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PRODUCTION OF CLAVULANIC ACID AND CEPHAMYCIN C BY *Streptomyces clavuligerus* UNDER DIFFERENT FED-BATCH CONDITIONS

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Abstract - The effect of carbon source and feeding conditions on the production of clavulanic acid (CA) and cephamycin C (CephC) by *Streptomyces clavuligerus* was investigated. In fed-batch experiments performed with glycerol feeding, production of CA exceeded that of CephC, and reached 1022 $mg.L^{-1}$. Highest CephC production (566.5 $mg.L^{-1}$) was obtained in fed-batch cultivation with glycerol feeding. In fed-batch experiments performed with starch feeding, the production of CephC was in general higher than that of CA. A dissociation index (DI) was used to identify feeding conditions that favored production of CephC relative to CA. In all cultures with glycerol, DI values were less than unity, indicating higher production of CA compared to CephC. Conversely, in cultures fed with starch, the DI values obtained were greater than unity. However, no carbon source or feeding condition was able to completely dissociate the production of CA from that of CephC.
Keywords: *Streptomyces clavuligerus*; Clavulanic acid; Cephamycin C; Fed-batch.

INTRODUCTION

Since the discovery of penicillin, thousands of molecules with antibiotic properties have been identified. Although a wide variety of microorganisms synthesize antibiotics, the majority of the clinically useful compounds are produced by actinomycetes (Khetan *et al.*, 1999). Among these organisms, members of the genus *Streptomyces* are responsible for the production of about 80% of the known secondary metabolites, especially antibiotics (Challis and Hopwood, 2003). *Streptomyces clavuligerus* is an actinomycete that produces clinically useful β-lactam compounds, such as cephamycin C and clavulanic acid (Paradkar *et al.*, 1996).

Cephamycin C (CephC) is highly resistant to the β-lactamase enzymes that cleave the β-lactam ring. Its molecular structure resembles that of cephalosporin C, but with a methoxy group at the 7α position of the β-lactam ring, which stabilizes the structure of the ring (Stapley *et al.*, 1972). CephC is currently used as an intermediate for important semi-synthetic antibiotics such as cefoxitin, cefmetazole, and cefotetan. Clavulanic acid (CA) has weak anti-bacterial activity; however, it is a potent β-lactamase inhibitor, and its combination with amoxicillin is the most successful example of the joint use of a β-lactam antibiotic sensitive to β-lactamase, together with an inhibitor of these enzymes (Mayer and Deckwer, 1996).

The biosynthetic routes of CephC and CA are totally different, and as for most bioactive compounds, they are rigorously controlled by intra-and extracellular factors through complex regulation

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mechanisms involving components of the culture medium and intermediate compounds of the primary metabolite, which participate directly or indirectly in the biosynthesis (Bibb, 2005). The presence of certain nutrients in the culture medium, such as sources of C, N, P, S, and salts, as well as substances produced in the biosynthetic route, can enhance the production of CA or CephC (Fang and Demain, 1995; Chen *et al.*, 2002, Oliveira *et al.*, 2009). Glycerol, for example, is preferentially used as carbon source for CA production (Chen *et al.*, 2002; Elson and Oliver, 1978; Romero *et al.*, 1984; Teodoro *et al.*, 2006, 2010), whereas starch is more suitable for the production of CephC (Lebrihi *et al.*, 1988; Rius and Demain, 1997).

The concentration of dissolved oxygen in the culture medium is another important aspect of the production processes of both CephC and CA. Previous work has shown that a high oxygen level is fundamental for the biosynthesis of CA and CephC (Rollins *et al.*, 1988a, 1988b, 1991; Rosa *et al.*, 2005; Yegneswaran *et al.*, 1988, 1991). The influence of carbon source and feeding conditions on production of both CA and CephC should therefore be studied under conditions that are not oxygen limited, which is possible using submerged bioreactor cultures.

In the CA and CephC production process, not only is the type of carbon source important, but also its uptake rate, which can be controlled by adjusting feeding conditions in fed-batch cultures. Fed-batch strategies can be used to overcome substrate inhibition and metabolic repression by an easily metabolized carbon source. These techniques have been optimized in order to control the growth rate and prolong the stationary phase.

