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Research Paper

Screening of wild type *Streptomyces* isolates able to overproduce clavulanic acid

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Abstract

The selection of new microorganisms able to produce antimicrobial compounds is hoped for to reduce their production costs and the side effects caused by synthetic drugs. Clavulanic acid is a β-lactam antibiotic produced by submerged culture, which is widely used in medicine as a powerful inhibitor of β-lactamases, enzymes produced by bacteria resistant to antibiotics such penicillin and cephalosporin. The purpose of this work was to select the best clavulanic acid producer among strains of *Streptomyces* belonging to the Microorganism Collection of the Department of Antibiotics of the Federal University of Pernambuco (DAUFPE). Initially, the strains were studied for their capacity to inhibit the action of β-lactamases produced by *Klebsiella aerogenes* ATCC 15380. From these results, five strains were selected to investigate the batch kinetics of growth and clavulanic acid production in submerged culture carried out in flasks. The results were compared with the ones obtained by *Streptomyces clavuligerus* ATCC 27064 selected as a control strain. The best clavulanic acid producer was *Streptomyces* DAUFPE 3060, molecularly identified as *Streptomyces variabilis*, which increased the clavulanic acid production by 28% compared to the control strain. This work contributes to the enlargement of knowledge on new *Streptomyces* wild strains able to produce clavulanic acid by submerged culture.

Key words: clavulanic acid, β-lactamases inhibitor, *Streptomyces* screening, submerged culture, growth kinetics.

Introduction

In spite of the antibiotics efficiency in the treatment of infections, the indiscriminate use of these compounds promoted an inevitable microbiological resistance (Spratt, 1994). One mechanisms of resistance shown by a variety of Gram-positive and Gram-negative bacteria is their ability to produce β-lactamases, enzymes that hydrolyze the β-lactam ring of penicillins and cephalosporins disabling them (Bush *et al.*, 1995).Several preventive measures have been

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taken to avoid the onset of antibiotic resistance, but still there is an urgent demand for new antimicrobial agents and new strategies to combat resistant pathogens (Hassan *et al.*, 2012). One way to overcome the antibiotic resistance of many bacteria is the introduction of novel β-lactams and the combination of classical penicillin with clavulanic acid (CA), a β-lactamase inhibitor (Líras and Martín, 2009). For instance, Augmentin™ is a synthetic drug that contains CA and amoxicillin (Santos *et al.*, 2011).

Actinomycetes are the most important group of antibiotic producers, and the genus *Streptomyces*, which is represented in the nature by a large number of species and varieties that differ in morphology along with physiological and biochemical activity, increases the economic importance of this group (Taddei *et al.*, 2005).

Overall, the productivity of microbial metabolites is closely related to the submerged culture process. Among the important variables for the process, types of nutrients, their concentrations and operating conditions have different effects on the accumulation of metabolites, which is mainly controlled by intracellular effectors (Kirk *et al.*, 2000; Gouveia *et al.*, 2001; Chen *et al.*, 2002). Therefore, the selection of the most suitable medium composition is of primary importance to increase the productivity and decrease the cost of any bioprocess (Ortiz *et al.*, 2007).

Although CA is produced by several species of *Streptomyces* (Butterworth, 1984), its industrial production is almost entirely dependent on *S. clavuligerus* cultivation in complex medium (Silva *et al.*, 2012). In view to produce clavulanic acid by *S. clavuligerus*, several components of industrial culture media such as starch and other carbohydrates are not essential (Efthimiou *et al.*, 2008), therefore alternative carbon sources are evaluated to synthesize this antibiotic. Lee and Ho (1996) obtained the highest productivity employing palm oil as carbon source, but other carbons sources such as maltose and dextrin were successfully employed (Pruess and Kellett, 1983). Mounir *et al.* (2010) reported that the use of olive oil as the only carbon and energy source could be a promising strategy for CA production by this species. Several studies are reported in the literature on the use of different nitrogen sources for CA production, among which bacteriologic peptone (Belmar-Beiny and Thomas, 1991), glutamic acid, ornitine or arginine and histidine (Romero *et al.*, 1984), soybean flour or soybean extract (Mayer, and Deckwer, 1996), and Samprosoy 90 NB, a protein hydrolyzate from soybean (Gouveia *et al.*, 1999).

