Brazilian Journal of Chemical Engineering

ISSN 0104-6632
Printed in Brazil

Vol. 19, No. 03, pp. 277 - 285, July - September 2002

DEVELOPMENT OF A BIOPROCESS FOR THE PRODUCTION OF ACTINOMYCIN-D

M.F.V.Q. Sousa[1], C.E.Lopes[1] and N.Pereira Jr.[2]

[1]Departamento de Antibióticos, Universidade Federal de Pernambuco,
Phone/Fax, (081) 3271-8346, 50670-901, Recife-PE, Brazil,, E-mail: mfvqs @npd.ufpe.br
[2]Departamento de Engenharia Bioquímica, UFRJ, Phone (021) 2562-7620,
21949-900, Rio de Janeiro - RJ, Brazil;
E-mail: nei@eq.ufrj.br

(Received: March 1, 2002 ; Accepted: June 25, 2002)

Abstract - *S. parvulus* was selected from three species of *Streptomyces* that produce actinomycins, as it has the greatest antibiotic activity (152 mg/L) and produces only actinomycin-D. Aiming at improving the antibiotic production in shaken flasks, the substitution of glucose by fructose in concentrations of 20, 30 and 40 g/L was investigated. In all cases this replacement led to an increase in the antibiotic production, reaching a maximum concentration of 635 mg/L for an initial fructose concentration of 30 g/L. In experiments conducted in a bioreactor at different degrees of aeration (0.5, 1.0 and 1.5 vvm) and stirring speeds (300 and 500 rpm), it was found that the greatest antibiotic production (1530 mg/L) occurred with a aeration of 1.5 vvm and a stirring speed of 500 rpm. The fermented medium showed the rheological behavior of a pseudoplastic fluid.
Keywords: actinomycin-D, *S. parvulus*, *S. felleus*, *S. regensis*, fermentation, rheology.

INTRODUCTION

Actinomycin-D is a chromopeptide antibiotic of indisputable therapeutic value, currently used in the treatment of several human neoplasias. This antibiotic can be synthesized by different species of *Streptomyces* as part of a mixture of several actinomycins (Korzybski et al., 1967).

Few studies have been done on the production of actinomycin-D (Williams and Katz, 1977; Dalili and Chau, 1988). Working with shaken flasks, Williams and Katz (1977) developed a chemically defined medium with a view to obtaining high levels of actinomycin-D (500 to 600 mg/L) with *S. parvulus* ATCC 12434. Using the same medium and the same strain immobilized in calcium alginate, Dalili and Chau (1988) achieved maximum actinomycin-D concentrations of around 50, 70 and 80 mg/L by employing batch, fed batch and continuous processes, respectively, in an airlift bioreactor.

Some studies have dealt with the rheological characterization of media fermented by actinomycete, though none refer to the production of actinomycin-D by *Streptomyces parvulus*.

The aim of this work was to maximize the production of this drug, addressing the following issues: selection of the most productive of the three species of *Streptomyces*; identification of the best composition of the production medium in shaken flasks; study of the most favourable aeration and agitation conditions in the bioreactor, using the selected species; and rheological characterization of the fermented medium.

MATERIALS AND METHODS

Microorganisms

The S*treptomyces* species used were *S. regensis* DAUFPE 3053, *S. felleus* DAUFPE 3079 and *S. parvulus* DAUFPE 3124 from the collection of

microorganisms at the Departamento de Antibióticos at the UFPE. *B. subtilis* ATCC 6633 was used to measure antibiotic activity, as recommended by Farmacopéia Brasileira (1988).

Conditions and Media for Maintenance and Sporulation

The slope medium used for the maintenance and sporulation of the species of *Streptomyces* had the following composition: glucose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L and agar medium 20 g/L, and the species were incubated at 30°C for five to seven days, after which time they were kept at 4°C. *B. subtilis* was maintained in slant tubes containing a nutrient agar medium (peptone 5 g/L, meat extract 3 g/L and agar 20 g/L), incubated at 37°C for 24 h and conserved at 4°C.

