Fermentation Technology
ISSN: 2167-7972

da Silva et al., Ferment Technol 2012, 1:3
DOI: 10.4172/2167-7972.1000105

Research Article Open Access

# The Effect of Varying Culture Conditions on the Production of Antibiotics by *Streptomyces* spp., Isolated from the Amazonian Soil

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## Abstract

The genus *Streptomyces* is considered to be of great industrial importance because of its ability to produce secondary metabolites that account for 80% of the antibiotics currently in use. To optimise the production of antimicrobial compounds from three strains of *Streptomyces* spp. isolated from the Amazon's soil, we investigated the influence of physical (temperature, pH, agitation and time) and chemical (concentrations of carbon and nitrogen) variables, according to a factorial statistical design consisting of three repetitions at the central point. During a period between five and twenty days of incubation, the temperature was varied between 20 and 40°C, the pH was varied between 4.5 and 8.5, and the agitation was varied between 100 and 300 rpm. The concentrations of carbon and nitrogen sources ranged from 5 to 15 g/L and 0.5 to 1.5 g/L, respectively, and the results were evaluated using the Response Surface Methodology (RSM). Our data showed that the most effective carbon sources were starch and glycerol and that the best sources of nitrogen were phenylalanine, ammonia sulphate, asparagine and peptone. The results of this study showed that the temperature, incubation time and the culture medium directly influenced the production of metabolites (antibiotics). These parameters can be modified for the optimisation and improvement of the fermentation process by increasing the production of the compound of interest. Each *Streptomyces* behaved differently, requiring specific conditions for the production of secondary metabolites.

**Keywords:** *Streptomyces*; Antibiotic; Secondary metabolites

## Introduction

*Streptomyces* spp. is a Gram-positive soil bacterium that grows as a vegetative mycelium with branching hyphae [1,2]. For dispersion, spores are formed on specialised reproductive structures called aerial hyphae, which emerge from the colony surface into the air [3]. Streptomycetes are renowned for their ability to produce clinically important antibiotics and other bioactive compounds. Recently, several studies were conducted to isolate new *Streptomyces* species from different habitats [4-6]. The biosynthesis of antibiotics is a specific property of microorganisms and depends on growth conditions. These microorganisms use a wide variety of substrates for growth; however, many of these substrates can have a negative effect on the production of some products of interest [7,8]. Under various nutrient limitations, the production of secondary metabolites is higher compared to non-limiting nutrient conditions [9]. Studies on the optimisation of antibiotic usually involve a search for ideal methods for their production. This is achieved by a systematic study of the adaptations of different physicochemical factors, such as nutrient limitation (carbon source, nitrogen source, and phosphate), oxygen, temperature, growth rate, feedback control, inactivation or induction [10]. The use of experimental factorial design and Response Surface Methodology (RSM) has been successfully applied in other fields, and it has been used to optimise media and culture conditions in some fermentation processes for the production of primary and secondary metabolites [11]. In the present study, we isolated *Streptomyces* from the Amazonian soil and determined the conditions for optimal antibiotic production.

## Materials and methods

### Microorganisms

The *Streptomyces* spp. were isolated from soil samples from the region of Manaus, Amazonas, Brazil. Pure cultures were grown in M9 medium (Sigma) plus starch (10 g/L) and stored at -70°C until needed.

### Evaluation of antimicrobial activity

The antimicrobial activity was evaluated by agar diffusion well assays. The microorganism indicators used to evaluate the production of antibiotics were *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *Staphylococcus aureus* antibiotic resistant. The indicator microorganisms were plated on Mueller-Hington agar plates containing 5 orifices of 6 mm, where 100 mL of filtrate from each *Streptomyces* isolate was placed in each well and standardised according to the McFarland scale (tube 0.5). The diameter of the zones of inhibition was measured after 16 hours of incubation at 37°C.