Kinetic modeling is an important tool for implementation of optimization techniques and control strategy development (Brass *et al.*, 1997). Several studies evaluating bioprocess modeling techniques are available in the literature, and different approaches are extensively described in text books such as that of Bailey and Ollis (1986). Tarbuck *et al.* (1985) proposed a kinetic model based on the Monod equation to describe CA production by *S. clavuligerus;* but it showed poor fitting to the experimental data. Baptista Neto *et al.* (2000) also utilized a Monod-type kinetic model to describe batch experimental data obtained using simultaneously peptone, soybean flour and Samprosoy 90 NB as nitrogen sources. For cultivation in peptone-containing medium, a pseudo-stoichiometric equation was proposed for cell growth; however, it was not possible to propose any stoichiometric equation for CA biosynthesis because the fraction of substrate converted into product was not available. Thus, additional kinetic efforts are needed to better elucidate CA production *vs.* cell growth.

On the basis of this background, 19 different *Streptomyces* strains were screened in this study, employing two different antimicrobial activity methods, for their ability to produce β-lactamases inhibitors in different culture media. Among these, five strains showed the largest β-lactamases inhibition zones; therefore, they were selected to investigate CA production in batch submerged culture, whose results were compared with those obtained with *S. clavuligerus* ATCC 27064, which was selected as a standard producer. Kinetic parameters of growth and CA production as well as yield coefficients were used to investigate the process.

Materials and Methods

Reagents

Potassium clavulanate from *Streptomyces clavuligerus* and imidazole were provided by Sigma Aldrich (São Paulo, Brazil). All the other reagents wereof analytical grade.

Microorganisms maintenance and screening media

Nineteen different *Streptomyces* spp. strains isolated from soil were provided by the Microorganism Collection of the Department of Antibiotics of the Federal University of Pernambuco (DAUFPE), Recife-PE, Brazil. They are going to be called DAUFPE-3004, DAUFPE -3006, DAUFPE-3007, DAUFPE-3009, DAUFPE-3012, DAUFPE-3016, DAUFPE-3018, DAUFPE-3036, DAUFPE-3053, DAUFPE-3060, DAUFPE-3061, DAUFPE-3094, DAUFPE-3095, DAUFPE-3098, DAUFPE-3125, DAUFPE-3126, DAUFPE-3131, DAUFPE-3132 and DAUFPE-3133. On the other hand, *S. clavuligerus* ATCC 27064 was used as a control strain for comparison purposes.

Klebsiella aerogenes ATCC 15380, a well-known β-lactamase producer, was used as target microorganism to test the antimicrobial activity. It was maintained in nutrient broth composed of (g/L) 10.0 peptone, 3.0 beef extract, 5.0 NaCl, pH 6.9-7.0. All *Streptomyces* strains and *K. aerogenes* ATCC 15380 were maintained at 4 °C on malt/yeast extract and nutrient agar plates, respectively, and stored in tubes (10% v/v glycerol) at -70 °C. The screening media were a) ISP-1 (Pridham and Gottlieb,

1948) composed of (g/L distilled water) 5.0 tryptone, 3.0 yeast extract, pH 7.0-7.2; b) Tryptone Soy broth (TSB) composed of (g/L distilled water) 17.0 hydrolyzed casein, 3.0 hydrolyzed soybean flour, 5.0 NaCl, 2.5 K_2HPO_4, 2.5 glucose, pH 7.0. The seed medium used for most of the strains was ISP-1 supplemented with TSB, with the exception of DAUFPE-3006 and DAUFPE-3053 for which only TSB was used and of DAUFPE-3061, DAUFPE-3126, DAUFPE-3131 and DAUFPE-3133 for which only ISP-1 was used.

Antimicrobial activity tests

The antimicrobial activity of all *Streptomyces* strains was initially explored by the agar block method. To this purpose, cylindrical pieces of mycelium were cut out from well grown and sporulated cultures on both Starch Casein Agar (SCA) and ISP-2 solid media. The SCA medium was composed of (g/L) 10.0 soluble starch, 0.3 vitamin free casein, 2.0 KNO_3, 2.0 NaCl, 2.0 K_2HPO_4, 0.05 $MgSO_4{\cdot}7H_2O$, 0.02 $CaCO_3$, 0.01 $FeSO_4.7H_2O$, 15.0 agar, while the ISP-2 one of (g/L) 4.0 yeast extract, 10.0 malt extract, 4.0 glucose, 15.0 agar (Pridham *et al.*, 1957). Nutrient agar (NA) was the culture medium for antimicrobial tests using *K. aerogenes* ATCC 15380 as target. The blocks were placed on Petri dishes with 10 mL of NA medium containing 10 μg/mL amoxicillin. Then, 1.0 mL of *K. aerogenes* ATCC15380 suspension was adjusted to McFarland turbidity No. 1 using optical density and poured on the plates (Brown *et al.*, 1976). The plates were placed for 10 min at 2-8 °C to allow the antimicrobial compound to diffuse and then incubated at 37 °C. After 24 h of incubation, the antimicrobial activity was measured as the growth inhibition zone diameter (mm).