Preparation of the Spore Suspensions

The spores were skimmed off the surface of the slant tubes with 3 mL of physiological solution containing 0.1% (v/v) Tween 80, using the technique employed by Hopwood et al. (1985). Each spore suspension was quantified – *S. parvulus* (8.0×10^7 CFU/mL), *S. regensis* (1.25×10^4 CFU/mL), *S. felleus* (2.7×10^8 CFU/mL) – and stored at -4°C. For *B. subtilis* the method described in the Farmacopéia Brasileira (1988) was adopted.

Growth and Production Media

The growth medium used in all the tests had the following composition: tryptone 5 g/L and yeast extract 5 g/L. To evaluate the actinomycin production of the *Streptomyces* species, a culture medium with the following composition was used: glucose 20 g/L, soy milk 20 g/L, NaCl 5 g/L, K_2HPO_4 1.5 g/L and $CaCO_3$ 2 g/L. For the experiments carried out in a bioreactor, an optimized medium with the following composition was used: fructose 30 g/l, soy milk 30 g/L and $CaCO_3$ 2 g/L. In all the media, the pH was adjusted to between 7.0 and 7.2. The soy milk used was a commercially available brand call Soymilk, produced by Olvebra Industrial S.A.

Experimental Procedure

250 mL Erlenmeyer flasks containing 25 mL growth medium were inoculated with 100 µL suspensions of the species *S. parvulus* and *S. felleus*, which had been standardized at 1.14×10^7 CFU/mL, and *S. regensis*, which had a lower quantity of spores (1.25×10^4 CFU/mL). These flasks were incubated at 30±2ºC and shaken at 200 rpm for 42 hours (growth phase). After this period, 5 mL of each of the suspensions was used to inoculate the 250 mL Erlenmeyer flasks, containing 50 mL of the selected medium. These were then submitted to the same temperature and agitation conditions for 192 hours (production phase). The performance of each of the species was evaluated for antibiotic activity after 96 h and thereafter at 24-hour intervals. To optimize the production medium for the selected species, glucose and fructose as well as their initial concentrations were varied for the tests in shaken flasks. The flasks were initially inoculated with a spore suspension of the selected species (8.0×10^7 CFU/mL) and submitted to the temperature and agitation conditions already described. The experiments in the bioreactor were carried out at different degrees of aeration (0.5, 1.0 and 1.5 vvm) and stirring speeds (300 and 500 rpm). Samples were removed at 24-hour intervals and assessed for concentration of actinomycin D, of total reducing sugars and of dry weight.

Description of the Bioreactor

A 14-liter bioreactor, operating with an 8.0-liter volume, was used. Sterile air was supplied continuously through a porous metal sparger, and the stirrers were turbines with four flat blades. Two turbines were placed in the following layout: the first was placed directly above the sparger, the second at a distance from the first corresponding to one-third of the reactor's diameter. Whenever necessary, antifoam was added automatically using a fixed-flow peristaltic pump connected to a sensor. The temperature was maintained at 30°C by controlling the temperature of the water bath in which the bioreactor was immersed. Figure 1 shows a diagram of the bioreactor.

Analytical Methods

The antibiotic activity of each species of *Streptomyces* was evaluated by the agar diffusion method (Isaacson and Kirschbaum, 1986), using *B. subtilis*. Using ethyl acetate as solvent, for each species of *Streptomyces* only the extracts of the samples corresponding to 144 h were submitted to thin layer chromatography. Five µL of the extracts was placed on the chromatographic plate (5.0 x 7.0 cm), the absorbent agent used was the silica gel

Polygram Sil G/UV_{254}, and the mobile phase was the toluene-acetone system at a ratio of 7:3. A spot of the commercially available actinomycin D, Dactinomycin, was also placed on the same plate to serve as a standard. In the other production runs, the antibiotic was quantified using the spectrophotometric method reported on by Katz and Weissbach (1963). The concentration of total reducing sugars was measured using the 3.5 dinitrosalicylic acid method (Miller, 1959), while the biomass concentration was measured by quantifying the dry weight. The samples were centrifuged at 2041 *g* for 20 minutes and the sediment was washed with a 0.1N HCl solution and dried at 105°C until a constant weight was obtained.

