Evaluation of Different Media for the Production of Cephalosporins by *Streptomyces clavuligerus* ATCC 27064

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ABSTRACT

The aim of this work was to compare the composition of a complex and soluble culture medium to eight other media described in the literature through the batch cultivation in a conventional bench-scale bioreactor for the production of cephamycin C by a wild strain of Streptomyces clavuligerus. *The proposed medium resulted in an antibiotic production 1.5 to 7.5 times higher than the other culture media.*

Key words: β-lactam antibiotic, *Streptomyces clavuligerus*, lysine metabolism, complex medium

INTRODUCTION

Various β-lactam compounds are produced by *Streptomyces clavuligerus* in submerged culture, which include the antibiotics of the cephalosporin C biosynthetic route, such as cephamycin C (CephC), and clavulanic acid (CA), a powerful β-lactamases inhibitor (Liras and Martín 2005). The cephalosporins and CA biosynthetic pathways are completely different from each other, and like most bioactive compounds, they are rigorously controlled by the intra-and extra-cellular factors (Santamarta et al. 2005). The presence of certain nutrients in the culture medium, such as the sources of C, N, P, or sulfur S, and salts, as well as the substances of the biosynthetic route can promote the production of CA or CephC (Bussari et al. 2008). Glycerol is preferably used as C source for the production of CA, whereas starch is more suitable for the production of CephC (Zhu et al. 2007; Sánchez et al. 2010).

Organic sources of N, such as amino acids and protein extracts, are more appropriate for the growth of *S. clavuligerus* than the inorganic ones and they should be carefully selected since they exert a strong influence on the secondary metabolism (Masurekar 2008). The positive effects of adding ornithine or high concentrations of lysine in synthetic culture medium of *S. clavuligerus* for the synthesis of CA or CephC, respectively, have been emphasized (Chen et al. 2003; Domingues et al. 2009). Several studies have shown that sulfur atom from the inorganic sources is more efficiently incorporated into the CephC molecule than sulfur from methionine or cysteine (Bussari et al. 2008). Nevertheless, the potential benefit of adding such compounds in the complex media with the aim of improving the antibiotic production has been suggested only in the technical publications, such as patents (Egel-Mitani et al. 1999). In this work, a complex and soluble culture medium was evaluated for the

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production of cephalosporins by the wild strain of *S. clavuligerus*, and its performance was compared to eight other culture media described in the literature in batch cultivations in a conventional bench scale bioreactor.

MATERIALS AND METHODS

Vegetative cells (8.0 g /liter) of *S. clavuligerus* ATCC 27064 were stored in cryotubes at -70°C using glycerol (20% v/v). The reactivation medium contained (g/l): tryptone 5, yeast extract 3, and malt extract 10. The basal inoculum medium was composed of (in g/l): yeast extract 1, malt extract 10, K_2HPO_4 0.8, $MgSO_4 \cdot 7H_2O$ 0.75, and salt solution 10 ml/l containing (in g/l): $MnCl_2 \cdot 4H_2O$ 1, $FeSO_4 \cdot 7H_2O$ 1, and $ZnSO_4 \cdot 7H_2O$ 1. The basal production medium contained (in g/l): yeast extract 0.5, K_2HPO_4 1.75, $MgSO_4 \cdot 7H_2O$ 0.75, $CaCl_2. 2H_2O$ 0.2, NaCl 2, sodium thiosulfate 1, added at 30 h post-inoculation according to Inamine and Birnbaum (1976), and salt solution described above (5 ml/l). The media were supplied with 21 g/l of the buffering agent 3-(N-morpholino) propanesulfonic acid (MOPS), and the initial pH was adjusted to 6.8.