The strains selected by the agar block method were then subject to further screening of the highest clavulanic acid (CA) production by the filter paper disk method. For this purpose, 500mL Erlenmeyer flasks containing 50mL of each medium described in Table 1 were inoculated with 5mL of the ISP-1 medium containing the selected strains and incubated in orbital shaker at 30 °C and 6000 *g* for 96 h. In particular, the Euromicin Production (EP) medium was modified in relation to carbon and nitrogen concentrations as indicated in the same table.

Aliquots were withdraw every 24h and used in filter paper disks to conduct the antimicrobial activity tests. The *K. aerogenes* ATCC 15380 suspension in NA medium containing 10.0μg/mL amoxicillin was distributed in Petri dishes, and, after solidification, filter paper disks with 6 mm diameter were soaked with 30 μL of different submerged culture media and placed upon the medium surface. Petri dishes were then incubated at 37 °C, and the inhibition zone diameter was measured every 24h aiming to select the *Streptomyces* strains able to produce and excrete CA.

Table 1 - Media used in screening experiments aiming at the production of clavulanic acid.

Component (g/L)	EP[1]	Modified EP	G[2]	SC[3]	SHG [4]
Glucose	20	10.0	-	-	-
Soybean flour	20	30.0	-	-	-
Glycerol	-	-	7.5	-	10.0
Sucrose	-	-	7.5		-
Soluble starch	-	-	-	10.0	-
Vitamins-free casein	-	-	-	0.3	-
Soybean hydrolyzed	-	-	-	-	30.0
Arginine	-	-	4.03	-	-
Proline	-	-	5.79	-	-
K_2HPO_4	-	1.5	2.0	2.0	1.5
$FeSO_4$	-	0.01	-	0.01	0.01
$CaCO_3$	2.0	-	-	0.02	-
NaCl	5.0	-	5.0	2.0	-
$CaCl_2$	-	-	0.4	-	-
$MnSO_4.H_2O$	-	-	0.1	-	-
$ZnCl_2$	-	-	0.05	-	-
$FeCl_3.6H_2O$	-	-	0.1	-	-
$MgSO_4.7H_2O$	-	-	1.0	0.05	-
KNO_3	-	-	-	2.0	-
pH	6.7-7.0	6.5	7.0	7.0-7.4	6.5

[1]Euromicin Production medium; [2]Glycerol medium; [3]Starch Casein medium; [4]Soybean Hydrolyzed medium containing Glycerol.

Culture media

The *Streptomyces* strains selected by the filter paper disk method were then used to evaluate their ability to produce CA in flasks. For this purpose, the strains were transferred to the seed medium that had the following composition (g/L distilled water) 15.0 glycerol, 10.0 bacto peptone, 10.0 malt extract, 1.0 yeast extract, 2.5 K_2HPO_4, 0.75 $MgSO_47H_2O$, 0.001 $MnCl_24H_2O$, 0.001 $FeSO_47H_2O$, 0.001 $ZnSO_47H_2O$, 21 3-(N-morpholino) propane sulfonic acid (MOPS) (Oritz *et al.*, 2007). The inoculum and production media had the same composition, namely (in g/L distilled water) 10.0 glycerol, 20.0 soybean flour (SF), 1.2 K_2HPO_4, 0.001 $MnCl_24H_2O$, 0.001 $FeSO_47H_2O$, 0.001 $ZnSO_47H_2O$, 21 MOPS (Maranesi *et al.*, 2005). The pH of media was adjusted to 6.8 with NaOH 5 M solution. All media were autoclaved at 121 °C for 15 min.

Submerged culture conditions

Cell fragments of *Streptomyces* spp. mycelia were withdrawn and cultivated in liquid culture medium for 96 h. After determination of initial biomass concentration by dry weight, cells were lyophilized and stored in 10% (v/v) glycerol. The seed culture was prepared by adding the lyophilized cells (6.6g/L dry weight) contained in a

cryotube with glycerol to 25 mL of seed medium in 250 mL-Erlenmeyer flasks that were incubated in an orbital shaker at 28 °C and 200 rpm for 24 h. Afterwards, 250 mL-Erlenmeyer flasks containing 45 mL of inoculum medium were inoculated with 5.0 mL of the seed culture and incubated under the same conditions. Aliquots of this suspension (5.0 mL) with cells grown for 24h were transferred to 250 mL-Erlenmeyer flasks containing 45 mL of the above production medium. Submerged cultivations were performed at 200 rpm at 28 °C for 168 h.