Although many reports have emphasized the important role of glycerol feeding in the CA production process, using either batch or fed-batch cultures, information remains scarce concerning CephC production. Saudagar and Singhal (2007) studied the effect of glycerol feeding on CA production by *S. clavuligerus* in shake flasks, and observed an increase of 18% in CA production compared to the control. Teodoro *et al.* (2006) studied the effect of feed medium glycerol concentration and volumetric flow rate in fed-batch cultures for CA production, and the best experimental conditions were found to be a volumetric flow rate of 0.01 $L.h^{-1}$ and a feed medium with glycerol concentration of 120 $g.L^{-1}$, with production of 719 $mg.L^{-1}$ of CA. Teodoro *et al.* (2010) obtained a production of 1.6 $g.L^{-1}$ of CA in fed-batch cultivation with glycerol feeding. Recent work has reported the production of CephC using solid-state fermentation. Kagliwal *et al.* (2009) studied the production of CephC by *Nocardia lactamdurans* in solid fermentation using different substrates, and observed an increase of the CephC yield from 8.4 to 27.6 $mg.g^{-1}$ with addition of 1,3-diaminopropane. Bussari *et al.* (2008) studied CephC production by *S. clavuligerus* using solid fermentation, and reported a maximum CephC production when cottonseed meal was used as substrate.

In the present study, the effect of carbon source and feeding conditions on production of both CA and CephC was investigated in cultures of *S. clavuligerus* performed under non-oxygen limitation conditions. Two batch cultures and fourteen fed-batch cultures were performed in a conventional bench-scale bioreactor, with different conditions of glycerol or starch feeding. The best conditions for CA and CephC production were defined, as well as the influence of the carbon source feeding strategy on the relative production of CA and CephC.

MATERIALS AND METHODS

Microorganism

Streptomyces clavuligerus DSM 41826 was used throughout this work, and was stored as vegetative cells at -80 ºC in cryotubes using glycerol (10% $v.v^{-1}$).

Culture Media and Experimental Conditions

The seed medium described by Kieser *et al.* (2000) was used in the present work. The inoculum medium contained (in $g.L^{-1}$ distilled water): glycerol, 10.0; soybean meal, 11.0; L-lysine, 18.3; yeast extract, 0.5; K_2HPO_4, 1.75; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.2; NaCl, 2.0; $FeSO_4.7H_2O$, 0.005; $MnCl_2.4H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.005; MOPS buffer, 21 (100 mM). The pH of the medium was 6.8. The production medium had the same composition as the inoculum medium, except that no MOPS buffer was used. Silicone antifoam solution (0.1 $mL.L^{-1}$) and sodium thiosulfate (1.0 $g.L^{-1}$) were added at the beginning and after 30 h of fermentation, respectively.

Batch and fed-batch cultivations were performed in a Bioflo II fermentor (New Brunswick Sci. Co. Inc., USA) with a 5 L total volume. All cultivations were conducted at 28 ºC, 800 rpm, and 0.5 vvm based on a 4 L working volume (2 $L.min^{-1}$). The pH was automatically controlled to remain at 6.8 ± 0.1 using additions of 2 M HCl or 1 M NaOH solutions.

Cryotube cell suspensions (3.5 mL) were inoculated into 50 mL of seed medium in a 500 mL

Erlenmeyer flask, and incubated in a rotary shaker (New Brunswick Scientific) for 24 h at 28 ºC and 250 rpm. Erlenmeyer flasks (500 mL) containing 45 mL of inoculum medium were inoculated with 5 mL of cultivated seed broth, and incubated in a rotary shaker for 24 h at 28 ºC and 250 rpm. Quantities of inoculum corresponding to 10% (v/v) were transferred to the fermentors.

The fed-batch operation was started at 24 h after inoculation by feeding the medium containing glycerol (Cs_{Fg}) or starch (Cs_{Fs}), at concentrations of 40.0-300.0 $g.L^{-1}$, at volumetric flow rates (F) of 0.005 and 0.01 $L.h^{-1}$.

The experimental conditions used for the batch cultures (B) were the following: Cs_0=15 $g.L^{-1}$ glycerol (B1) and Cs_0=15 $g.L^{-1}$ starch (B2). The experimental conditions of the fed-batch (FB) cultures are presented in Table 1.

Table 1: Experimental conditions of the fed-batch cultures.