In order to avoid the excessive increase in viscosity of the broth and the problems related to oxygen limitation during the shake-flask process, the broth volume was 10% (v/v) in relation to the nominal volume of the flask, and the concentration of the C sources (glycerol and/or starch) was established to reach a total of 10 g/l, considering a mean cell yield coefficient based on substrate consumption of $Y_{X/S} = 0.6 \pm 0.1$ g/g (Gouveia et al. 2001). The main N sources were composed of cottonseed extract (Proflo, Traders Protein), casein hydrolysate enzymatic, casein hydrolysate acid, or soybean protein hydrolysate (Soytone Bacto™, BD) - at a mass ratio of C:N = 5:1, and 100 mM of L-lysine. The final concentration of the N sources resulted in a mass proportion of C:N = 1.4:1 in the production medium.

Fermentation runs were carried out in shake flasks (500 ml nominal volume, 260 rpm, 28°C) at 10% (v/v, total volume per nominal volume), in triplicate. In bench scale bioreactors (New Brunswick Bioflo 2000, 5 l working volume), the runs were performed at pH 6.8 and 28°C and dissolved oxygen (DO) level was controlled at

40% of air saturation by varying the stirrer speed at specific air flow rate of 1 vvm. The main fermentation inoculum was obtained from two subsequent cultures (ca. 24 h each) in the reactivation and inoculum media, respectively, maintaining a 10% (v/v) inoculum ratio. The concentration of CephC was expressed in terms of the total cephalosporins' concentration, which was determined by agar diffusion bioassay using *Escherichia coli* ESS 2235 and cephalosporin C as standard (Liras and Martín 2005). Penicillin N was removed by adding 0.25 µl/ml of sample of BD Difco™ Penase (power 1977 UI/min/ml, pH 7, 25°C; 1 UI $\equiv$ 6.27 x 10^{-4} mg of PenG), followed by reaction at 25°C for 20 min.

Biomass was determined as DCW/l (105°C, overnight). Glycerol was determined as formaldehyde after reaction of the samples with periodic acid. Starch was quantified as total reducing sugars by using the DNS method, after hydrolysis (HCL 10 M). Lysine was determined by the post column orthophthaldialdehyde (OPA) derivatization method. Enzymatic activity of lysine ε-aminotransferase (LAT) and cadaverine aminotransferase (CAT) was determined according to Khethan et al. (1999).

RESULTS

Figures 1A and 1B show the consumption of glycerol and starch in culture media containing different proportions of these C sources, and cottonseed extract (Proflo) as complex N source. The initial content of phosphate (10 mM) was low enough to avoid the repression and/or inhibition of the synthesis of antibiotics (Martín 2004). The cell concentration was similar in all the media, and the highest production of cephalosporins was obtained in the medium containing only starch (Figs. 2A and 2B).