Analytical methods

The fermented broth was centrifuged at 5500 x *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. Clavulanic acid concentration in the fermented broth was determined spectrophotometric ally by its reaction with imidazole (Bird *et al.*, 1982). For this purpose, the increase in the optical density at 311 nm consequent to the formation of the product [1-(8-hydroxy-6-oxo-4-azooct-2-enol)-imidazole] was determined using a UV/Vis spectrophotometer, model Ultrospec 3000 pro (GE Healthcare, Life Sciences, Uppsala, Sweden). Glycerol concentration was determined according to Hae Bok and Demain (1977).

Kinetic parameters

The specific growth rate (μ_x), expressed in h^{-1}, was defined as:

$$\mu_x = \frac{1}{t} \ln \frac{X}{X_o} \quad (1)$$

where t is the time (h) and X and X_o are the cell concentrations (g/L) at time *t* and at the beginning of the run, respectively.

The CA specific production rate (μ_{CA}), mean CA productivity (P_{CA}) and yield of biomass on product ($Y_{X/P}$), expressed in h^{-1}, mg/L.h and dimensionless, respectively, were defined as:

$$\mu_{CA} = \frac{1}{X} \frac{dC_{CA}}{dt} \quad (2)$$

$$P_{CA} = \frac{C_{CA}}{t} \quad (3)$$

$$Y_{X/P} = \frac{dX}{dT} \frac{dC_{CA}}{dt} \quad (4)$$

where C_{CA} is the maximum CA concentration (mg/L) at time *t*.

Molecular identification

The best *Streptomyces* CA producer, namely *Streptomyces* DAUFPE-3060, was submitted to DNA sequencing. Its DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Amplification of 16S DNA was performed by polymerase chain reaction using universal primers for eubacteria FD1 (5' AGAGTTTGATCCTGGCTCAG-3') and RD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991). The reaction mixture was composed of10 to 50 ng of DNA, 5 p moles of each primer, 200 mM dNTP, 1.5 mM $MgCl_2$, 1X buffer, 1 U Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) to a final volume of 25 μL. The reaction took place with 5 min denaturation at 94 °C; 25 cycles were carried out for 1 min at 94 °C, 30 s at 52 °C and 2 min at 72 °C. These cycles were followed by a final elongation period of 10 min at 72 °C. The amplification product was sequenced and the resulting sequence compared with others available in GenBank using the BLAST software from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

Statistical analyses

All the analyses and experiments were performed in triplicate, and the results expressed as mean values. The errors of experimental data from the mean values were expressed as standard deviations using the Microsoft Excel 2000 program (MapInfo Corporation, Troy, NY, USA) and illustrated as error bars.

Results

Screening of producers of β-lactamase inhibitors

In a first screening, the 19 *Streptomyces* isolates were cultivated in Starch Casein (SC) and ISP-2 agar media to select producers of β-lactamase inhibitors by the agar block method. Among these strains, 21% (DAUFPE-3036, DAUFPE-3060, DAUFPE-3094, DAUFPE-3098 and DAUFPE-3133) showed antimicrobial activity in the SC medium and 16% (DAUFPE-3060, DAUFPE-3133 and DAUFPE-3094) also in the ISP-2 medium. Table 2 shows the inhibition zone diameters obtained culturing these 5 strains in both media.

Table 2 - Results of antimicrobial activity tests of *Streptomyces* strains made in Starch Casein Agar (SCA) and ISP-2 media according to the agar block method.

Strain	Inhibition zone in SCA medium (mm) *	Inhibition zone in ISP-2 medium (mm) *
DAUFPE-3060	11	18
DAUFPE-3133	11	19
DAUFPE-3094	12	15
DAUFPE-3036	0	37
DAUFPE-3098	0	32

*Maximum standard deviation estimated from triplicate experiments was ± 1 mm.

After this step, the 5 active strains of *Streptomyces* spp. were submitted to tests according to the filter paper disk method in different media in order to select the best producers of β-lactamase inhibitors. The results obtained from cultivations performed in SC, Euromicin Production (EP) and modified EP media are listed in Table 3. The strains that showed β-lactamaseinhibition activity were DAUFPE-3036 after 24 h and DAUFPE-3098 after 48 h of incubation, while the other three strains (DAUFPE-3060, DAUFPE-3133 and DAUFPE-3094) showed activity only in the EP medium. The best result as a whole were obtained after 72 h of incubation in the EP medium with the strain DAUFPE-3133 that exhibited an inhibition zone of 18 mm.