## Identification of Isolates

The identification of isolated strains was performed according to Bergey's Manual of Determinative Bacteriology [12]. Genomic DNA was extracted by a bead beating lysis method with 10% sodium dodecyl sulphate and phenol-chloroform. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1401R (5'-CGGTGTGTACCCGGCCCGGAACG-3').

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**Received** July 19, 2012; **Accepted** July 26, 2012; **Published** July 28, 2012

**Citation:** da Silva IR, Martins MK, Carvalho CM, de Azevedo JL, de Lima Procópio RE (2012) The Effect of Varying Culture Conditions on the Production of Antibiotics by *Streptomyces* spp., Isolated from the Amazonian Soil. Ferment Technol 1:105. doi:10.4172/2167-7972.1000105

The PCR conditions consisted of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s, with a final step at 72°C for 10 min. Subsequently, the DNA amplification product was purified (GFX PCR kit, Amersham Pharmacia Biotech) and sequenced. The partial 16S rDNA sequence obtained was submitted to GenBank for BLAST searching, and phylogenetic analyses were conducted using the MEGA4 software program [13].

## Culture Media and Growth Conditions

The following carbon sources were evaluated: corn starch, rice starch, glycerol, glucose and cellulose. To determine the best nitrogen source for antibiotic production, we assessed the following compounds: alanine, arginine, asparagine, cysteine, glycine, guanidine, glutamine, histidine, isoleucine, leucine, lysine, methionine, proline, phenylalanine, valine, tyrosine, aspartic acid, tryptophan, serine, threonine, yeast extract, malt extract, peptone, casein and ammonium sulphate. *Streptomyces* was grown for 7 days on medium containing AC-starch-casein [14]. The spores were then inoculated into a 500 mL Erlenmeyer flask containing 50 mL of liquid medium with a starch-casein composition (g/L). For the optimisation of antibiotic production, a pre-inoculum was used, whereby a volume of 2.5 mL of broth was inoculated into Erlenmeyer flasks (250 mL) containing 22.5 mL of production medium. As described below, carbon sources (10.00 g/L), casein (0.30 gL), $KNO_3$ (2.00 g/L), NaCl (2.00 g/L), $K_2HPO_4$ (2.00 g/L), $KH_2PO_4$ (1.00 g/L), $CaCO_3$ (0.10 g/L), $MgSO_4$ (0.10 g/L), $FeSO_4$ (0.01 g/L), $ZnSO_4$ (0.01 g/L), nitrogen sources (1.00 g/L) and distilled water (1 L) were mixed and the pH was adjusted to 6.5. The flask was incubated at 28°C for two days. Using statistical design, the concentrations and conditions of the carbon and nitrogen sources were defined for the cultivation and production of antimicrobial compounds.

## Experimental Design

A statistical factorial design was conducted by the RSM with three replicates at the central point for building the statistical model by MINITAB. Twenty-seven experiments were performed where the isolates were grown in liquid culture medium under different conditions of pH, agitation, temperature and rise time. The pH was varied from 4.5 to 8.5, stirring was varied between 100 to 300 rpm, temperature was varied between 20 to 40°C and incubation time was varied between 5 to 20 days. To determine the optimal conditions for improving the production of antimicrobial metabolites, we performed another statistical design, as described above using the RSM. We evaluated the influence of two carbon sources (starch and glycerol) and four sources of nitrogen (phenylalanine, asparagine, peptone and ammonium sulphate). The concentrations of starch and glycerol ranged from 5 to15 g/L and nitrogen varied from 0.5 to 1.5 g/L. This resulted in 27 media formulations from different cultures, each corresponding to an experimental test.

## Results and Discussion

### Antimicrobial activity

Using the methodology described, 371 *Streptomyces* were isolated from the soil in the region of Manaus, Amazonas, Brazil. The antimicrobial activity for all 371 isolates on solid medium by the agar diffusion method was determined. The predominant antimicrobial activity was against Gram-positive bacteria (46 isolates), and only five isolates had activity against Gram-negative bacteria. Table 1 shows the isolates exhibiting antimicrobial activity against microorganisms. There was a high rate of inhibition of Gram-positive bacteria by antibiotics produced by *Streptomyces*. Low activity against Gram-negative bacteria may be due to the complexity of the outer membrane of these bacteria [15,16].