Therefore, four media containing different complex and soluble N sources were tested using starch as the main source of C and energy. Figures 3A and 3B show the time profiles of the biomass and production of total cephalosporins obtained in these cultures. The cottonseed extract (Proflo) led to a higher production of the antibiotic, and thus it was used as the complex N source in the media in the subsequent experiments.
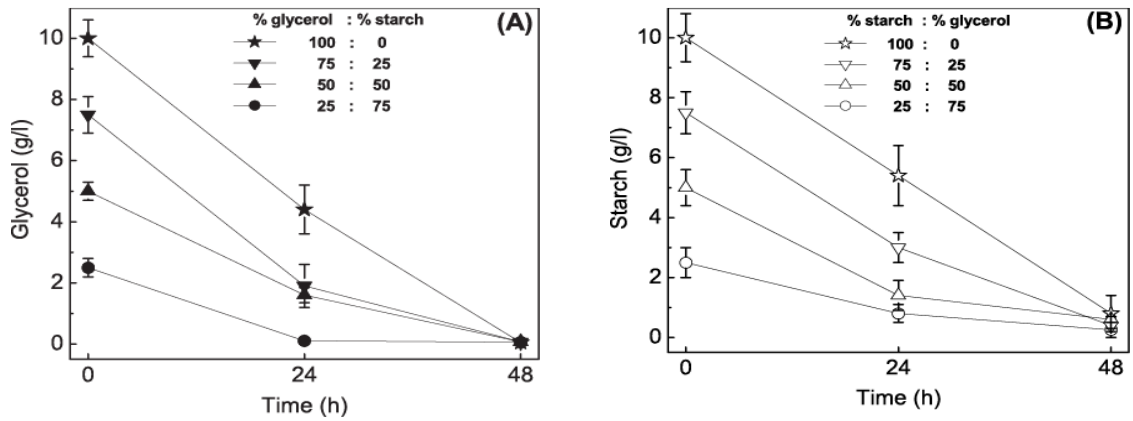
Figure 1 - Profile of consumption of glycerol (A) and starch (B) in shake flasks experiments using cottonseed extract as N source.
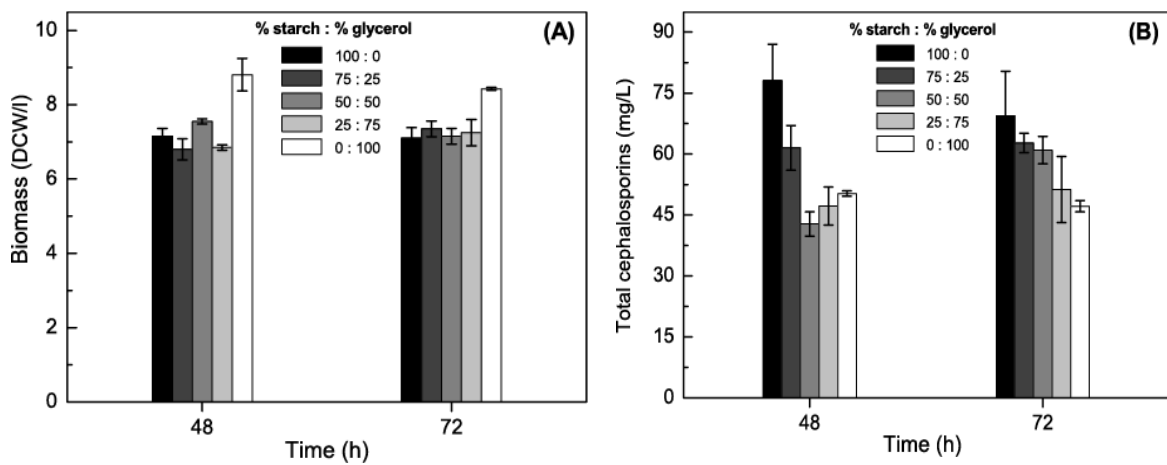

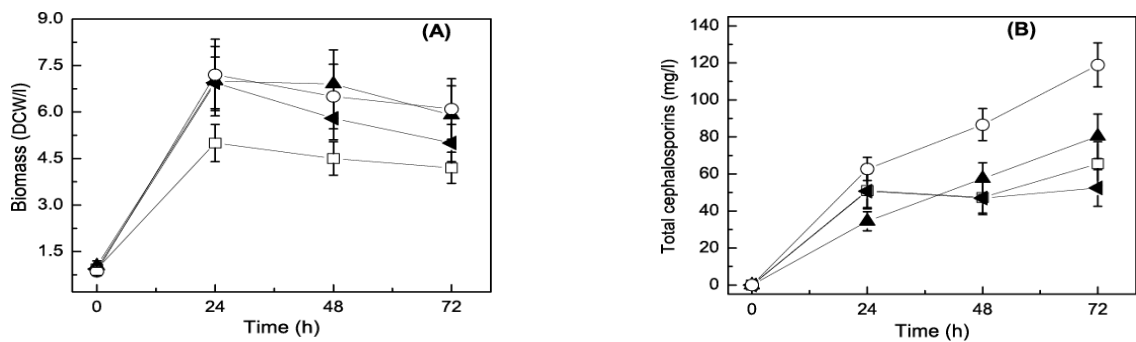**Figure 2 -** Biomass (A) and total cephalosporins (B) in shake flasks experiments.