Biomass growth and clavulanic acid production

The factors required for microbial growth can be divided into two classes, the physical and chemical. Physical factors include temperature, pH and osmotic pressure while chemical factors include sources of carbon, nitrogen, sulfur, phosphorus, oxygen, trace elements and growth factors (Tortora *et al.*, 2012). Furthermore, according to Santos-Ebinuma *et al.* (2013) carbon and nitrogen sources may influence not only cell growth but also product formation. As in the present work different *Streptomyces* strains are being evaluated for their capacity to produce CA, it is important to select the best producer before carrying out studies devoted to optimization of conditions.

Figure 1A shows the results of growth of the five active *Streptomyces* strains (DAUFPE-3036, DAUFPE-3060, DAUFPE-3094, DAUFPE-3133 and DAUFPE-3098) and of *Streptomyces clavuligerus* ATCC 27064 (control) during submerged cultures in flasks for 168 h. The exponential phase was detected for all strains between 24 and 72 h; however, whereas DAUFPE-3133, DAUFPE-3094, DAUFPE-3060, DAUFPE-3098 and ATCC 27064 did not exhibit any lag phase and quickly reached the exponential one, DAUFPE-3036 took a comparatively long adaptation period and displayed a short exponential phase. This situation was the likely result of premature microbial growth already in the inoculum medium and subsequent withdrawal of a portion of biomass to inoculate the production medium.

The results of CA production by the 5 *Streptomyces* isolates and the control strain are illustrated in Figure 1B. The best producer was DAUFPE-3060 that accumulated no less than 575 mg/L CA after 96 h and then showed a progressive decrease in CA concentration. Such production was 22% higher than that observed with the control strain after 120 h and much higher than those achieved with the other strains.

Kinetic study

Although kinetic modeling is essential to estimate costs of an optimized bioprocess as well as to develop control strategies (Baptista-Neto *et al.*, 2000), there are only a few papers in the literature dealing with the kinetics of CA production *vs.* growth; therefore, it was one of the issues investigated in this study.

Consistently with the above results of CA production, the best producer showed also the highest CA productivity (5.99 mg/L h) after 96 h of cultivation (Figure 2), while DAUFPE-3133 and DAUFPE-3036 exhibited the lowest values (1.63 and 1.78 mg/L h, respectively), and the control strain a value close to that ofDAUFPE-3060 (5.66 mg/L h).

As shown in Figure 3, the DAUFPE-3098 and the control strain showed the lowest maximum specific rates of CA formation (μ_{CA}). Although a higher value of this kinetic parameter was obtained with the strain DAUFPE-3036 (2.01 h^{-1}), the overall performance of DAUFPE-3060 should be considered the best one being able to ensure almost the same μ_{CA} value (1.96 h^{-1}) but in quarter the time (24 instead of 96 h). In contrast with the other strains that suffered a progressive decrease of μ_{CA} with time, DAUFPE-3133 showed lower μ_{CA} after 72 h of cultivation and reached a maximum value of 1.39 h^{-1} after 120 h, likely due to both an increase in cell concentration and a CA degradation during the run. Taking all results together, μ_{CA} varied from 0.06 h^{-1} (standard strain after 144 h) to 2.01 h^{-1}

Table 3 - Results of antimicrobial activity tests of *Streptomyces* strains made in Starch Casein (SC), Euromicin Production (EP) and modified EP media according to the filter paper disk method.

Strain	Time (h)	Inhibition zone in SC medium (mm)*	Inhibition zone in EP medium (mm)*	Inhibition zone in modified EP medium (mm)*
DAUFPE-3060	48	-	14	17
DAUFPE-3133	72	-	18	-
DAUFPE-3094	72	-	16	12
DAUFPE-3036	24	10	-	-
DAUFPE-3036	48	-	-	13
DAUFPE-3098	48	9	-	15

* Maximum standard deviation estimated from triplicate experiments was ± 1 mm.