Measurement of Rheological Properties

These properties were measured only at the end of the fermentation process at 25±1ºC, using a Brookfield Synchro-Lectric rotary viscometer, model RVT with spindle number 2 and 300mL of the sample at different rotation frequencies (5, 10, 20, 50 and 100 min^{-1}).

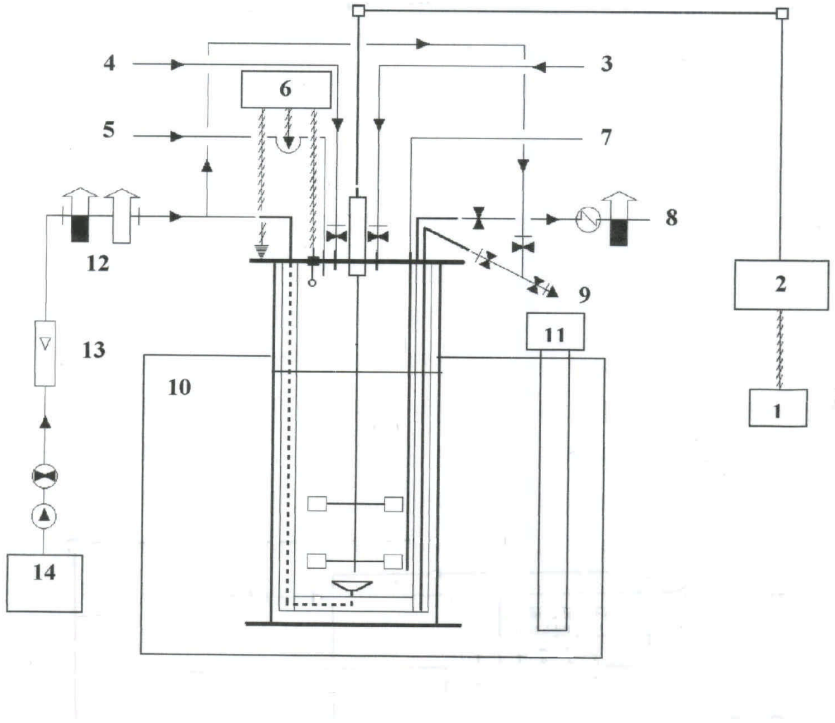

Figure 1: Diagram of the bioreactor: (1) engine regulator, (2) engine, (3) inoculum, (4) fructose, (5) antifoam, (6) antifoam controller, (7) thermometer, (8) air exhaust system with filter and condensator, (9) sampling, (10) water bath, (11) temperature controller, (12) air filters, (13) flow meter, (14) air compressor.

RESULTS AND DISCUSSION

Figure 2 shows the production of actinomycin by the different species of *Streptomyces*. *S. parvulus* had the highest level of antibiotic activity (152 mg/L), followed by *S. felleus*, which had a maximum concentration of around 20 mg/L, and *S. regensis*, which did not exceed 12 mg/L. The same figure also shows that the antibiotic production curves reached their maximum values at around 144 h, which is consistent with the findings of Williams and Katz (1977). According to Martin et al. (1979), this is due to the fact that the extent of antibiotic production equals the tolerance of the cells to the antibiotic. In agreement with to Martin and Demain (1980), the cessation of antibiotic biosynthesis may be attributed to one of the following factors: a shortage of intermediate precursors, the feedback effect of the accumulated antibiotic or the irreversible decay of one or more enzymes.

