0 | 0.0 | - | - | Influence of ornithine in batch cultivations. B1 is the batch control run (without ornithine). |
| B2 | 15.0 | 0.66 | - | - | |
| B3 | 15.0 | 0.99 | - | - | |
| B4 | 15.0 | 1.32 | - | - | |
| FB1 | 15.0 | 0.0 | 120.0 | 0.0 | Effect of ornithine feeding in fed-batch cultivations. Feeding media with same composition as the main medium. |
| FB2 | 15.0 | 0.0 | 120.0 | 3.7 | |
| FB3 | 15.0 | 0.0 | 120.0 | 7.4 | |
| FB4 | 15.0 | 0.0 | 120.0 | 11.1 | |
| FB5 | 15.0 | 0.0 | 120.0 | 3.7 | Influence of the global composition of the feed medium. In FB6, the feeding medium is composed by glycerol, ornithine and distilled water. |
| FB6 | 15.0 | 0.0 | 120.0 | 3.7 | |
| FB6 | 15.0 | 0.0 | 120.0 | 3.7 | Influence of glycerol feeding in fed-batch experiments. Feeding medium containing glycerol, ornithine and distilled water. |
| FB7 | 15.0 | 0.0 | 150.0 | 3.7 | |
| FB8 | 15.0 | 0.0 | 180.0 | 3.7 | |
| FB9 | 15.0 | 0.0 | 240.0 | 3.7 | |
| FB10 | 15.0 | 0.0 | 180.0 | 3.7 | Validation of the best results on a higher scale (10 L). |
| FB11 | 15.0 | 0.0 | 180.0 | 5.5 | |

B: batch experiments, FB: fed-batch experiments, $C_{S\text{-}0}$: initial glycerol concentration, $C_{orn\text{-}0}$: initial ornithine concentration, $C_{S\text{-}F}$: glycerol concentration in feeding medium, $C_{orn\text{-}F}$: ornithine concentration in feeding medium.

The batch and fed-batch cultivations were performed in a Bioflo III fermentor (New Brunswick Sci. Co. Inc., USA) with a 5-L total volume. All cultivations were conducted at 28°C, 800 rpm, and 0.5 vvm based on a 4-L working volume, and the pH was automatically controlled at 6.8±0.1 by adding 1 M HCl and/or 2 M NaOH solutions. To confirm the best results obtained in 5-L fermentor, cultivations, with the best conditions found, were performed in a Bioflo 310 fermentor (New Brunswick Sci. Co. Inc., USA) with a 10-L total volume. Cell suspensions from cryotubes (3.5 mL), having a concentration of 5 g.L$^{-1}$ dry weight, were inoculated into 45 mL seed medium in a 500 mL Erlenmeyer flask and incubated in a rotary shaker (New Brunswick Scientific) at 28°C and 250 rpm for 24 hours. Next, 5 ml of the cultivated broth were inoculated into Erlenmeyer flasks (500 mL) containing 45 mL inoculum medium. The flasks were incubated in a rotary shaker at 28°C and 250 rpm for 24 hours. The contents of inoculum corresponding to 10% v/v were transferred to the fermentors resulting in initial fermentation volumes of 4.0 L for batch and 3.4 L for fed-batch (5-L bioreactor) or 6.5 L for fed-batch (10-L bioreactor) cultivations, respectively.

The fed-batch operation started 24 hours after inoculation at a volumetric flow rate (F) of 0.01 L.h$^{-1}$ (5-L bioreactor) or 0.02 L.h$^{-1}$ (10-L bioreactor) settling dilution rates (D=F/V) in the range from 0.002 to 0.003 h$^{-1}$. Samples of 20 mL were withdrawn every 6 h approximately, and 10 mL aliquots were centrifuged at 3.720×g and 5°C for 20 min. Fed-batch cultivations were standardized in such a way that, at the end of the process (120 h), the

working volumes completed 4 L (5-L bioreactor) and 8 L (10-L bioreactor), respectively.

Assays were performed in duplicate and the results of CA concentration presented standard deviations ranging from 5 to 10%.

**Analytical Methods**

Cell growth was evaluated indirectly by measuring the broth rheological parameter K (consistency index) of the "power law" model using a Brookfield concentric-cylinder rheometer. The literature reports that the consistency index (K) is the most appropriate parameter to infer cell growth in a broth containing insoluble particles such as is the case with complex fermentation broths. It can also be related to the morphology of filamentous microorganisms (Badino et al., 1999; Neves et al., 2000). As cultivation proceeded, the amount of insoluble particles could be neglected and the cell growth was evaluated as dry matter obtained by centrifugation at 3.720×g for 20 min and drying at 85°C for 12 h.