Only five isolates exhibited activity, under the conditions tested, against Gram-negative bacteria (*Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC700603). In most cases the antimicrobial activity was detected against the Gram-positive bacteria (*Staphylococcus aureus* ATCC25923, ATCC49619 *Streptococcus pneumoniae*, *Enterococcus faecalis* ATCC29212). All three (01, 325 and 355) isolates selected demonstrated activity against antibiotic-resistant *Staphylococcus aureus* (erythromycin, penicillin, gentamicin, oxacillin, cefotaxime, cefoxitin, norfloxacin, ceftriaxone, aztreonam, cephalothin, amikacin, clindamycin, tobramycin and ceftazidima). Among the pathogens, *Staphylococcus aureus* represents one of the main agents of nosocomial infection. Because *S. aureus* is difficult to control with antibiotics, spreads easily and causes high mortality rates, there is a need to develop new compounds for therapeutic treatment against infections caused by these bacteria. It was found that a *Streptomyces* spp. isolated from marine sediment that showed activity against *Staphylococcus aureus* (MRSA) [17].

### Molecular characterisation of *Streptomyces*

Using the 16S rRNA gene of three *Streptomyces* (01, 325 and 355), in this study it was possible to identify and evaluate the similarity with other strains at the National Center for Biotechnology Information (NCBI). Considering the importance of *Streptomyces* in biological terms, the production of secondary metabolites and adaptation to the environment is important in understanding its relationship to other species and the diversity within this genus. This indicates that the chemical diversity is associated with the biological diversity of complex microbial communities and is distributed heterogeneously [18]. The three strains studied did not form a single phylogenetic group, as seen in Figure 1. Despite the differences between the *Streptomyces* spp.,

| Isolates | Pathogens | | | | |
|---|---|---|---|---|---|
| | *S aureus* | *S pneumoniae* | *E faecalis* | *K pneumoniae* | *E coli* |
| Nº 01, 315,325, 355, 234 | +++ | +++ | +++ | - | - |
| N º 166 | +++ | ++ | ++ | - | - |
| Nº 279 | +++ | - | +++ | + | - |
| Nº 121, 167, 282 | +++ | - | +++ | - | - |
| Nº 371 | +++ | ++ | - | - | - |
| Nº 107, 305, 372 | +++ | - | - | - | - |
| Nº 129, 184, 189,, 252 | - | - | +++ | - | - |
| Nº 168, 204, 210, 211, 343, 353, 356, 357, 358, 361 | - | +++ | - | - | - |
| Nº 335, 379 | ++ | - | ++ | - | - |
| Nº 113 | - | - | - | - | ++ |
| Nº 181, 256, 265, 294 | - | - | - | ++ | - |
| Nº 02, 81, 84, 365, 376 | ++ | - | - | - | - |
| Nº 359 | - | ++ | - | - | - |
| Nº 377, 383, 392, 393, 394 | + | - | - | - | - |

Low (+): inhibition zone diameter between 07 and 12 cm;
Moderate (+ +): inhibition zone diameter between 13 and 16 cm;
High (+ + +): inhibition zone diameter greater than 17 cm;
Neg (-): no inhibition zone

**Table 1:** Classification of antimicrobial activity displayed by *Streptomyces*.

the similarity is high (0.005, or less than 1%), which is common for this genus [19]. There remains the difficulty of finding patterns in the sequences and strains in most studies that describe the strain as *Streptomyces* spp. [20,21]. Using only the 16S rRNA sequence, it is almost impossible to identify the level of a *Streptomyces* species, six strains of *S. hygroscopicus* evaluated were unable to form a group using the phylogenetic 16S rRNA [18]. Other authors suggest that the 16S rRNA gene is an important tool for phylogenetic analysis of Gram-negative bacteria but not for *Streptomyces* [19]. Despite the difficulty in classifying each strain to the species level using the 16S rRNA gene, all three strains were confirmed to belong to the genus *Streptomyces*.