Run	Carbon Source	F ($L.h^{-1}$)	Cs_F ($g.L^{-1}$)
FB1	glycerol	0.005	100.5
FB2	glycerol	0.005	201.0
FB3	glycerol	0.005	301.5
FB4	glycerol	0.01	44.3
FB5	glycerol	0.01	88.5
FB6	glycerol	0.01	132.8
FB7	glycerol	0.01	177.0
FB8	glycerol	0.01	221.3
FB9	glycerol	0.01	265.5
FB10	starch	0.005	100.5
FB11	starch	0.005	201.0
FB12	starch	0.01	44.3
FB13	starch	0.01	88.5
FB14	starch	0.01	132.8

All experiments were performed in duplicate. Samples of about 20 mL were withdrawn at approximately 6 h intervals. A fraction of each sample (10 mL) was used for determination of rheological parameters, namely the consistency index, K (in $Pa.s^n$), and the flow index, n (dimensionless), using the power law model. The remainder of the sample (~10 mL) was centrifuged at 3720×g and 5 °C for 20 min, and the supernatants were used for the determination of antibiotic and substrate concentrations. The fed-batch cultures were standardized in such a way that, at the end of the process (after 120 h), the working volume was 4 L (in a 5 L bioreactor).

Analytical Methods

Fermentation broths containing filamentous microorganisms exhibit non-Newtonian (pseudo-plastic) behavior. Due to the presence of insoluble particles in the broth, cell growth was evaluated indirectly by determining the consistency index and flow index of the broth from the power law model, using a Brookfield concentric-cylinder rheometer. It has been found previously that the consistency index (K) is the most appropriate parameter to use to infer cell growth in a fermentation broth containing insoluble particles, as in the present case. This parameter is related to the cell concentration and to the morphology of the filamentous microorganisms (Badino *et al.*, 1999).

Glycerol was determined by high performance liquid chromatography (HPLC), with Milli-Q water as the mobile phase. The equipment was operated at 80 ºC, using a flow rate of 1 $mL.min^{-1}$, and a calibration curve was constructed for glycerol concentrations ranging from 0.1 to 1.0 $g.L^{-1}$. A Shodex KS-802 (Lonpak) column and a W410 refractive index detector (Waters) were used.

Starch was determined by the dinitrosalicylic acid (DNS) colorimetric assay method, after previous hydrolysis with 10 M HCl at 100 ºC for 30 min.

CA was determined by HPLC, as described by Foulstone and Reading (1982). The pharmaceutical product Clavulin (Glaxo-SmithKline Farmacêutica, Rio de Janeiro, Brazil) was used for the CA standards. The imidazole derivative of CA was injected onto C-18 µ-Bondapack (Waters) column, used as the stationary phase; the mobile phase was composed of 94% 0.1 M KH_2PO_4 (pH 3.2) and 6% methanol (v/v). The CA derivative was detected at 311 nm.

Cephamycin C concentrations were measured by HPLC using a Sinergy MAXPP 12 (Phenomenex) column, with 0.01 M acetic acid as the mobile phase. The equipment was operated at 28 ºC, with a flow rate of 2 $mL.min^{-1}$. The calibration curve was obtained by relating the area of the peak to the diameter of the inhibition halo obtained in the bioassay, using cephalosporin C (Sigma-Aldrich) as standard (Liras and Martin, 2005).

RESULTS AND DISCUSSION

Batch Cultures

Firstly, two batch experiments were carried out using either glycerol (B1) or starch (B2) as the carbon source. These cultures were considered as control runs in order to compare the CA and CephC production results with those obtained in the fed-batch experiments. Figure 1 illustrates the evolution of cell growth, lysine and substrate consumption, and CA and CephC production during the course of cultures B1 and B2.

