Appl Biochem Biotechnol (2010) 160:1797–1807
DOI 10.1007/s12010-009-8671-3

Screening of Variables Influencing the Clavulanic Acid Production by *Streptomyces* DAUFPE 3060 Strain

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Received: 10 March 2009 / Accepted: 13 May 2009 /
Published online: 28 May 2009

Abstract Clavulanic acid (CA) is a β-lactam antibiotic, which has a potent β-lactamase inhibiting activity. The influence of five variables, namely pH (6.0, 6.4, and 6.8), temperature (28°C, 30°C, and 32°C), agitation intensity (150, 200, and 250 rpm), glycerol concentration (5.0, 7.5, and 10 g/L) and soybean flour concentration (5.0, 12.5, and 20 g/L), on CA production by a new isolate of *Streptomyces* (DAUFPE 3060) was investigated in 250-mL Erlenmeyer flasks using a fractional factorial design. Temperature and soybean flour concentration were shown to be the two variables that exerted the most important effects on the production of CA at 95% confidence level. The highest CA concentration (494 mg/L) was obtained after 48 h at 150 rpm, 32°C, pH 6.0, 5.0 g/L glycerol, and 20 g/L soybean flour concentrations. Under these conditions, the yields of biomass and product on consumed substrate were 0.26 g_X/g_S and 64.3 mg_P/g_S, respectively. Fermentations

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performed in 3.0-L bench-scale fermenter allowed increasing the CA production by about 60%.

Keywords *Streptomyces* · Soybean flour · Glycerol · Clavulanic acid · Screening of variables · Fermentation

Introduction

Since the advent of the antibiotic era in the 1930s, medical science has witnessed the successful therapeutic application of numerous classes of antibiotics, including penicillins, cephalosporins, and tetracyclines [1]. Today, the β-lactam antibiotics, particularly penicillins and cephalosporins, represent the major biotechnology products with worldwide dosage sales at around 65% of the total market of antibiotics [2].

Clavulanic acid (CA) is a β-lactam antibiotic produced by *Streptomyces clavuligerus* having a powerful inhibitory activity against β-lactamases from a variety of Gram-positive and Gram-negative bacteria [3]. The treatment of bacterial resistance to the therapeutic efficacy of β-lactams and other antibiotics is nowadays more difficult than in the past. The discovery and application of CA demonstrate to be an effective approach to face antibiotic resistance that has been successfully applied in the clinic field [4]. CA is a potent antibiotic inhibitor of "serine" (or classes A, C, and D) β-lactamases, which is nowadays used as potassium salt in conjunction with amoxycillin and prescribed clinically as co-amoxiclav (Augmentin™) or with ticarcillin as Timentin™ [5].

Streptomyces is the largest antibiotic-producing genus in the discovered microbial world. Species belonging to this genus still remain an important source of antibiotics [6]. In recent years, screening of natural products, particularly microbial products, has fallen out. Nevertheless, it is becoming increasingly apparent that 99% of the diverse bacterial species is still unexplored [1, 2, 5]. Thus, the discovery of new *Streptomyces* species is a challenge for the improvement of CA production.

The productivity of microbial metabolites is, in general, closely related to the fermentation process. The types of nutrients, their concentrations, and the operating conditions have different effects on the accumulation of metabolites, which is controlled by intracellular effectors [7–9]. Selection of the most suited culture medium composition is of primary importance to increase the productivity of any bioprocess. Besides, the culture medium should provide nutrients easily available in the market and, if possible, of low cost [10].

Statistical design of experiments is a widely used tool for process optimization and control. The multivariate design of experiments is a very efficient method for studying the influence of a certain number of variables on a given response of interest. By a fractional factorial design, the significant factors and their effects can be studied using only a few experiments, thus reducing the operating costs [11]. Most of previous studies were devoted to the improvement of CA production by *S. clavuligerus* [6], by selection of the optimum operating conditions, namely pH [12], temperature [13], agitation [14], concentrations of glycerol as a carbon source [8], and of soybean flour as a nitrogen source [15].