Figure 3 - Biomass (A) and total cephalosporins (B) in shake flasks experiments using starch, L-lysine, and cottonseed extract (○), soybean protein hydrolysate (▲), casein hydrolysate acid (□), and triptone (◄).

The concentration of 100 mM lysine initially used in the production medium was based on the best results obtained by Fang et al. (1996) in synthetic medium cultures. Increases in the production of cephalosporins by *S. clavuligerus* in the presence of high concentrations of exogenous lysine were first reported by these authors. In order to establish an appropriate initial concentration of lysine in the proposed medium, the cultures were carried out varying the concentration from 10 to 150 mM

amino acid. Figures 4A and 4B show the concentration-time profiles of cell and antibiotics, respectively. The activities of CAT and LAT measured in samples withdrawn at 24 h of fermentation are shown in Figure 4C, and Figure 4D shows the consumption of lysine.

Figure 5 shows the activity of LAT and CAT measured in the samples withdrawn at different time intervals during the fermentations carried out with culture media initially containing 10 and 100 mM lysine. Figures 6A and 6B show the concentration- time profiles of C sources and total cephalosporins, respectively, in all the tested media.
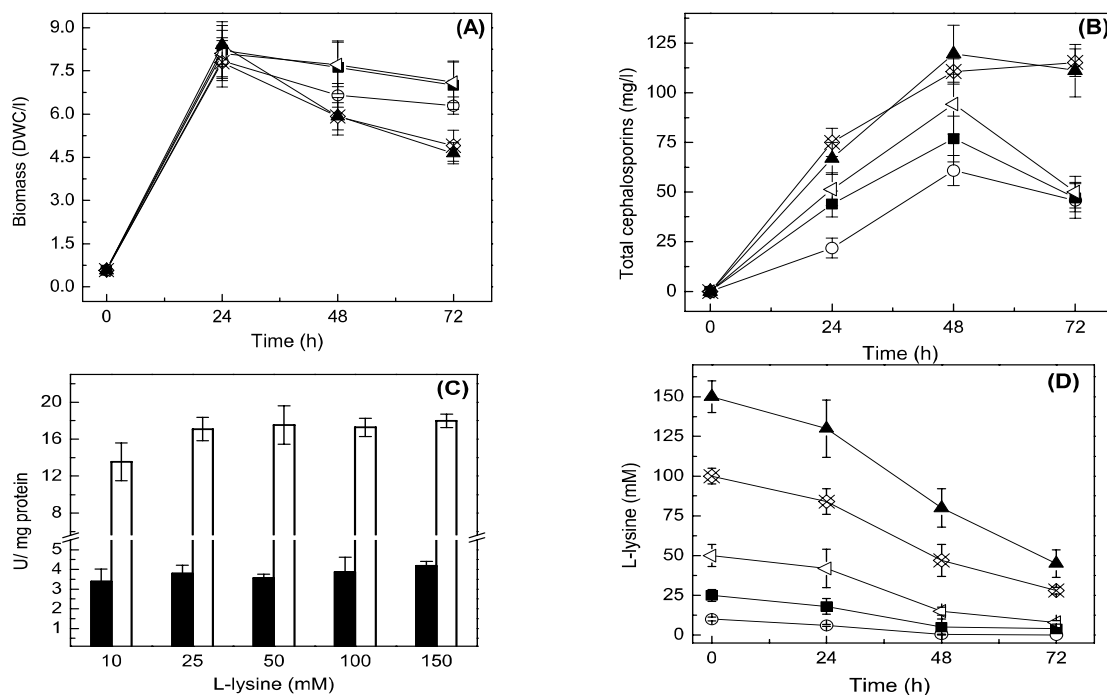