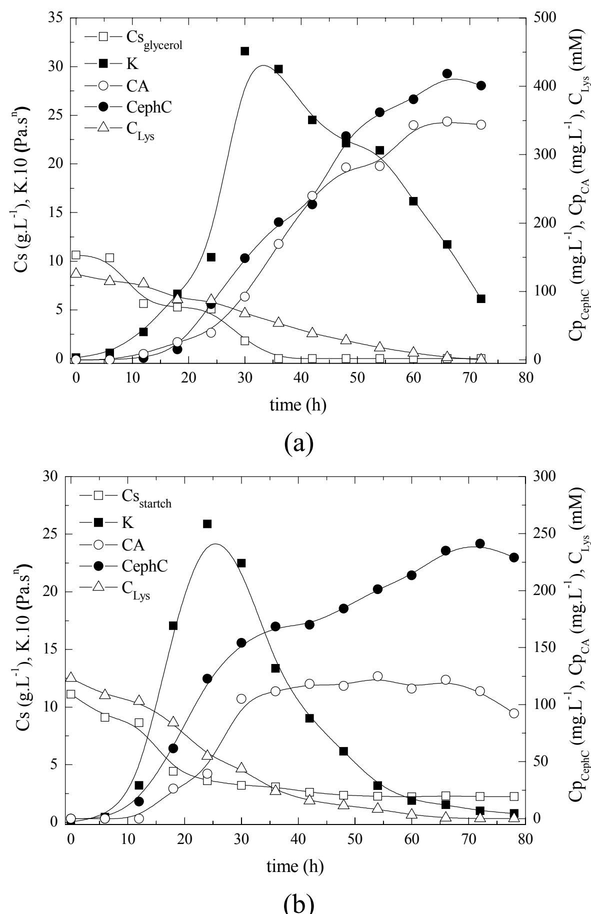

Figure 1: Time course of the concentrations of carbon source, lysine, CA, CephC, and consistency index (K) in batch cultures: (a) culture B1 (glycerol as carbon source) and (b) culture B2 (starch as carbon source).

The minimum dissolved oxygen concentrations (DO) in the two cultures were 50 and 70% of the saturation with air, respectively. Both cultures showed similar cellular growth, evaluated by the consistency index of the broth (K), and lysine consumption. The consistency index (K) reached average maximum values, at the end of carbon source consumption (24 h), of 3.2 and 2.6 Pa.s^n in cultures B1 and B2, respectively. In culture B2, a residual concentration of reducing sugars of approximately 2.2 g.L^{-1} was observed. *S. clavuligerus* is able to consume oligosaccharides; however, it is unable to assimilate glucose, which accumulated in the fermentation broth at the end of the cultivation (Ives and Bushell, 1997). In culture B1 (glycerol), the maximum concentrations (Cp_{max}) of CA and CephC (348.5 and 418.5 mg.L^{-1}, respectively) were obtained after 66 h of cultivation. In culture B2 (starch), the maximum concentrations of CA (125.2 mg.L^{-1}) and CephC (241.0 mg.L^{-1}) were reached after 54 and 72 h, respectively. In both batch cultures, with glycerol or starch, there was production of CephC and CA, with production of CephC exceeding that of CA. However, in the presence of starch, CephC production was about two-fold higher than that of CA. Zhang and Demain (1992) observed that the use of 2% (v/v) of glycerol as carbon source resulted in less than 50% of the specific CephC production obtained with 0.5% (v/v) of glycerol.

As expected, CA production was higher using glycerol as carbon source, in agreement with earlier work (Elson and Oliver, 1978; Romero *et al.*, 1984). Nevertheless, glycerol was more effective than starch for CephC production, in disagreement with the literature. Lebrihi *et al.* (1988) carried out cultures of *S. clavuligerus* in culture media with either starch or glycerol added as carbon source, and observed that the final biomass obtained with glycerol was higher than with starch, although CephC production was lower. In the present work, CA production occurred using starch as carbon source (culture B2), in contrast to the findings of Romero *et al.* (1984), where no formation of CA was observed in the absence of glycerol, suggesting that under the conditions employed the biosynthesis of CephC was dissociated from that of CA. The difference between the results reported previously (using shake flasks) and in the present work (using a bench-scale bioreactor) could be due to the different mixing and oxygen transfer conditions in the two systems.

Fed-Batch Cultures

Fourteen fed-batch cultures were performed using glycerol (FB1 to FB9) and starch (FB10 to FB14) as carbon source. The ranges of the fed-batch conditions Cs_F and F were chosen based on the average substrate uptake rate at the end of the batch cultivation periods (r_S=-dCs/dt), considering the "quasi-steady state" condition, when the substrate uptake rate is controlled by the substrate feeding conditions (r_S=F·Cs_F/V). In both batch cultures, with glycerol or starch, the average substrate uptake rate (r_S) was approximately 0.25 g.L^{-1}.h^{-1}.

Fed-Batch Cultures with Glycerol Feeding

In the fed-batch experiments performed with glycerol feeding, the consistency index (K) of the broths varied from 3.8 (FB1) to 5.8 Pa.s^n (FB9). However, no correlation was observed between cellular concentration and CA and CephC production.

With respect to the dissolved oxygen concentration (DO), in all cultures the minimum DO values were above 40% of the saturation with air, showing that no oxygen limitation occurred.