However, there are only a few works that deal with high CA producing mutants of this species or other *Streptomyces* species [16, 17] as well as the influence of process variables on CA production by statistical design [18].

On the basis of this ground, a 2^{5-2} fractional two-level factorial design was used in this work to study the influence of pH, temperature, agitation intensity, glycerol concentration, and soybean flour concentration on CA production by the new isolate *Streptomyces* DAUFPE

3060, selecting the CA and biomass concentrations as the responses. In fact, these variables were shown to be the most influencing CA production by other strains [8, 12–15]. Although such an isolate was not yet characterized, its high performance in CA production with respect to common *S. clavuligerus* strains constitutes the main novelty of this study.

Materials and Methods

Reagents

Potassium clavulanate from *S. clavuligerus* was used for the calibration curve needed to determine the concentration of CA. The imidazole used in the CA determination was provided by Sigma Aldrich (São Paulo, Brazil). The salts, glycerol, bacto-peptone, malt extract, and yeast extract used to prepare the media were analytical grade reagents.

Microorganism

The *Streptomyces* spp. DAUFPE 3060 strain was kindly provided by the Microorganism Collection of the Department of Antibiotics of the Federal University of Pernambuco, Recife, PE, Brazil. This strain was isolated directly from soil samples. For each collected sample, 3 g of soil were diluted in 100 mL of saline solution (0.85% NaCl) and allowed to stand for 15 min. Three different dilutions (1:10, 1:100 and 1:1,000) were prepared using sterile saline solutions in a total volume of 10 mL. According to Lawrence [19], aliquots of 0.1 mL of each dilution were plated using glucose–asparagine–agar (Difco Laboratories, Detroit, MI, USA) at pH 6.5 as selective medium. Plates were incubated at 28°C and monitored after 48, 72, and 96 h. Representative colonies were selected and streaked on new plates of this medium. The isolated *Streptomyces* species were preserved on yeast malt extract plates at 4°C until further use. Preliminary characterization was carried out by optical microscopy in order to determine the morphology of the spore chains, the colony color, and the release of pigments in the cultivation medium. The microorganism, which is still at the stage of molecular characterization, was stored in cryotubes (glycerol 10%, *v*/*v*) at −70°C and used throughout the present work.

Culture Media

The seed medium had the following composition (in g/L distilled water): glycerol, 15; bacto-peptone, 10; malt extract, 10; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.75; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.001. The pH of the medium was adjusted to 6.8 with NaOH 5.0 M before autoclaving at 121°C for 15 min. The inoculum medium used in the cultivations, based on that proposed by Maranesi et al. [20], had the following composition (in g/L distilled water): glycerol, 10; soybean flour (SF), 20; K_2HPO_4, 1.2; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.001, pH 6.8. The composition of the production medium was similar to that used for the inoculum, except for the concentrations of SF and glycerol that were varied according to the selected experimental design.

Fermentation Conditions

The seed culture was prepared by adding the spore suspension (6.6 g/L dry weight) contained in a cryotube (3.0 mL) to 25 mL of seed medium in 250-mL Erlenmeyer flasks and incubated in

an orbital shaker at 28°C and 200 rpm for 24 h. Afterward, 250-mL Erlenmeyer flasks containing 45 mL of inoculum medium were inoculated with 5.0 mL of seed culture and incubated in an orbital shaker at 28°C and 200 rpm for 24 h. Then, 5.0-mL aliquots of this medium were transferred to 250-mL Erlenmeyer flasks containing 45 mL of production medium. The production runs, which lasted 168 h, were performed in an orbital shaker under different conditions according to the experimental design described later (Table 1). One flask was drawn every 24 h and utilized to determine cell concentration, pH, and CA concentration.