Figure 4 - Biomass (A), total cephalosporins (B), enzymatic activity - LAT (solid columns) and CAT (open columns) (C), and profile of consumption of L-lysine (initial concentration, in mM): 10 (○), 25 (■), 50 (◄), 100 (⊗), 150 (▲) (D), in shake flasks experiments.
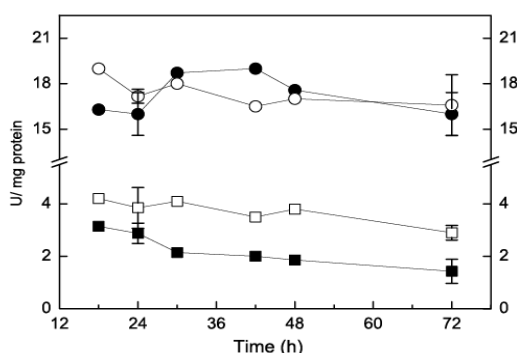

Figure 5 - Enzymatic activity of – LAT (square) and CAT (circle) –in shake flasks experiments with initial concentrations of 10 mM (solid symbols) and 100 mM (open symbols) L-lysine.
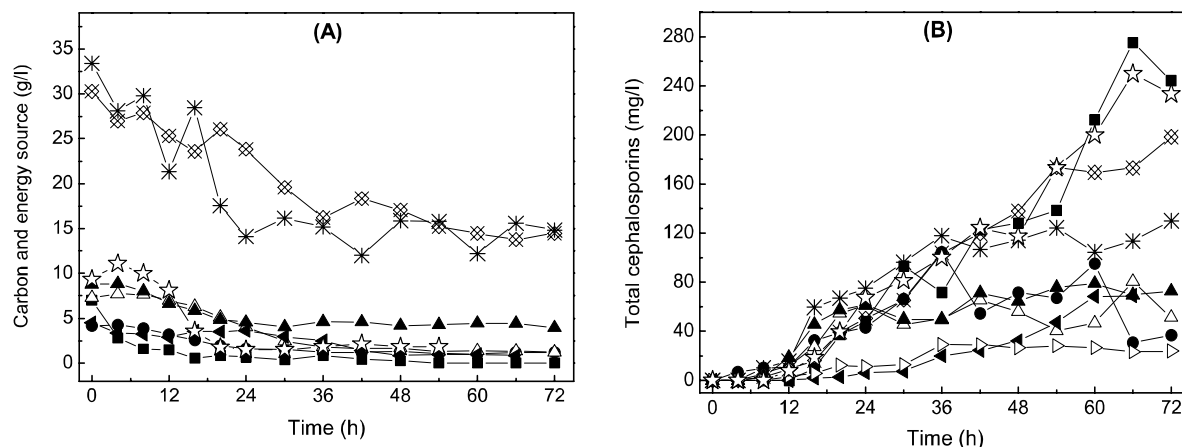
Figure 6 - Main C source consumption (A) and total cephalosporins (B) in batch cultivation in agitated and aerated bench-bioreactor in the cephamycin C production media (initial concentration of sources of C and energy, and N, in g/l): Park et al. (1994) (soybean oil, 7; soybean flour, 20; casamino acid, 10; lysine, 1) (■); Dévi and Sridhar (1999) (soluble starch, 5; corn steep liquor, 5; sunflower oil cake, 2.5) (●); Sarada and Sridhar (1998) (cassava flour, 10; corn steep liquor, 10; sunflower oil cake, 5) (△); Rollins et al. (1988a) (soluble starch, 10; tryptone, 17; peptone, 3) (▲); Kamogashira et al. (1982) (soluble starch, 30, and sucrose, 10; cottonseed extract, 20; dry yeast, 10) (✳); Aharonowitz et al. (1978) (soluble starch, 5; asparagine, 2) (◀); Park et al. (1994) (soybean oil, 30; peptone, 5; (NH$_4$)$_2$ SO$_4$, 1) (▷); Kamogashira et al. (1982), modified by the addition of 5 g/l of sodium thiosulphate (soluble starch, 30; sucrose, 10; cottonseed extract, 20; dry yeast, 10; lysine, 1) (⊗); this study (soluble starch, 10.0; cottonseed extract, 8.5; lysine ,18.3) (☆).