The fed-batch cultures with glycerol feeding were separated into two sets, with dilution rates (D) of either 0.0013 h^{-1} (FB1 to FB3) or 0.0026 h^{-1} (FB4 to FB9). During the glycerol feeding period, no residual glycerol was detected in the broths, which showed that the "quasi-steady state" condition was reached and maintained in all cultures. Figures 2 and 3 illustrate the maximum CA (Cp_{CAmax}) and CephC ($Cp_{CephCmax}$) concentrations as a function of the average substrate uptake rate (r_S), at dilution rates (D) of 0.0013 and 0.0026 h^{-1}, respectively.

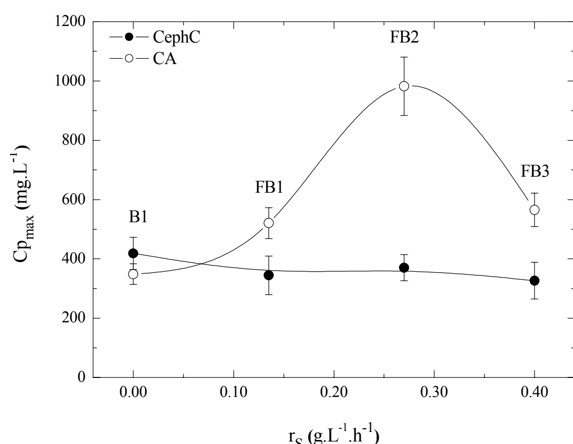

Figure 2: Maximum concentrations of CA and CephC as a function of the average substrate uptake rate (r_S) in fed-batch cultures with glycerol feeding, at a dilution rate (D) of 0.0013 h^{-1}.

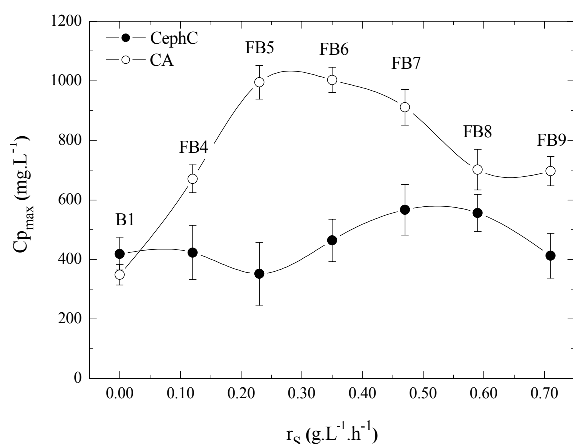

Figure 3: Maximum concentrations of CA and CephC as a function of the average substrate uptake rate (r_S) in fed-batch cultures with glycerol feeding, at a dilution rate (D) of 0.0026 h^{-1}.

During all glycerol feeding periods (stationary phase), maximum CA production (Cp_{CAmax}) was greater than maximum CephC production ($Cp_{CephCmax}$), independent of the dilution rate (D).

In Figures 2 and 3, it can be observed that the maximum CephC production seemed not to be affected by glycerol feeding in the range studied. In fed-batch cultures at D=0.0013 h^{-1}, the $Cp_{CephCmax}$ values were lower than for the control run. The highest $Cp_{CephCmax}$ value (566.5 $mg.L^{-1}$) was obtained at r_S=0.47 $g.L^{-1}.h^{-1}$ and D=0.0026 h^{-1} (FB7). However, at D=0.0026 h^{-1} the standard deviations of the results showed that the $Cp_{CephCmax}$ values were similar for the range of r_S values. A different result was observed for CA production. The maximum CA concentration (Cp_{CAmax}) was strongly dependent on the glycerol feeding conditions. Similar values of Cp_{CAmax} were obtained at similar values of r_S, independent of the dilution rate used. A value of Cp_{CAmax} of 982.1 $mg.L^{-1}$ was obtained at r_S=0.27 $g.L^{-1}.h^{-1}$ when the bioreactor was operated at D=0.0013 h^{-1} (FB2), and Cp_{CAmax} values in the range from 995.2 to 1002.6 $mg.L^{-1}$ were obtained at $0.24<r_S<0.35$ $g.L^{-1}.h^{-1}$ and D=0.0026 h^{-1} (FB5 and FB6). It is known from the literature that the carbon source most commonly used for CA production by *S. clavuligerus* is glycerol, which in the biosynthetic route of CA is converted to D-glyceraldehyde-3-phosphate, the primary metabolic precursor of CA (Khaleeli *et al.*, 1999).