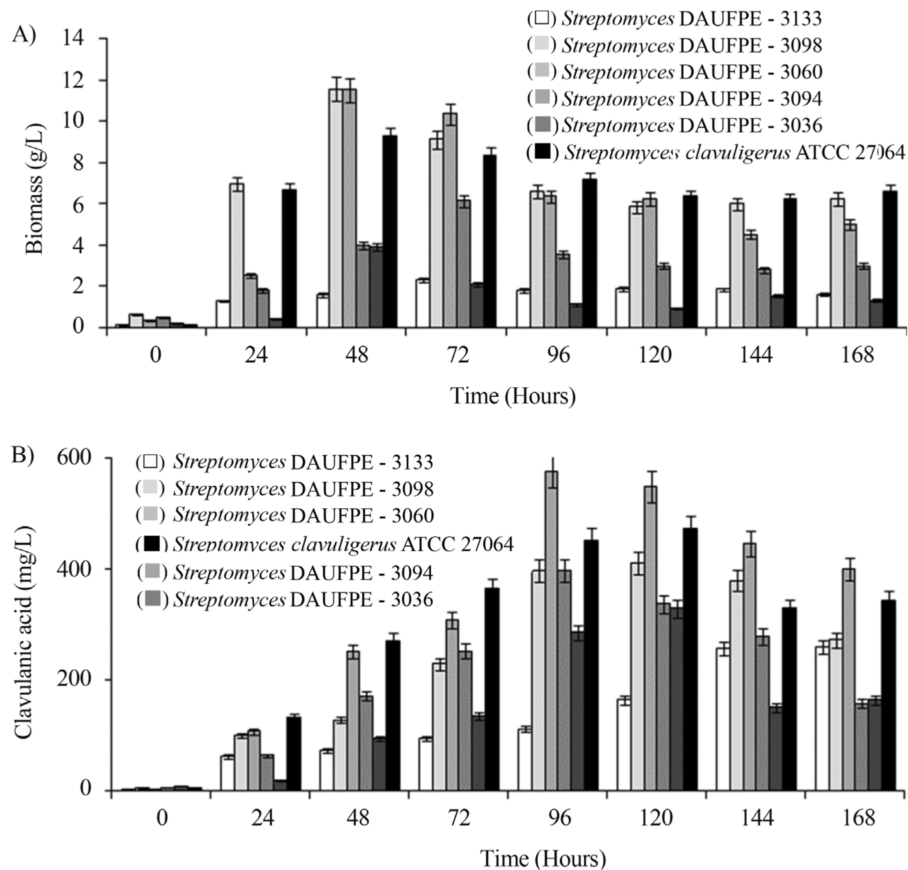

Figure 1 - Time behavior of biomass (A) and clavulanic acid (B) concentrations along 168 h-submerged cultures of *Streptomyces* DAUFPE-3133, DAUFPE-3098, DAUFPE-3060, DAUFPE-3094, DAUFPE-3036 and *Streptomyces clavuligerus* ATCC 27064.

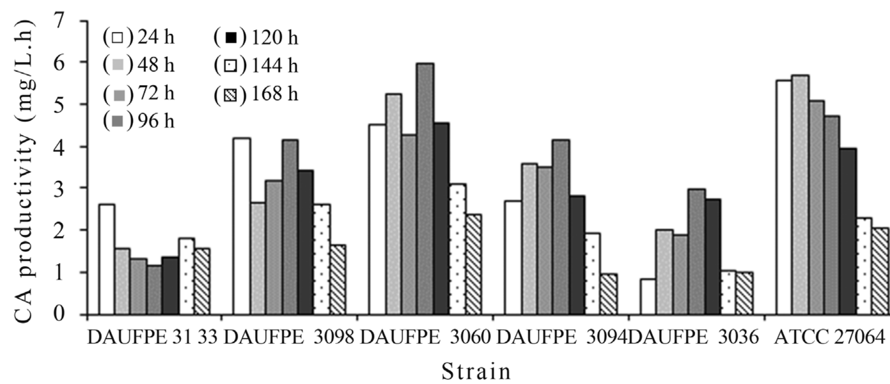

Figure 2 - Time behavior of clavulanic acid productivity along 168 h-submerged cultures of different *Streptomyces* strains. Time (h): 24; 48, 72, 96, 120, 144 168 h.

(DAUFPE-3036 after 96 h). These specific productivities are higher than that reported by Elibol and Mavituna (1999) for the actinorhodin production by *Streptomyces coelicolor* in bioreactor.

As it can be observed in Table 4, the strains DAUFPE-3060 and DAUFPE-3098 had the highest specific growth rate (μ_x = 0.062 h^{-1} for both) after 48 h, while the control strain grew about 8% less quickly.

Figure 4 shows that the highest values of the yield of growth on product ($Y_{X/P}$) were obtained within 24 h with DAUFPE-3098 (0.15) and DAUFPE-3036 (0.11), both preferring to grow rather than to produce CA, while, as expected, the opposite took place with the best CA producer and the control strain ($Y_{X/P}$ = 0.05 for both). Even lower yields were obtained with the other strains due to a too slow growth in addition to a poor production.