DISCUSSION

In this work, a complex soluble production medium was chosen, which presented advantages in relation to culture media with insoluble components, mainly the easier control of operating variables and downstream processing. The best results in terms of cephalosporin production were obtained using starch as the main source of C and energy. It is consistent with various studies which have suggested that the continuous and gradual hydrolysis of starch can avoid mechanisms of repression and /or inhibition in the production of β-lactam antibiotics that are normally triggered by C sources that are more easily metabolized by the microorganism, such as glucose or glycerol (Alexander et al. 2007; Sánchez et al. 2010). It has been observed that these easily assimilated sources can lead to an accumulation of intracellular phosphorylated compounds that drastically inhibit the expandase activity, which is the enzyme responsible for the expansion of the penicillin N ring of the β-lactam antibiotics biosynthetic pathway (Martín 2004; Zhu et al. 2007).

Moreover, the negative effect of the excess of glycerol on antibiotic production is more evident when this substrate is added at the beginning of the fermentation, both alone or with starch.

At first glance, the results of lysine consumption obtained in the present work were inconsistent with the need to add high concentrations of that amino acid to provide significant increases in the production of the antibiotic, as suggested in the literature. This was justified based on the higher demand of lysine in the primary metabolism (via CAT), when compared to the demand required for the antibiotic biosynthetic route (via LAT), in which the amino acid flow was much less favorable (Leitão et al. 2001). Earlier studies have demonstrated significant increases in the production (200 to 500 % in relation to medium without lysine) and significant effects on the cell growth in synthetic media containing 50 to 150 mM of lysine. Fang et al.(1996) evaluated the production of cephalosporins in synthetic medium containing from 50 to 150 mM of lysine and obtained the highest production in the medium with 100 mM amino acid, around 125 mg

cephalosporins /l, which is approximately 500% higher than that obtained without lysine.

The LAT activity seemed to be independent of the initial lysine concentration in the culture media, presenting the same level of expression in 24 h of fermentation. These results were consistent with the previous data from the literature which demonstrated that LAT was preferably synthesized in the exponential growth phase and presented higher activity at this stage of the process (Khethan et al. 1999). However, in addition to playing the role of positive regulator of the LAT expression, it was observed that the excess of lysine in the medium, initially containing 100 mM of amino acid, supported the maintenance of the LAT activity at slightly higher values along the fermentation, when compared with those observed in 10 mM supplemented medium. This suggested that the excess of lysine provided an increase in the enzyme loading of the cell, thus leading to more intracellular concentration of the enzyme-substrate complex, when compared to that observed in the low amino acid concentration media. It could be worth mentioning that the conditions of the analytical method used were established to provide the maximum activity (*in vitro*) of the sample enzymes. Hence, the substrate excess, the pH values, and suitable temperatures, as well as a period of time during which the maximum reaction rate was kept, were guaranteed. These conditions, besides the fact that the cell concentration of the suspension from which the protein material was extracted, was the same in all the samples, enabled concluding that the difference in the results of the samples were due to the difference in the enzyme loading of the cell. On the other hand, CAT activity was quite stable along the fermentations using both 10 and 100 mM of lysine.

With relation to the comparison between the proposed medium and other culture media described in the literature, the maximum value of antibiotic yield based on substrate consumption ($Y_{P/S}$) was obtained by Park et al. (1994). However, in this medium, the soy oil should not be considered as the only source of C and energy in this culture medium, since it also contained 20 g/l of soy flour, which presented ca. 38% (w/w) carbohydrate in its composition. Therefore, the medium used by Park et al. (1994) and proposed medium were the most suitable for cephalosporins production among the tested media.

The results showed that although limited by the productive capacity of a wild-strain microorganism, the manipulation of the medium formulation in order to increase the production of a specific metabolite could be still an efficient method to intervene in the strain performance.

ACKNOWLEDGMENTS

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