Fed-Batch Cultures with Starch Feeding

Fed-batch cultures with starch feeding were separated into two sets, with dilution rates (D) of 0.0013 h^{-1} (FB10 to FB11) and 0.0026 h^{-1} (FB12 to FB14). The consistency index (K) of the broths varied between 2.4 (FB10) and 6.2 $Pa.s^n$ (FB14). Again, no correlation was observed between cellular concentration and CA and CephC production. The dissolved oxygen concentration remained between 54 and 75% of the saturation with air, showing that there was no oxygen limitation. The negative effect of low DO concentration on CephC and CA production has been recognized previously (Rollins *et al.*, 1988a; Rosa *et al.*, 2005; Yegneswaran *et al.*, 1991). However, in the present work the production of CephC and CA was not affected, since high DO levels were always present.

In all cultures, a residual concentration of reducing sugars, of approximately 2.5 $g.L^{-1}$ (data not shown), was observed in the feeding phase, due to the inability of *S. clavuligerus* to consume glucose, which therefore accumulated in the broth. Figures 4 and 5 illustrate the maximum concentrations of CA

(Cp_{CAmax}) and CephC ($Cp_{CephCmax}$) as a function of the average substrate uptake rate (r_S) in the fed-batch cultures with starch feeding, at dilution rates (D) of 0.0013 h^{-1} and 0.0026 h^{-1}, respectively. In contrast to the previous results for the cultures with glycerol feeding, CephC production in the cultures fed with starch was dependent on the feeding conditions. A $Cp_{CephCmax}$ value of 356.7 $mg.L^{-1}$ at r_S=0.13 $g.L^{-1}.h^{-1}$ was obtained when the bioreactor was operated at D=0.0013 h^{-1} (FB10), while a value of 379.6 $mg.L^{-1}$ was obtained at r_S=0.27 $g.L^{-1}.h^{-1}$ and D=0.0026 h^{-1} (FB13). Contradicting the literature, both CA and CephC formation occurred in the cultures fed with starch, as observed using batch cultivation. As in the cultures using glycerol, maximum CA production was also influenced by the starch feeding conditions. A Cp_{CAmax} value of 209.7 $mg.L^{-1}$ was obtained at r_S=0.13 $g.L^{-1}.h^{-1}$ and D=0.0013 h^{-1} (FB10), while a value of 469.0 $mg.L^{-1}$ was obtained at r_S=0.35 $g.L^{-1}.h^{-1}$ and D=0.0026 h^{-1} (FB14). In fed-batch cultures with starch, for the different feeding conditions the values of $Cp_{CephCmax}$ and Cp_{CAmax} were much lower than those obtained using glycerol as carbon source. Additionally, maximum production of CephC was higher than that of CA, except for r_S=0.35 $g.L^{-1}.h^{-1}$ and D=0.0026 h^{-1}, when there was probably an inhibition of CephC production by excess of substrate, due to the high level of feeding. Starch did not appear to be a good source of carbon for the production of either CA or CephC. In the batch culture (control run) that used glycerol as carbon source (B1), the maximum levels of production of CephC and CA were similar to or higher than those obtained using fed-batch culture with starch feeding. The production of CA in fed-batch cultures has been widely reported in the literature (Chen *et al.*, 2002, 2003; Mayer and Deckwer, 1996; Teodoro *et al.*, 2006, 2010). On the other hand, there is only one work reporting CephC production in fed-batch culture with a wild strain of *S. clavuligerus*, in which a maltose-fed culture of *S. clavuligerus* showed a 2-fold reduction in deacetoxycephalosporin C synthase (DAOCS) activity and CephC production, compared to a control run without maltose feeding (Rollins *et al.*, 1988a). The absence of accumulation of the intermediate penicillin N, together with repression of the ring-expansion enzyme DAOCS, indicated that maltose interfered in CephC production by directly regulating enzymes in the earlier part of the pathway (such as AVC synthetase) and the later ring-expansion step. Furthermore, in relation to the regulation of CephC biosynthesis, Lebrihi *et al.* (1988) observed that excess starch reduced CephC production in cultures of *S. clavuligerus*, although the effect was slight compared to that of glycerol. Such behavior was not observed in the present work using cultures fed with either glycerol or starch.