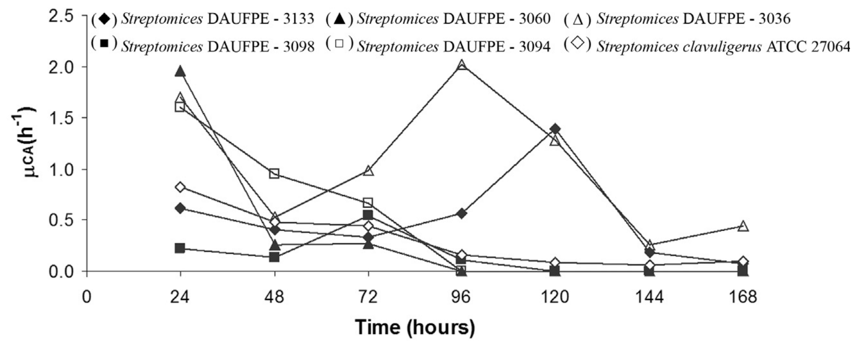

Figure 3 - Time behavior of specific clavulanic acid productivity (μ_{CA}) along 168 h-*s*ubmerged cultures of *Streptomyces* DAUFPE-3133, DAUFPE-3098, DAUFPE-3060, DAUFPE-3094, DAUFPE-3036 and *Streptomyces clavuligerus* ATCC 27064.

Table 4 - Specific growth rate of different *Streptomyces* isolates and *S. clavuligerus* ATCC 27064 (control strain) during submerged cultures.

Time (h)/strain	DAUFPE-3133 (h^{-1})	DAUFPE-3098 (h^{-1})	DAUFPE-3060 (h^{-1})	DAUFPE-3094 (h^{-1})	DAUFPE-3036 (h^{-1})	ATCC 27064 (h^{-1})
48	0.049	0.062	0.062	0.040	0.039	0.057
72	0.038	0.038	0.040	0.032	0.017	0.036
96	0.026	0.025	0.025	0.018	0.006	0.026
120	0.021	0.019	0.019	0.013	0.003	0.020
144	0.017	0.016	0.014	0.011	0.006	0.016
168	0.014	0.014	0.013	0.010	0.005	0.014

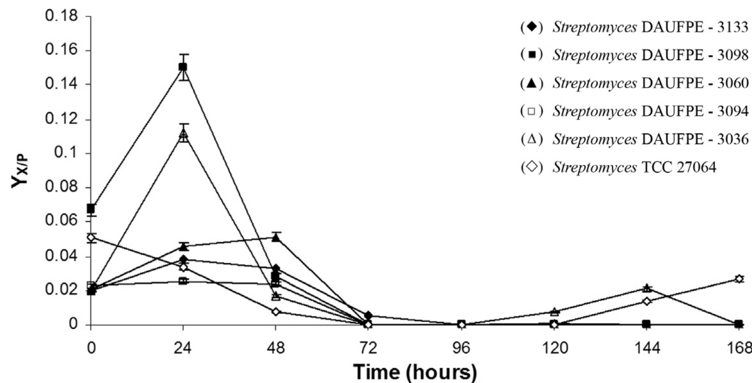

Figure 4 - Time behavior of the yield of growth on product ($Y_{X/P}$) along 168 h-submerged cultures of *Streptomyces* DAUFPE-3133, DAUFPE-3098, DAUFPE-3060, DAUFPE-3094, DAUFPE-3036) and *Streptomyces* ATCC 27064.

Molecular identification of the best CA producer

Molecular identification was finally performed for DAUFPE-3060 that behaved as the best CA producer. The 1550 bp sequenced fragment showed 96% identity with the strain *Streptomyces variabilis* 7525 (accession number: gi|341793371|JN180216.1); so, it was identified as the action bacterium *Streptomyces variabilis* DAUFPE 3060.

Discussion

In this study, we tested different *Streptomyces* strains in order to find a novel and alternative microorganism able to overproduce CA, for which distinct media were investigated (Figure 1). The results obtained with EP and modified EP media were quite different, in that the CA production was considerably quicker in the latter medium, where the antimicrobial activity was observed only after 12 h of incubation with all the strains studied. The diameter of the inhibition zone made from samples collected between 12 and 24 h ranged from 13 to 17 mm with DAUFPE-3036, 3060, 3094 and 3098, but it considerably decreased after 48 h. Sometimes, a decrease in activity was observed after 48-72 h probably due to degradation of CA released during the

submerged culture. For instance, Chen *et al.* (2003) reported that a decrease in CA concentration after 36 h of *S. clavuligerus* submerged culture was the likely consequence of increasing pH and cell autolysis, and similar occurrence was observed by Mayer and Deckwer (1996) and Romero *et al.* (1984) with *S. clavuligerus* and by Viana Marques *et al.* (2009) with *Streptomyces* DAUFPE-3060. The strains DAUFPE-3094, DAUFPE-3036 and DAUFPE-3060 exhibited positive response with inhibition zones narrower than 10 mm in Soybean Hydrolyzed medium with Glycerol (SHG). These results suggest that the other strains (DAUFPE-3133 and DAUPE-3098) probably preferred glucose as carbon source to grow.