According to the literature, *S. parvulus* has the advantage of producing predominantly actinomycin-

D (Meienhofer and Atherton, 1973). To prove this statement, thin layer chromatography was prepared for the three microbial species, using Dactinomycin donated by LAFEPE (*Laboratório Farmacêutico do Estado de Pernambuco*) as a standard. The results of the thin layer chromatography are given in Table 1 and they show that *S. parvulus* had only a single band for actinomycin-D, while the other species had five different compounds as well as the antibiotic in question. For this reason the species selected to continue this study was *S. parvulus*.

In previous research by Sousa et al. (1997) using the selected species, the production medium initially used was optimized, giving the following composition: soy milk 30 g/L, glucose 20 g/L and $CaCO_3$ 2 g/L, for which a maximum concentration of 530 mg actinomycin-D per liter was obtained. When glucose was substituted by fructose in the 20, 30 and 40 g/L concentrations, all the antibiotic concentrations were higher after 144 h production than with glucose, as can be seen in Figure 3. Furthermore, the optimum concentration was found to be 30 g fructose per liter, which yielded a maximum value of 635 mg/L.

Figures 4 and 5 show the concentration profiles for actinomycin-D obtained in the bioreactor using stirring speeds of 300 and 500 rpm, respectively, and different degrees of aeration (0.5 ,1.0 and 1.5vvm). The maximum values registered after 144h fermentation are higher than any that appear in the literature, as detailed below: 740 mg/L (500 rpm; 0.5 vvm), 1152 mg/L (500 rpm; 1.0 vvm), 1530 mg/L (500 rpm; 1.5 vvm), 617 mg/L (300 rpm; 0.5 vvm), 882 mg/L (300 rpm; 1.0 vvm) and 1422 mg/L (300 rpm; 1.5 vvm), resulting in the maximum yields and productivity of actinomycin-D, as can be seen in Table 2. The same table shows that at both stirring speeds (300 and 500 rpm) the aforementioned parameters increase as aeration is increased and that the values obtained at 500 rpm are greater than those at 300 rpm. Figures 4 and 5 also show that the concentration profile for the antibiotic produced at 300 rpm and 1.5 vvm has the highest actinomycin-D values until shortly before 144 h, although at this time, the greatest antibiotic concentration was found for 500 rpm and 1.5 vvm (1530 mg/L). The reason for this could be that at 500 rpm, there is a greater breaking up of air bubbles, resulting in a greater specific contact area for gas-liquid mass transfer, while the increased turbulence in the medium results in the diminished thickness of the gas and liquid films that provide resistance to the aforementioned oxygen mass transport.

The typical results obtained in the bioreactor can be seen in Figure 6 (300 rpm and 1.5 vvm), where mycelial growth occurs concomitantly with antibiotic production, rather than following the typical standard for a trophophase-idiophase fermentation of a secondary metabolite (Bu'Lock, 1961). This was also observed by Williams and Katz (1977) in their work with *S. parvulus* ATCC 12434 in a chemically defined medium.

So as to make an overall assessment of the results obtained, the histogram in Figure 7 was prepared. It shows an increase of around ten times the antibiotic concentration (from 152 to 1530 mg/L) in a complex medium by applying classic batch production technology, using optimized compositions for the media. This demonstrates how entirely dependent the selected species is on the composition of the culture medium and the operating conditions of the bioreactor in which the experiments are conducted.

Figure 8 shows the variation of shear stress with the shear rate in a typical experiment, where the concentration of dry weight at the end of a 168h fermentation was 29.1g/L. The rheogram clearly shows that the shape of the curve is typical of a pseudoplastic fluid, therefore obeying the Ostwald-de Waele power law model, in which the values of rheological parameters n and K are as follows: n=0.252 and K=28.423dyn.cm^{-2}.s$^{0.252}$.