All the experiments were performed once according to the selected experimental design, but run 3 ensured the highest CA production, and then was carried out in triplicate. For comparison, an additional fermentation was carried out in triplicate in a 3.0-L fermenter, model Z61103CT04 (Applikon, Schiedam, The Netherlands). An electronic device, model ADI1030 (Applikon), allowed performing the fermentation under the best conditions determined in shaken flasks (32°C, pH 6.0±0.1, 5 g/L glycerol, and 20 g/L soybean flour). According to Ortiz et al. [10], the rotational speed and the aeration were set at 800 rpm and 0.5 vvm, respectively. Dissolved oxygen concentration was monitored by a sterilized galvanic electrode, InPro6000 Series (Mettler-Toledo, Novate Milanese, Italy).

Analytical Methods

The fermentation broth was centrifuged at 5,500×*g* for 20 min at 4°C, and the cell pellet was washed twice with distilled water and dried to constant weight at 80°C. The spectrophotometric assay by reaction with imidazole, selected in this study, was described in detail by Bird et al. [21] and utilized by most of the researchers working in this field [10, 13, 20]. According to this method, the CA concentration in the fermented broth was determined by measuring the increase in the optical density at 311 nm due to the release of the product [1-(8-hydroxy-6-oxo-4-azooct-2-enol)-imidazole] of the reaction between CA and imidazole. Glycerol concentration was determined according to Hae Bok and Demain [22]. All the analyses were performed in duplicate.

Experimental Procedure

Batch fermentations were carried out according to a 2^{5-2} fractional factorial design, which is a one-fourth fraction of the full two-level factorial design [11]. It was used to evaluate the relative influence of several variables on the production of CA. In such a fraction, the effects are confounded in groups of four, which is why the calculated results are called "contrasts," to distinguish them from the effects themselves. The experimental design was composed of eight runs and four repetitions in the central point, needed to calculate the pure

Table 1 CA production by *Streptomyces* spp. DAUFPE 3060 under different conditions.

Levels	Lower	Central	Higher
Coded values	−1	0	+1
(1) Temperature (°C)	28	30	32
(2) Agitation (rpm)	150	200	250
(3) pH	6.0	6.4	6.8
(4) Glycerol concentration (g/L)	5	7.5	10
(5) Soybean flour concentration (g/L)	5	12.5	20

error. We selected, as the two response variables, biomass and CA concentrations after 48 h, i.e., the time at which the highest CA concentration was obtained. A statistical evaluation of the results was carried out by the Statistica 6.0 statistical program package.

Results and Discussion

Soybean Flour and Glycerol as Nitrogen and Carbon Sources

The main results obtained by *Streptomyces* DAUFPE 3060 fermentations according to the fractional factorial design are listed in Table 2.

The effect of different combinations of the concentrations of the nitrogen source (SF) and the carbon source (glycerol) at different pH values on CA production after 48 h are summarized in Fig. 1. It is noteworthy that the two highest values of CA concentration (494 and 129 mg/L) are located on the top face of the diagram, which corresponds to pH 6.0, to opposite level combinations (−1 and +1) for glycerol concentration (5.0 and 10 g/L, respectively), and to 20 g/L SF concentration.

A study of the influence of the type of soybean derivatives as nitrogen sources on CA production by *S. clavuligerus* was already performed by Ortiz et al. [10], who utilized two different media, the one containing SF (20 g/L) as nitrogen source and soybean oil (23 g/L) and glycerol (10 g/L) as carbon sources and the other containing soy protein isolate (SPI) (20 g/L) instead of SF. These authors found that cell growth and CA production were higher in the former medium, which contained more soluble nutrients. According to Chen et al. [8], soybean derivatives, such as soy meal flour and soybean protein hydrolyzates, are excellent components of media for CA production because they contain arginine, the precursor of CA. Teodoro et al. [23], who have recently investigated CA production by *S. clavuligerus* using three different nitrogen sources, specifically 1% of malt extract, 1% of yeast extract, and 2% of commercial soybean derivative (Samprosoy 90NB), observed that

Table 2 Influence of the independent variables on biomass and clavulanic acid production.