The glycerol concentration (Cs) was determined by high performance liquid chromatography (HPLC) calibrated with 25, 50, and 75% (v/v) dilutions of 0.5 g.L$^{-1}$ glycerol solution. Milli-Q water was used as the mobile phase. The equipment was operated at 80°C with a 1mL.min$^{-1}$ flow rate. A Shodex KS 802 (Lonpak) column was utilized.

The CA concentration (Cp) was determined by HPLC as described by Foulstone and Reading (1982). The CA from the Pharmaceutical product Clavulin (Glaxo-SmithKline Farmacêutica, Rio de

Janeiro, Brazil) was used as the standard. The imidazole derivative of CA was injected into a HPLC unit. The C-18 μ-Bondapack (Waters) column was utilized as the stationary phase, and the mobile phase was composed of 94% $KH_2PO_4$ 0.1 M (pH 3.2) and 6% methanol, v/v. The detection of the CA derivative occurred at 311 nm.

The amino acids ornithine and arginine were determined by HPLC based on the method proposed by Henrikson and Meredith (1984). A Pico-tag (Waters) column was utilized. Standards of amino acids were kindly donated by Ajinomoto Interamericana Ltda (Brazil).

## RESULTS AND DISCUSSION

### Effect of Ornithine Concentration on Batch Cultures

Glycerol ($C_S$) and the consistency index (K) presented similar profiles along all the batch cultivations (data not shown), indicating that the presence of ornithine had no effect on the cellular growth and carbon source consumption. The consistency index (K), which is an indirect way to represent the cellular concentration, increased exponentially in the first 24 h of cultivation but, after the glycerol exhaustion, the K values decreased showing that the microorganism did not make use of other carbon sources to grow.

Figure 1 illustrates the maximum CA concentration ($Cp_{max}$) and the maximum volumetric productivity ($Pp_{max}$) along the batch cultivations (B1 to B4). The maximum CA production varied within a very narrow range from 460 to 560 mg.$L^{-1}$ in cultivations B1 to B3. The $Cp_{max}$ obtained in the control run (B1) presented $Cp_{max}$ of 509 mg.$L^{-1}$, which is slightly lower than that obtained only in cultivation B2 (560 mg.$L^{-1}$) with an ornithine concentration of 0.66 g.$L^{-1}$. Differently from the results found by Chen et al. (2003), the maximum CA production ($Cp_{max}$) did not seem to be influenced positively by the presence of ornithine under the experimental conditions investigated. However, the maximum volumetric productivity ($Pp_{max}$) was considerably affected by the ornithine concentration. In all cultivations with ornithine, the volumetric productivities were higher than those found in the control run without ornithine (B1). The value of $Pp_{max}$ of 13.7 mg.$L^{-1}$.$h^{-1}$ found in batch B2, with an ornithine concentration of 0.66 g.$L^{-1}$, was 44.2% higher than the $Pp_{max}$ obtained in the control run (9.5 mg.$L^{-1}$.$h^{-1}$). In all cultivations, the highest production rate occurred when the glycerol was exhausted (data not shown). These results were also different from

those obtained by Chen et al. (2003) in shake flasks. These authors observed that the CA production of 200 mg.$L^{-1}$ was nearly two-fold higher than that obtained without the addition of ornithine (115 mg.$L^{-1}$). The different CA production results found in the present work when compared with those of Chen et al. (2003) are probably due to the fact that the cultivations were performed under different conditions of oxygen mass transfer. In other words, under conditions of no oxygen limitation, the presence of ornithine affects remarkably only the maximum CA productivity ($Pp_{max}$), with practically no effect on the maximum CA production ($Cp_{max}$).