## Optimisation of production conditions

Development of efficient fermentation processes for the production of secondary metabolites by *Streptomyces* requires examination of a diverse array of species-specific features, including physical and chemical factors. Carbohydrates and nitrogen play key roles as structural and energy compounds in cells. Thus, to determine the optimal medium for antibiotic production, various carbon and nitrogen sources were tested. The results showed that the most effective carbon sources were starch and glycerol, and the best sources of nitrogen were phenylalanine, ammonia sulphate, asparagine and peptone [22]. Therefore, these compounds were chosen for the subsequent optimisation study. Although cultures of *S. noursei* ATCC 11455 supplied with glucose as a carbon source achieved high nystatin titres, due to the high biomass concentration, glucose tended to negatively influence the production of nystatin, independent of specific growth rates when phosphate and ammonium were in excess. Glycerol was a better carbon source than glucose, yielding higher nystatin production. Several studies have been performed cultivating *Streptomyces* under different growth conditions, such as fermentation liquid and solid, static culture or under agitation, to optimise the production of antibiotics. These studies demonstrate the significant influence of external conditions on the biosynthesis of these compounds [23]. In (Table 2), the optimal physical conditions for the production of antimicrobial compounds by each isolate are shown. Values demarcated with an (s) were statistically significant, with a confidence level of 95% or p value = 0.05.

Statistical analysis showed that varying the time had a significant effect on the production of the antimicrobial compounds produced by strain No. 01. The antimicrobial compound produced by isolate No. 01 showed the largest inhibition zones and highest stability when exposed to different growth conditions. The production of its antimicrobial compound was observed on the first day of cultivation. Cultivation temperature generally affects metabolite biosynthesis. In this study, the change in temperature was only significant for strain No. 325, based on the diameter of the inhibition zone, the production of this antimicrobial agent was greatest at 30°C. Temperature and soybean flour concentration were shown to be the two most important variables that affect the production of clavulanic acid, at a 95% confidence level [24]. Validamycin-A biosynthesis by *S. hygroscopicus* 5008 was investigated between 28°C and 42°C. An interesting threshold of temperature for biosynthesis was found between 35°C and 37°C [25].

The significant variables for isolate No. 355 were time and pH. The production of the antimicrobial compound was greatest after incubation for about 10 to 15 days at pHs between 7-8.5. The production of antimicrobial compounds also significantly change in response to the carbon source in the culture growth medium. Table 3 illustrates the ideal nutritional conditions for each isolate. Values with an (s) were statistically significant with a confidence level of 95% or p value=0.05.

The three independent variables of concentration of the carbon source (glucose), nitrogen source (soybean meal) and temperature of incubation were found to be the most important for the production of antifungal antibiotics by the *S. chattanoogensis* MTCC 3423 isolate. The optimal combination of the three factors for a maximum yield (263.63 U) of the antibiotic was 5% glucose (carbon source), 1% soybean meal (nitrogen source) and an incubation temperature of 30°C [26].

For isolate No.01, according to the diameter of the inhibition zone, the production of the antimicrobial compound was greater when the microorganism was grown in glycerol rather than in starch. Similar results were reported [27], who employed the RSM to optimise the composition of the medium for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A. Corn starch and yeast extract were significant variables, yielding maximum values of production with 149.57 g/L of corn starch and 8.92 g/L of extract yeast. Yeast extract