Run	Agitation (rpm)	Temperature (°C)	pH	Glycerol (g/L)	Soybean flour (g/L)	Clavulanic acid (mg/L)[a]	Biomass (g/L)
2	250	28	6.0	5.0	5.0	23	1.6
4	250	32	6.0	10	5.0	95	2.2
7	150	32	6.8	5.0	5.0	121	1.9
5	150	28	6.8	10	5.0	39	1.1
12 (C)[a]	200	30	6.4	7.5	12.5	62	2.3
6	250	28	6.8	5.0	20.0	73	5.3
9 (C)	200	30	6.4	7.5	12.5	62	2.4
1	150	28	6.0	10	20.0	129	4.1
8	250	32	6.8	10	20.0	104	7.6
3	150	32	6.0	5.0	20.0	494	2.8
10 (C)	200	30	6.4	7.5	12.5	63	2.3
11 (C)	200	30	6.4	7.5	12.5	61	2.3

[a] Pure error estimated from the replicate runs at the central point (C) was ±0.54. All values were statistically significant at 95% confidence level

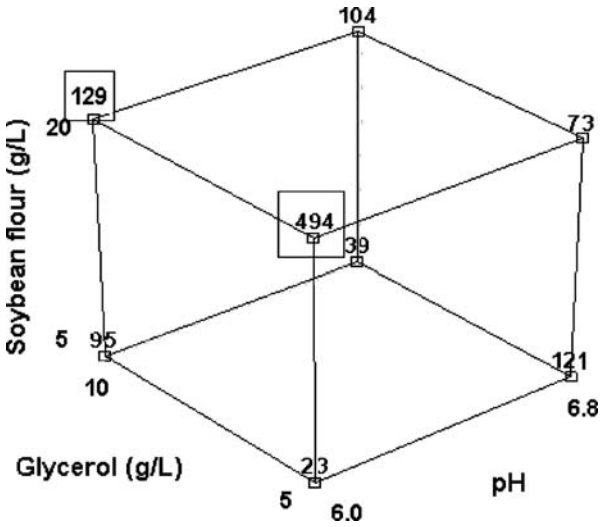

Fig. 1 Cubic plot of the average concentration of clavulanic acid at different levels of pH, soybean flour concentration, and glycerol concentration according to the fractional factorial design of Table 1

cell growth was favored by the large nitrogen source availability to the detriment of CA production.

The results obtained in the present work demonstrate that the use of SF (20 g/L) as nitrogen source in mixture with 5.0 g/L glycerol as the only carbon source, i.e. a carbon source level quite lower than that used by Ortiz et al. [10], was enough to guarantee satisfactory production of CA. The main potential of these results arises from the fact that SF is a raw material about five times cheaper (US$ 0.43 kg^{-1}) than the SPI (US$ 2.15 kg^{-1}) (rates of 2006) [10]. Even taking into account that the nitrogen content of SF is about half that of SPI, the use of SF is technically and economically preferable for CA production under these fermentation conditions.

Influence of the Independent Variables on the Clavulanic Acid Fermentation

The contrast values calculated for all the five variables by the selected fractional factorial design are given in Table 3. All the contrasts were statistically significant at the 95% confidence level. The main effects of temperature (1) and SF concentration (5) as well as their interaction (1–5) were significant and positive, thereby indicating that the increment of anyone of them resulted in a corresponding increase in the CA concentration. On the contrary, the effects of the others variables and the interaction 1–3 were all significant and negative, the most important being that shown by the agitation (2).

As shown in Fig. 2, the run 3, performed at 150 rpm, 32°C, pH6.0, 5.0 g/L glycerol, and 20 g/L SF, exhibited by far the highest production of CA (494 mg/L) with respect to the other conditions, whereas the highest biomass growth (7.6 g/L) was observed with run 8 performed at highest levels of agitation (250 rpm) and glycerol concentration (10 g/L).