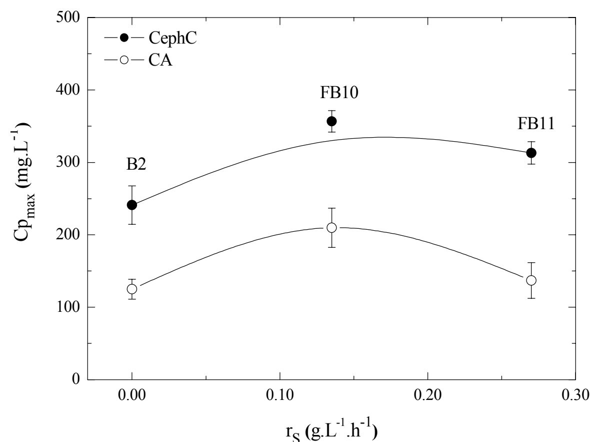

Figure 4: Maximum concentrations of CA and CephC as a function of the average substrate uptake rate (r_S) in fed-batch cultures with starch feeding, at a dilution rate (D) of 0.0013 h^{-1}.

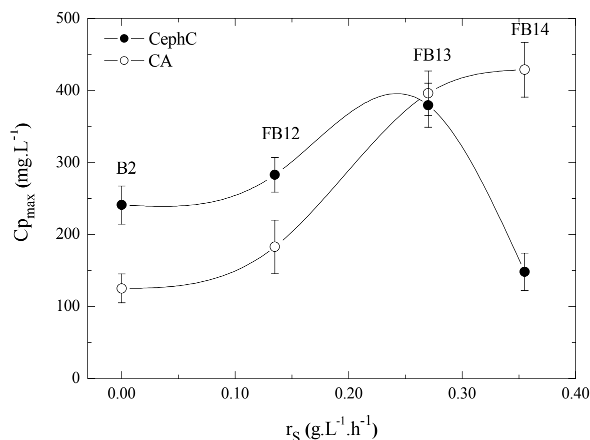

Figure 5: Maximum concentrations of CA and CephC as a function of the average substrate uptake rate (r_S) in fed-batch cultures with starch feeding, at a dilution rate (D) of 0.0026 h^{-1}.

Table 2 shows the CephC and CA yield coefficients ($Y_{P/S}$) obtained for both batch and fed-batch cultivations performed under different experimental conditions. Values of $Y_{P/S}$ (CephC) for batch cultures fed with glycerol or starch were higher than the $Y_{P/S}$ values obtained for fed-batch cultures, indicating that the carbon source was not positive for CephC production by *S. clavuligerus*. Moreover, due to higher maximum CephC production ($Cp_{CephCmax}$), $Y_{P/S}$ (CephC) values for glycerol-fed cultures were higher than those obtained for feeding with starch. The $Y_{P/S}$ (CA) values obtained for glycerol-fed cultures

varied within the range from 0.013 to 0.041 $g_P.g_S^{-1}$, close to the range of values obtained by Teodoro *et al.* (2010) of 0.025 to 0.039 $g_P.g_S^{-1}$ for dilution rates (D) between 0.002 and 0.003 h^{-1}. Bushell *et al.* (2007) obtained CA yields for glycerol ranging from $0.40x10^{-4}$ $g_P.g_S^{-1}$ (D=0.10 h^{-1}) to $5.8x10^{-4}$ $g_P.g_S^{-1}$ (D=0.03 h^{-1}) in chemostat cultures of *S. clavuligerus* fed with a synthetic culture medium supplemented with amino acids. It is therefore clear that $Y_{P/S}$ is strongly affected by the culture medium composition and by glycerol feeding conditions. Again, due to higher maximum CA production, $Y_{P/S}$ (CA) values for glycerol-fed cultures were higher than those obtained for feeding with starch.

A dissociation index (DI) was elaborated in order to try to find a feeding condition that favored CephC production over CA production. This was defined as the relationship between the maximum production of CephC and CA ($Cp_{CephCmax}/Cp_{CAmax}$). Table 2 shows DI values for different feeding conditions. In all cultures fed with glycerol, the DI values were less than unity (DI<1), indicating that production of CA was higher than that of CephC. In cultures fed with starch, DI values higher that the unity were found, indicating that starch promoted the production of CephC (with the exception of culture FB14, where r_S=0.35 $g.L^{-1}.h^{-1}$, when CephC production was possibly inhibited by the high starch feeding conditions). However, it can be observed that for the wild strain of *S. clavuligerus* used in the present work, no carbon source or feeding condition completely dissociated the production of CephC from that of CA.