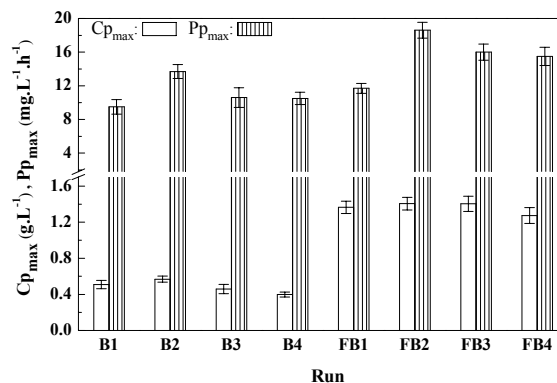**Figure 1:** Influence of ornithine feeding on maximum CA concentration ($Cp_{max}$) and maximum volumetric productivity in CA ($Pp_{max}$) in batch and fed-batch cultivations

Figure 2 shows the time course of arginine ($C_{arg}$) and ornithine ($C_{orn}$) concentrations along the batch cultivations. In all cultivations, ornithine was consumed in the first 20 h of cultivation. According to Townsend and Ho (1985b), ornithine and arginine are better utilized than the other amino acids in the urea cycle. On the other hand, the time course of arginine concentration during the cultivations varied. Arginine accumulated in the broth, probably due to the hydrolysis of the soy protein isolate (SPI), containing about 8% w/w arginine. After ornithine exhaustion, a change in arginine behavior occurred. In the idiophase, the arginine concentration varied as a function of the hydrolysis of SPI by proteases liberated in the broth by both its consumption as nitrogen source and utilization as CA precursor.

Chen et al. (2003) reported that ornithine and arginine have been considered to be the precursors of clavulanic acid. However, adding arginine initially or intermittently did not enhance the clavulanic acid production. In contrast, the addition of ornithine, either at the beginning or intermittently, revealed a substantial enhancing effect. When ornithine was added, the unusual presence of the urea cycle in a

prokaryote provided an abundant supply of arginine, the $C_5$ precursor to clavulanic acid (Valentine et al., 1993). The authors observed that not only did it reduce the anaplerotic carbon flux of $C_3$ to the synthesis of the $C_5$-amino acid, but also it was required for the removal of ammonia from the cells to avoid toxic intracellular concentrations.

## Effect of Ornithine Feeding on Fed-Batch Cultures

The influence of ornithine feeding on CA production was evaluated in four fed-batch cultivations (FB1 to FB4) with a volumetric flow rate of 0.01 $L.h^{-1}$ of a supplementary medium containing 120 $g.L^{-1}$ glycerol and variable ornithine concentration, as shown in Table 1.

In the fed-batch experiments performed without ornithine (FB1) or with the lowest ornithine concentration (FB2), values of K above 20 $dyn.cm^{-2}.s^n$ were observed during the cultivations. However, the high cellular concentrations in FB1 and FB2 did not affect CA production. In fed-batch experiments performed at high levels of ornithine concentration (FB3 and FB4), the K profiles displayed similar behavior, with a rapid decrease to very low values that were reached at the end of the cultivations (data not shown). Figure 1 also illustrates the maximum CA concentration ($Cp_{max}$) and the maximum volumetric productivity ($Pp_{max}$) during the fed-batch cultivations. It can be observed that the maximum production of CA in fed-batch cultivations was nearly three times higher than that obtained in the batch cultivation control (B1) without ornithine (509 $mg.L^{-1}$). Higher CA productivity values were obtained in fed-batch cultivations with ornithine feeding (FB2, FB3, and FB4) than those obtained in the cultivation without the feeding of ornithine (FB1). In cultivation FB2, the highest values of $Cp_{max}$ (1.405 $g.L^{-1}$) and

$Pp_{max}$ (18.6 $mg.L^{-1}.h^{-1}$) were reached. It can be noted that the $Cp_{max}$ in fed-batch experiments FB1, FB2, and FB3 was practically independent of ornithine feeding, in contrast with the results reported in the literature. Chen et al. (2003) observed an increase of 270% in CA production with ornithine feeding in shake flask cultivations. Again, the difference between the results obtained by the authors and those obtained in the present work seem to be due to the different mixing and oxygen transfer conditions in the shaker and in the bench scale bioreactor. In all cultivations in bioreactor the dissolved oxygen concentration stayed above 50% of the saturation with air.

In batch cultivation B4 with the $C_{orn-0}$ of 1.32 $g.L^{-1}$, a decrease in CA production was observed in relation to cultivation B2 with 0.66 $g.L^{-1}$ of ornithine. In the fed-batch cultivation FB4 with $C_{orn-F}$ of 11.1 $g.L^{-1}$, a decrease in CA production was also observed in relation to the cultivation FB2 with $C_{orn-F}$=3.7 $g.L^{-1}$. These results suggest that under higher ornithine concentration a possible inhibition of the CA production by ornithine can be occurring.