On the basis of these results, we focused in study on the simultaneous effects exerted by temperature and agitation because of their well-known importance in the scale-up of bench-scale results to the industrial scale. To this purpose, we considered together all the runs carried out under the same conditions of temperature (32°C) and agitation (150 rpm), which yielded an average maximum CA concentration of 308 mg/L. Most of the studies reported in the literature did not take into consideration this interaction, simply investigating separately the effects of temperature

Table 3 Contrast values calculated according to the 2^{5-2} fractional design for the concentration of clavulanic acid by *Streptomyces* DAUFPE 3060 fermentation.

Variables and interactions	Contrasts[a]
(1) Temperature (°C)	249
(2) Agitation (rpm)	−211
(3) pH	−164
(4) Glycerol concentration (g/L)	−150
(5) Soybean flour concentration (g/L)	220
Interaction 1–3	−134
Interaction 1–5	95

[a] Pure error estimated from the replicate runs at the central point (C) was ±0.54. All values were statistically significant at 95% confidence level.

and agitation in the ranges 28–30°C and 200–250 rpm [7–10, 20, 23, 24], respectively. The values of temperature and agitation that ensured the highest production of CA in this study were out of these ranges, hence confirming the importance of a combined investigation of these two variables by a factorial design rather than through a step-by-step variation protocol.

Regression analysis of the fractional factorial design in Table 4 shows that both temperature and soybean flour concentration were significant at the probability levels of 95% and 97% for CA production, respectively, and that they were the most important independent variables. This table also shows the values of the regression coefficients, which were calculated by the following first-order equation:

$$\mathrm{CA} = 106.71 + 65.05x_1 - 55.21x_2 - 42.78x_3 - 39.10x_4 + 57.57x_5$$

where CA is the CA concentration and x_i are the variable terms. The model explained 90% of the variability data (R^2=0.90), and its statistical significance was also confirmed by the

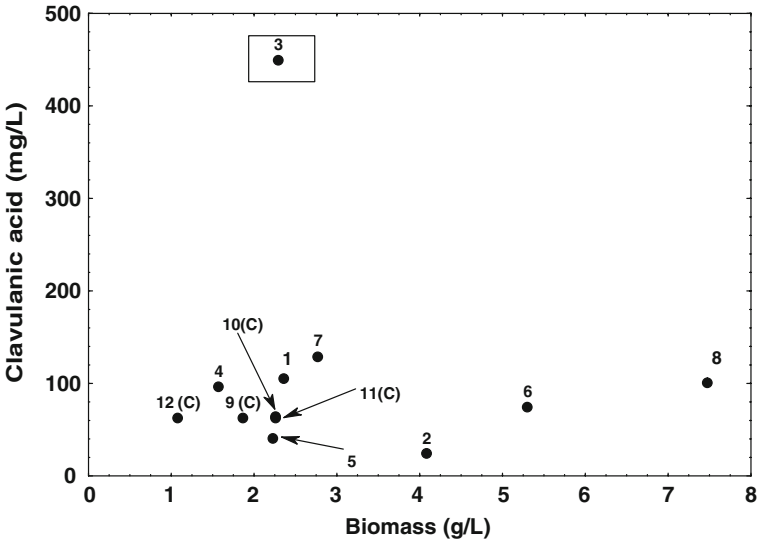

Fig. 2 Representation of the relationship between biomass and CA concentrations obtained after 48 h of *Streptomyces* DAUFPE 3060 fermentations carried out according to the fractional factorial design of Table 1

Table 4 Fractional factorial design regression results for clavulanic acid production.