The maximum CA value obtained in this work was 1002.6 $mg.L^{-1}$ (FB6), lower than the 1.6 $g.L^{-1}$ found by Teodoro *et al.* (2010) using cultures of *S. clavuligerus* ATCC 27064, which is the highest value ever published in the literature for a wild strain. However, Teodoro *et al.* (2010) fed their cultures with glycerol and ornithine, and used soybean protein isolate as nitrogen source, without the carbohydrates present in soybean meal that can inhibit CA production. In the case of CephC, a good production of 566.5 $mg.L^{-1}$ (FB7) was obtained here. The literature reports a maximum CephC production of 300 $mg.L^{-1}$ using a wild strain of *S. clavuligerus* NRRL 3585 in a 10 L working volume bioreactor (Rollins *et al.*, 1988a).

Table 2: Experimental conditions and main results obtained in batch and fed-batch cultures fed with glycerol and starch for CA and CephC production.

Run	Carbon Source	$r_s = \frac{F.Cs_F}{V}$ ($g.L^{-1}.h^{-1}$)	$C_{CephCmax}$ ($mg.L^{-1}$)	C_{CAmax} ($mg.L^{-1}$)	$Y_{P/S}$ (CephC) ($g.g^{-1}$)	$Y_{P/S}$ (CA) ($g.g^{-1}$)	$\frac{C_{CephC\,max}}{C_{CA\,max}}$
B1	glycerol	0.00	418.5	348.5	0.039	0.033	1.20
B2	starch	0.00	241.0	125.0	0.024	0.012	1.93
FB1	glycerol	0.13	344.5	520.7	0.023	0.029	0.66
FB2	glycerol	0.27	370.3	982.1	0.013	0.035	0.38
FB3	glycerol	0.40	326.5	565.5	0.009	0.013	0.58
FB4	glycerol	0.12	423.0	670.8	0.025	0.039	0.63
FB5	glycerol	0.24	351.8	995.2	0.015	0.041	0.35
FB6	glycerol	0.35	463.9	1002.6	0.014	0.027	0.46
FB7	glycerol	0.47	566.5	911.1	0.012	0.019	0.62
FB8	glycerol	0.59	556.0	701.2	0.011	0.016	0.79
FB9	glycerol	0.71	411.9	696.6	0.007	0.013	0.59
FB10	starch	0.13	356.7	209.7	0.017	0.012	1.70
FB11	starch	0.27	313.0	136.9	0.014	0.011	3.23
FB12	starch	0.12	283.0	183.0	0.010	0.006	1.55
FB13	starch	0.27	379.6	396.1	0.007	0.010	1.03
FB14	starch	0.35	148.1	429.0	0.006	0.017	0.32

CONCLUSIONS

In batch cultures employing glycerol and starch, there was production of both CephC and CA, and CephC production exceeded that of CA. In the presence of starch, CephC production was about two times higher than that of CA.

The maximum CephC production was not affected by glycerol feeding conditions in the experimental range studied, but maximum CA production was strongly dependent on these conditions. On the other hand, for the cultures fed with starch the maximum CephC production was dependent on the carbon source feeding conditions.

In all experiments where glycerol feeding was employed, CA production was greater than CephC production. In fed-batch experiments performed with starch feeding, there was greater production of CephC than CA, except for r_S=0.35 $g.L^{-1}.h^{-1}$, where there was probably an excess substrate inhibition of CephC production.

For batch cultures using glycerol or starch, the product yield coefficient ($Y_{P/S}$) values for CephC were higher than the values obtained for fed-batch cultures, and $Y_{P/S}$ values for glycerol-fed cultures varied within a wide range, reflecting a strong influence of the glycerol feeding conditions.

Dissociation index values lower than unity (DI<1) for all glycerol-fed cultures indicated higher production of CA than CephC, while conversely DI values higher than unity were obtained for cultures fed with starch. However, no carbon source or feeding condition was able to dissociate the production of CA and CephC by the wild strain of *S. clavuligerus* used in this work.

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NOMENCLATURE

CephC	cephamycin C	
CA	clavulanic acid	
DI	dissociation index	(-)
C_{SFg}	feed medium containing glycerol	$g.L^{-1}$
C_{SFs}	feed medium containing starch	$g.L^{-1}$
F	volumetric flow rate	$L.h^{-1}$
B	batch	
FB	fed-batch	
K	consistency index	$Pa.s^n$
n	behavior index	(-)
DO	dissolved oxygen concentration	%
r_S	substrate uptake rate	$g.L^{-1}.h^{-1}$
$Cp_{Cephmax}$	maximum CephC concentration	$g.L^{-1}$
Cp_{CAmax}	maximum CA concentration	$g.L^{-1}$
D	dilution rate	h^{-1}
$Y_{P/S}$	product yield coefficient	$g_P.g_S^{-1}$

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