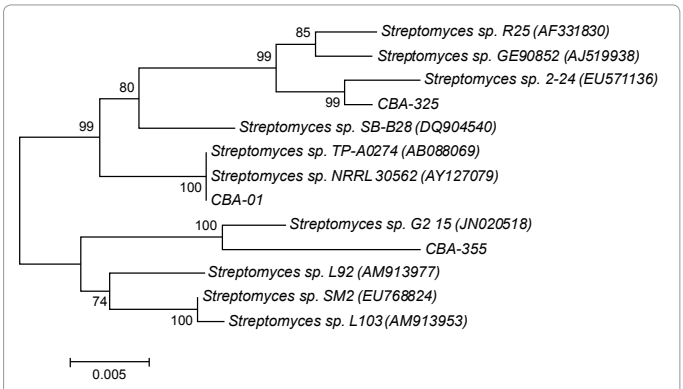


**Figure 1:** Phylogeny showing relationships between *Streptomyces*, based on clustering of the sequences of the 16S rRNA gene (coefficient of Neighbour-joining) The software package MEGA 4 was used for analysis.

| Isolates | Temperature | Time | pH | Agitation |
|---|---|---|---|---|
| intervals | 20 to 40°C | 1 to 20 days | 4,5 to 8,5 | 100 to 300 rpm |
| **01** | 20 to 35°C (ns) | 10 to 20 days **(s)** | 6,5 to 7,5 (ns) | 150 to 200 (ns) |
| **325** | 30°C **(s)** | 10 days (ns) | 6,5 (ns) | 100 to 200 (ns) |
| **355** | 25°C (ns) | 15 days **(s)** | 7,5 **(s)** | 150 (ns) |

**(ns)**: not significant
**(s):** significant at the level of 95%

**Table 2:** Improved physical conditions for the production of antimicrobial compounds using isolates No 01, 325 and 355.

| Isolates | Source of nitrogen | Starch | Source of nitrogen and starch | Glycerol | Source of nitrogen and glycerol |
|---|---|---|---|---|---|
| Intervals | 20 Amino Acids | 5 to 15 g | 0,5 to 1,5 g | 5 to 15 g | 0,5 to 1,5 g |
| **01** | Phenylalanine and Sulf ammonium | 12,5 to 15 g **(s)** | 1 to 1,25 g (ns) | Minor Production | Minor Production |
| **325** | Phenylalanine and Sulf ammonium | Minor Production | Minor Production | 7,5 g (ns) | 1,25 g **(s)** |
| **355** | Asparagine peptone | 7,5 g (ns) | 0,75 g (ns) | No production | No production |

(ns): Not significant
(s): A significant 95%

**Table 3:** Nutritional conditions ideal for the production of the antimicrobial compound using isolate No 01, 325 and 355.

increased the mycelium biosynthesis but did not affect the growth rate [28].

The production of antimicrobial compounds by strain No. 325 was higher when the organism was cultured in medium with glycerol as the carbon source. The variable inorganic nitrogen (ammonium sulphate) was only significant for trials with glycerol. Nutrition plays an important role in the onset and intensity of secondary metabolism. Control of antibiotics biosynthesis was demonstrated to be a multifunctional process in which limiting nutrients, such as nitrogen, affects the metabolism [29]. The best conditions for isolate No. 355 were established by adjusting the physical variables (Table 2). Supplementing the culture media to enhance antimicrobial activity did not alter the production. The nitrogen sources usually favourable to growth, such as ammonium salts in nonlimiting amounts, repress the enzymes involved in the assimilation of other nitrogen sources such as amino acids with an inhibitory effect on the secondary metabolism[30].

The results of this study showed that the temperature, incubation time and the culture medium directly influence the production of metabolites (antibiotics). These parameters can be modified for the optimisation and improvement of the fermentation process by increasing the production of the compound of interest. It is important to establish a mathematical relationship among the medium components and length of incubation period for achieving maximum antibiotic yield [31]. The three strains tested here behaved differently, with each *Streptomyces* spp. requiring specific conditions for the production of secondary metabolites. The three selected isolates have the potential for producing antibiotics that are able to inhibit many clinically important strains, such as *Staphylococcus aureus* (MRSA). These *Streptomyces* strains may be studied in the future to discover new and more effective antibiotics.

## Acknowledgements

**Financial support:** This work was supported by the Fundação de Amparo a Pesquisa do Estado Amazonas (FAPEAM) and the Centro de Biotecnologia da Amazônia (CBA).

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