Term	Intercept	Independent variables				
		Temperature (°C)	Agitation (rpm)	pH	Glycerol concentration (g/L)	SF concentration (g/L)
Coefficient	106.71	65.05	−55.21	−42.78	−39.10	57.57
t value	5.54	2.75	−2.34	−1.81	−1.66	2.44
p value	0.001	0.032	0.060	0.112	0.148	0.050

R^2 =0.90; F=11.55>$F_{5,6}$=5.01

quite high F value (11.55). The model was found to be adequate to fit the data at a probability level of 95%. These results confirm that temperature and soybean flour concentration are the important independent variables governing the CA production by *Streptomyces* DAUFPE 3060.

Fermentations under the Best Conditions

Figure 3 shows the time behavior of the best run (3) performed at 150 rpm, 32°C, pH 6.0, 20 g/L SF, and 5.0 g/L glycerol. The CA production fermentation was shown to be dependent on the fermentation time. In fact, the maximum CA concentration (494 mg/L) was obtained after 48 h, likely due to conditions of low carbon source availability (5 g/L glycerol) at the beginning of fermentation. Because of the lack of carbon source, after this period the microorganism grew utilizing soybean proteins, thus releasing mainly amino acids and other compounds [25, 26]. The consequent release of NH_3 and other byproducts progressively increased the pH (results not shown) and was likely responsible for CA degradation. Resuming, according to this behavior, too long fermentation times should be avoided in an industrial process to get satisfactory performance.

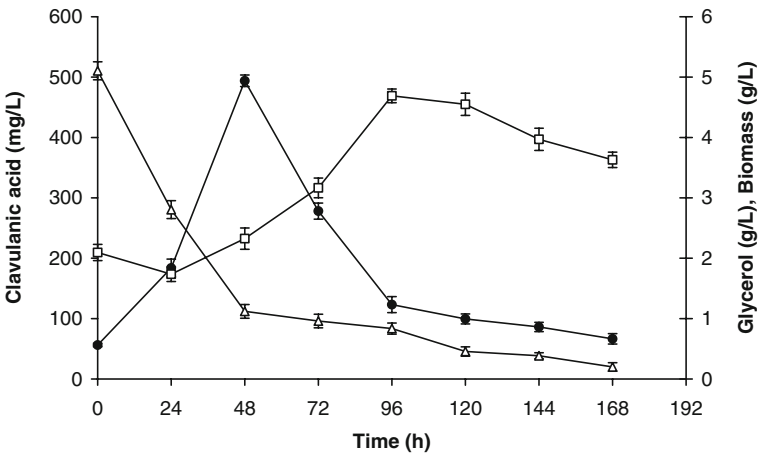

Fig. 3 Time course of the concentrations of biomass (*empty square*), glycerol (*open triangle*), and clavulanic acid (*filled circle*) during run 3 in shake flasks

These results agree with those of Mayer and Deckwer [27], who observed that growth and production occurred simultaneously in the trophophase when a soluble nitrogen source was utilized. The CA production associated to cell growth was also observed by Lynch and Yang [25] and Kenji et al. [28] both utilizing soybean derivatives as nitrogen sources. On the other hand, Teodoro et al. [23] observed that CA synthesis occurred during the trophophase but the highest CA concentration was obtained during the stationary phase. Moreover, they demonstrated that the concentration of the nitrogen source has dramatic influence on the production of CA because, at high nitrogen levels, it is inhibited by the ammonia released due to the catabolism of amino acids [29].

The maximum CA production (494 mg/L after 48 h) obtained in shake flasks with *Streptomyces* DAUFPE 3060 (Fig. 3) was higher than that obtained in the same medium containing SPI instead of SF (338 mg/L after 108 h) [10] or soluble starch instead of glycerol (458 mg/L of CA after 96 h) [21]. However, it was lower than that observed by Ortiz et al. [10] and Maranesi et al. [20] using *S. clavuligerus* in mixtures of SF, soybean oil, and glycerol (698–753 mg/L) after a much longer time (130–132 h). The main difference between these results was likely due to the different carbon sources employed. The use in this work only of glycerol as a carbon source made the growth and the CA production very quick, whereas the very slow β-oxidation of fatty acids was likely to be the main metabolic route after the complete glycerol uptake in the work of those authors.