Figure 3 illustrates the ornithine concentration during the fed-batch cultivations. An accumulation of ornithine with concentrations between 50 and 70 $mg.L^{-1}$ can be observed, four hours after the feeding start-up, no matter the quantity of ornithine fed, showing slow ornithine consumption along 96 hours of cultivation. This indicates that the ornithine uptake rate is controlled by the mass flow rate of the ornithine feed, so the CA production rate is affected by the amino acid feeding condition, while the maximum CA production ($Cp_{max}$) is not. This suggests that CA production has been influenced by other limiting nutrients or inhibited by compounds accumulated in the broth, by-products, or even by the main product, clavulanic acid.
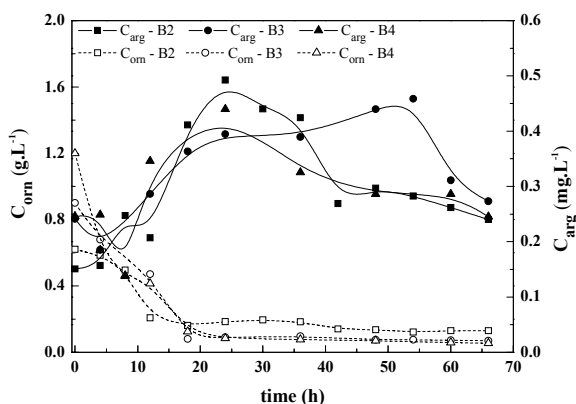


**Figure 2:** Time course of ornithine and arginine in cultivations B2 ($C_{orn-0}$=0.66 $g.L^{-1}$), B3 ($C_{orn-0}$= 0.99 $g.L^{-1}$), and B4 ($C_{orn-0}$= 1.32 $g.L^{-1}$)
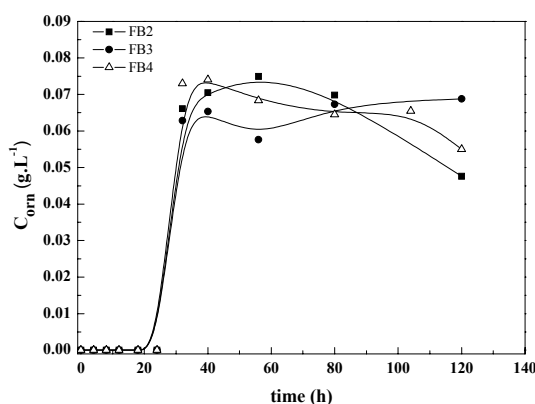


**Figure 3:** Time course of the ornithine concentration in fed-batch cultivations FB2 ($C_{orn-F}$=3.70 $g.L^{-1}$), FB3 ($C_{orn-F}$=7.40 $g.L^{-1}$), and FB4 ($C_{orn-F}$= 11.10 $g.L^{-1}$)

## Effect of Feed Medium Composition and Glycerol Feeding on Fed-Batch Cultivations

The results of the CA concentration along fed-batch cultivations with complex and defined feeding media (FB5 and FB6) are illustrated in Figure 4. It can be noted that, in terms of CA production, no differences were found for the maximum CA productions of about 1.1 g.L$^{-1}$ in 102 h. Therefore, the presence of other nutrients besides glycerol and ornithine in the complex feeding medium had no influence on the CA production in the cultivation conditions studied. Perhaps, the concentration of nutrients coming from the medium is enough for the microorganism to grow and to produce CA. Consequently, the less expensive defined feeding medium containing ornithine, glycerol, and distilled water was chosen in the subsequent cultivations.
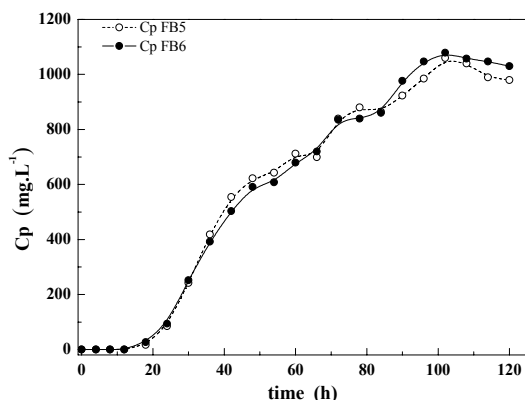


**Figure 4:** Time course of the CA concentration in FB5 (complex medium) and FB6 (defined medium) cultivations with F=0.01 L.h$^{-1}$, $C_{orn-F}$=3.70 g.L$^{-1}$, and $C_{S-F}$=120 g.L$^{-1}$

The effect of glycerol feeding on the biosynthesis of CA was evaluated in four fed-batch cultivations (FB6 to FB9) in a 5-L working volume bioreactor. Figure 5 illustrates the maximum CA concentration ($Cp_{max}$) and the maximum volumetric productivity ($Pp_{max}$) along the fed-batch cultivations. Differently from the ornithine effect, it can be observed that the glycerol feeding conditions influenced both the maximum CA production ($Cp_{max}$) and the maximum volumetric productivity ($Pp_{max}$).