Resuming, this part of the study pointed out different behaviors of the five strains possessing β-lactamase inhibition activity, all of them being able to produce CA in the EP medium, DAUFPE-3036, DAUFPE-3060 and DAUFPE-3094 also in the modified EP and SHG media, and DAUFPE-3098 in the former.

Considering biomass growth and CA production, all the strains reached maximum biomass concentration (4-12 g/L) after 48 h of cultivation, with the exception of the strains DAUFPE-3133 and DAUFPE-3094 that lasted longer (72 h). These times were relatively short taking into account that the exponential phase of Streptomycetes usually stops after 96 h (Viana *et al.*, 2010; Viana Marques *et al.*, 2011) and that all these submerged cultures were performed in flasks, *i.e.* under non-optimized conditions. Bushell *et al.* (2006) studied the CA production by *S. clavuligerus* NRRL 3585 in 2.5 L bioreactor and obtained a biomass concentration of 5 g/L within 150 h. A similar result was obtained after 72 h by Gouveia *et al.* (1999) with S. *clavuligerus* NRRL 3585 in a medium enriched with amino acids. Viana *et al.* (2010) reported for DAUFPE-3060 a maximum CA production of 494 mg/L after 48 h and a subsequent CA consumption after glycerol depletion in the medium using soybean proteins. Lower CA concentration (338 mg/L after 108 h) was reported for *S. clavuligerus* ATCC 27064 submerged culture in flasks using soy protein as nitrogen source and soybean oil and glycerol as carbon sources (Ortiz *et al.*, 2007); however, Costa and Badino (2012) achieved no less than1543 mg/L with the same microorganism using pulses of glycerol at constant temperature (20 °C).

A literature survey reveals that the nitrogen source has a strong influence on CA production by *S. clavuligerus*. A comparison of the results obtained in the present work in flasks using a cheap nitrogen source like soybean flour with those reported in the literature using proline and glutamic acid (Romero *et al.*, 1984), malt extract and bacteriological peptone (Belmar-Beiny and Thomas, 1991), soy extract and peptone from meat (Mayer and Deckwer, 1996), soybean extract and bacteriological peptone (Gouveia *et al.*, 1999) and Samprosoy (Teodoro *et al.*, 2006) stands out DAUFPE-3060 as a promising CA producer at an industrial level.

All the strains quickly grew in the first hours of submerged culture. Similar results were obtained by Saudagar and Singhal (2007), who studied the influence of different amino acids as nitrogen source on CA production by *S. clavuligerus* and found μ_x values of 0.057 and 0.055 h^{-1} using arginine and threonine, respectively. Kim and Lee (1995) studying the continuous production of leupeptin (a protease inhibitor) by *Streptomyces exfoliatus* SMF13 obtained $\mu_x = 0.08\ h^{-1}$ and a productivity as high as 0.24 g/L.h between 48 and 72 h of cultivation. A μ_{max} of 0.207 h^{-1} was reported by Baptista Neto *et al.* (2000) for CA production by *S. clavuligerus* NRLL 3585 in bioreactor using peptone and Samprosoy 90 NB as nitrogen sources, which suggests the potential of future optimization of our process at least in a bench-scale fermenter. In general, the large variability of $Y_{X/P}$ observed in this study under all the conditions tested is the result of the well-known presence of two separate phases of growth (trophophase) and production (idiophase).

Conclusions

A screening study was made, which suggested that clavulanic acid production could be greatly improved using new strains of *Streptomyces.* Five new isolates with β-lactamase inhibiting activity screened in this study showed potential to be used as CA producers; however, the strain DAUFPE-3060 showed the best results and was subsequently identified as the novel actinobacterium *Streptomyces variabilis* DAUFPE 3060. Thus, further optimization studies devoted to possible industrial exploitation are of great interest. This strain did in fact show a 28% increase in CA production compared to *S. Clavuligerus* ATCC 27064 (control strain). Soybean flour was shown to be an interesting alternative nitrogen source for CA production, being cheaper than others reported in the literature. Therefore, its use is expected to substantially reduce the production cost of such a β-lactamase inhibitor.

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