Higher CA production (672 mg/L) after 72 h was reported for *S. clavuligerus* under optimum conditions using soy meal powder, glycerol, and ornithine [18]. However, this medium is much more expensive than the soybean flour and likely would be unsuitable for the scale-up to industrial purposes.

Glycerol exhaustion occurred between 24 and 48 h, but never was it complete, and the biomass reached its maximum value (4.7 g/L) after 96 h. The yield of cell mass on consumed substrate ($Y_{x/s}$) was 0.26 g/g and that of the product ($Y_{P/S}$) was 64.3 mg/g, which means that, under the conditions adopted in this work, the yield of CA on produced biomass ($Y_{P/X}$=247 mg/g) was about two orders of magnitude higher than that determined by Lynch and Yang [25].

These promising results suggested performing a triplicate fermentation in stirred tank reactor under conditions as much as possible similar to the best ones in shake flasks. As expected, the results of these fermentations (Fig. 4) were quite better than those obtained in

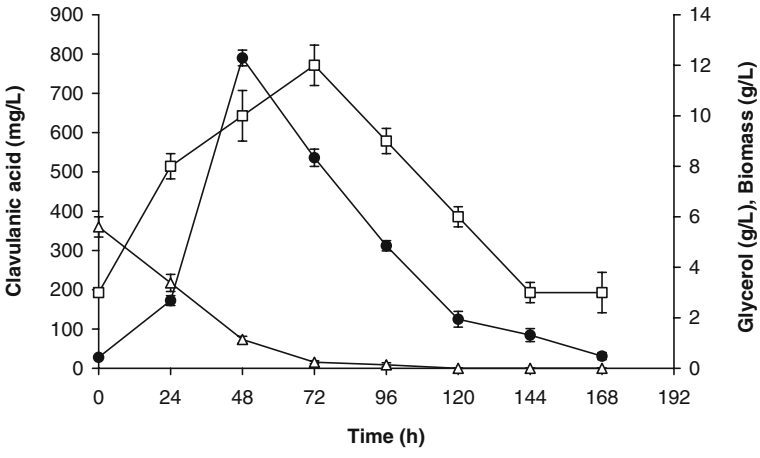

Fig. 4 Time course of the concentrations of biomass (*empty square*), glycerol (*open triangle*) and clavulanic acid (*filled circle*) during fermentation in stirred tank reactor

flasks, likely because of better oxygen transfer in the fermenter. In fact, the maximum CA concentration (790 mg/L) after 48 h was about 60% higher and the maximum cell concentration about 2.5-fold that obtained in flasks. Finally, glycerol was completely consumed after only 120 h.

Conclusions

The influence of some process variables, specifically temperature, pH, agitation, and soybean flour and glycerol concentrations, on the CA production by the new strain *Streptomyces* DAUFPE 3060 has been investigated using a 2^{5-2} fractional factorial design. The results collected in this study demonstrated that soybean flour concentration and temperature were the variables that most influenced the CA concentration. This strain exhibited, in the cheap medium selected for this work (20 g/L of soybean flour and 5.0 g/L of glycerol), a promising and particularly fast CA production (494 mg/L after 48 h) with respect to *S. clavuligerus*, the species commonly used for this purpose. When the fermentation was carried out under the best conditions in a fermenter, the maximum CA concentration (790 mg/L) after 48 h was about 60% higher and the maximum cell concentration about 2.5-fold that obtained in flasks. Further work on additional variables is needed to optimize this process and scaling up it for industrial purposes.

Acknowledgement The authors gratefully acknowledge the financial support of the Brazilian Research Funding Institution FAPESP (process no. 05/60158-0).

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