In a previous work, Teodoro et al. (2007) defined F=0.01 L.h$^{-1}$, $Cs_F$=120 g.L$^{-1}$ and the product (F·$Cs_F$=1.2 g.h$^{-1}$) as the best experimental conditions for CA production in cultivations without the presence of ornithine in the culture medium. In the present work, the maximum CA production of 1.506 g.L$^{-1}$ with

$Cs_F$=180 g.L$^{-1}$ and F·$Cs_F$=1.8 g.h$^{-1}$ was reached in the presence of ornithine in the feeding medium at the concentration of 3.7 g.L$^{-1}$ ($C_{orn-F}$) showing that there is a combined positive effect of ornithine and glycerol in the biosynthesis of CA. It can be observed that $Cp_{max}$ increased with the glycerol concentration in the feeding medium up to $C_{S-F}$=180 g.L$^{-1}$ in the FB8 cultivation, showing that at values of $C_{SF}$ higher than 180 g.L$^{-1}$ (F·$C_{S-F}$=1.8 g.h$^{-1}$) the CA production seems to be inhibited by the glycerol.
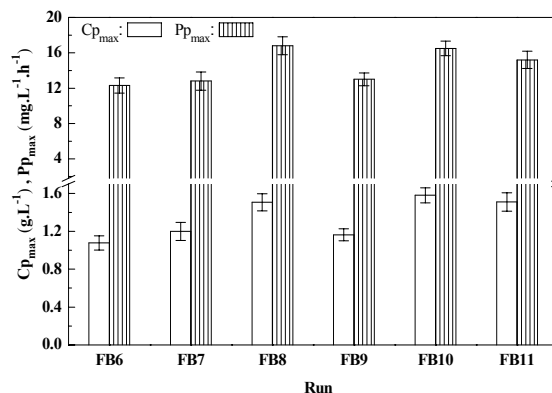


**Figure 5:** Influence of glycerol feeding on maximum CA concentration ($Cp_{max}$) and maximum volumetric productivity of CA ($Pp_{max}$) in fed-batch cultivations

The best results in terms of CA production were confirmed by running two fed-batch cultivations in a 10-L bioreactor (FB10 and FB11). In fed-batch FB10, the glycerol and ornithine feeding conditions were the same utilized in cultivation FB8 in a 5-L bioreactor ($Cs_F$=180 g.L$^{-1}$ and $C_{orn-F}$=3.7 g.L$^{-1}$). In fed-batch FB11, the ornithine concentration was set at 5.5 g.L$^{-1}$ in order to keep the same $C_{S-F}$/$C_{orn-F}$ ratio utilized in fed-batch FB2, in which the highest value of maximum productivity was reached. It can be observed in Figure 5 that the highest maximum CA production of approximately 1.6 g.L$^{-1}$ with $Pp_{max}$ of 16.5 mg.L$^{-1}$.h$^{-1}$ was obtained in fed-batch FB10 in accordance with the best result obtained in fed-batch FB8 in a 5-L bioreactor utilizing $C_{SF}$/$C_{ornF}$=180/3.7=48.6. The low demand for ornithine indicates that it is utilized mainly as CA precursor.

The maximum CA concentration and the CA productivity found in the present work, 1.6 g.L$^{-1}$ and 18.6 mg.L$^{-1}$.h$^{-1}$, respectively, are the highest values ever published in the literature in batch and fed-batch cultivations utilizing a wild strain of *S. clavuligerus* in cultivations with similar complex media, i.e., with soybean derivatives and glycerol or soybean oil as nitrogen and carbon sources, respectively (Mayer and Deckwer, 1996; Chen et al., 2002; Chen et al.,

2003; Wang et al., 2005; Teodoro et al., 2006; Ortiz et al., 2007). Recently, Saudagar and Singhal (2007) reported a high CA production (about 1.8 g.L$^{-1}$) in shake flasks utilizing *S. clavuligerus* MTCC 1142 cultivated in synthetic culture and feeding L-threonine. The cultivation conditions proposed by these authors were assayed utilizing *S. clavuligerus* ATCC 27064 in shake flasks and in a bench scale bioreactor. Nevertheless, the results in terms of CA production were much lower than those reported by the authors, which is about 50 mg.L$^{-1}$. Indeed, Gouveia et al. (1999) cultivated *S. clavuligerus* NRRL 3585 in GSPG medium and obtained a maximum CA production of 25 mg.L$^{-1}$ and around 90 mg.L$^{-1}$ in GSPA medium. Table 2 shows the results in terms of yield coefficients obtained for both batch and fed-batch cultivations performed under different experimental conditions.

**Table 2: Yield coefficients and efficiency of CA production in relation to ornithine consumed obtained in batch and fed-batch cultivations performed under different experimental conditions**

| Run | $Y_{P/S}$ (g/g) | $Y_{P/Orn}$ (g/g) | $\eta_P$ (%) |
|---|---|---|---|
| B1 (control) | 0.032 | - | - |
| B2 | 0.035 | 1.056 | 46.7 |
| B3 | 0.027 | 0.523 | 23.1 |
| B4 | 0.025 | 0.337 | 14.9 |
| FB1 | 0.033 | - | - |
| FB2 | 0.034 | 0.807 | 35.7 |
| FB3 | 0.037 | 0.696 | 30.8 |
| FB4 | 0.039 | 2.472 | 109.4 |
| FB5 | 0.029 | 1.467 | 64.9 |
| FB6 | 0.028 | 1.431 | 63.3 |
| FB7 | 0.025 | 1.616 | 71.5 |
| FB8 | 0.029 | 1.993 | 88.2 |
| FB9 | 0.025 | 1.003 | 44.4 |
| FB10 | 0.027 | 1.779 | 78.8 |
| FB11 | 0.026 | 1.144 | 50.6 |

The product (CA) yield coefficients in relation to the glycerol consumption ($Y_{P/S}$) and to the ornithine consumption ($Y_{P/Orn}$) were calculated based on the mass of glycerol and ornithine consumed and the mass of the CA produced. Based on the stoichiometry proposed by Bushell et al. (2007) and on the fact that ornithine is converted to arginine and incorporated into the CA molecule, 2 moles of ornithine originates 3 moles of CA, so the stoichiometric coefficient ($Y_{St}$) is 1.5 moles CA/mol ornithine or 2.26 g CA/g ornithine on a mass basis. In their work, Bushell et al. (2007) obtained clavulanic acid yields in relation to glycerol from $0.40 \cdot 10^{-4}$ g$_P$.g$_S^{-1}$ (D=0.10 h$^{-1}$) to $5.8 \cdot 10^{-4}$ g$_P$.g$_S^{-1}$ (D=0.03 h$^{-1}$). In the present work, the values of $Y_{P/S}$ for batch and fed-batch cultivations varied within the narrow

range of 0.025 and 0.039 g$_P$.g$_S^{-1}$ for smaller dilution rates (D), from 0.002 to 0.003 h$^{-1}$. Therefore, it is clear that $Y_{P/S}$ is strongly affected by the culture medium composition and by glycerol feeding conditions. With respect to clavulanic acid yields in relation to ornithine ($Y_{P/Orn}$), the values of efficiency ($\eta_P$) calculated in relation to the stoichiometric value ($Y_{St}$) as being $\eta_P=100 \times Y_{P/Orn}/Y_{St}$, are also shown in Table 2. High values of $\eta_P$, above 50%, were obtained in fed-batch cultivations performed with low ornithine concentration in the feeding medium (3.70 g.L$^{-1}$). The highest value (109.4%) was obtained in the run FB4. The percentage above 100% can be explained by the additional conversion of arginine, initially present in the complex medium, into clavulanic acid. Independent of the bench scale bioreactor (5 and 10-L), the CA efficiencies in relation to ornithine were associated with the highest CA productions in fed-batch cultivations performed with $C_{S-F}$=180 g.L$^{-1}$ and low ornithine concentration in the feeding medium (3.70 g.L$^{-1}$) (FB8 and FB10).

**CONCLUSION**

In batch bioreactor cultivations, CA productivity increased in the presence of ornithine. However, CA production was little affected by ornithine, in contrast with reports in the literature. Unlike CA productivity, the $Cp_{max}$ was little affected by the presence of ornithine in fed-batch cultivations with ornithine feeding. It can be said that, in batch and fed-batch cultivations, the ornithine feeding condition affects the CA production rate, but has no effect on $Cp_{max}$, suggesting that the CA production was influenced by other limiting nutrients or inhibited by compounds such as by-products, or even by clavulanic acid itself.

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