In their work with three actinomycetes (*Saccharopolyspora erythraea*, *Actinomadura roseorufa* and *Streptomyces rimosus*) that synthesize secondary metabolites of commercial value (erythromycin, anti-coccidiostatic antibiotic and oxytetracycline, respectively), Warren et al. (1995) noted that in submerged cultivation, the rheology of the three broths obeyed pseudoplastic fluid behavior, obtaining values for the power law index (n) of 0.20 to 0.25 and for the consistency index (K) varying from 20 to 70 dyn.cm^{-2}.s^{n} (*S. erythraea*), 5 to 95 dyn.cm^{-2}.s^{n} (*A. roseorufa*) and 5 to 70 dyn.cm^{-2}.s^{n} (*S. rimosus*). The same authors observed that the value of this latter rheological parameter increases with the concentration of biomass, although it decreases at the end of the antibiotic fermentation. Atkinson and Mavituna (1983) report variations in the pseudoplastic characteristics of the medium during the fermentation with *Streptomyces aureofaciens*, which produces tetracycline, showing values for n of between 0.25 and 0.45 and values for the consistency index K of between 30 and 90 dyn.cm^{-2}.s^{n}.

Banks (1977) explains that the occurrence of pseudoplastic behavior in mycelial suspensions is due to the alignment of the hyphae, which brings about a decrease in the apparent viscosity when high shear rates are applied.

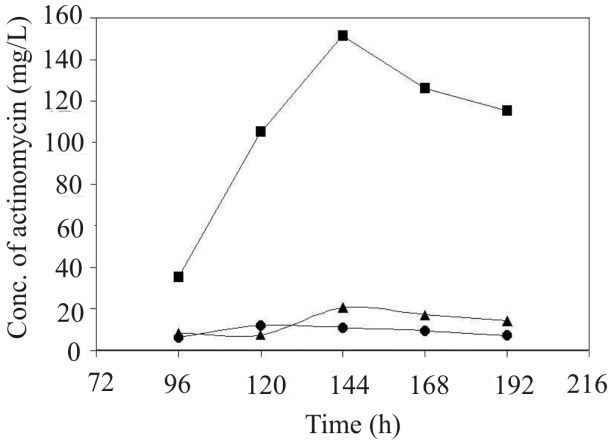

Figure 2: Concentration profiles for actinomycin obtained by the different species of *Streptomyces*: (■) *S. parvulus*, (▲) *S. felleus* and (2) *S.regensis*.

Table 1: R_f values in thin layer chromatography for the species of *Streptomyces* and for the Dactinomycin standard.

S.regensis	*S.felleus*	*S.parvulus*	Dactinomycin Standard
0.93	0.90		
0.87	0.48		
0.42	0.38	0.37	0.36
0.35	0.28		
0.26	0.18		
0.04	0.08		

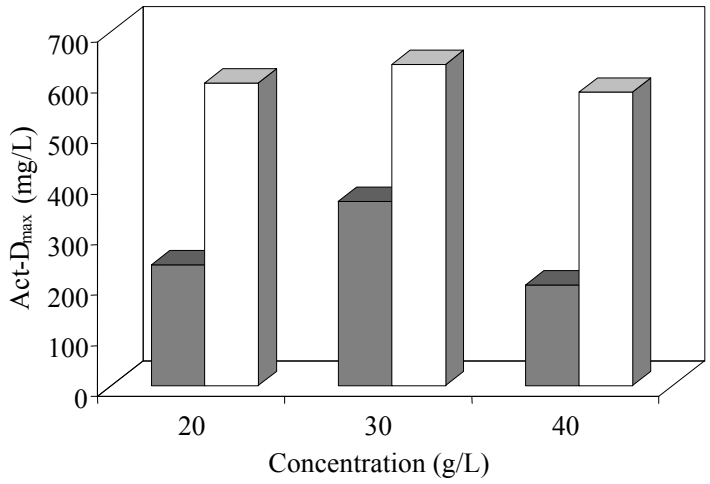

Figure 3: Maximum concentrations of actinomycin-D obtained by *S. parvulus* in shaken flasks, using the following medium: soy milk 30 g/L, $CaCO_3$ 2g/L and varied concentrations of (4) glucose and (ÿ) fructose (20, 30 and 40g/L).

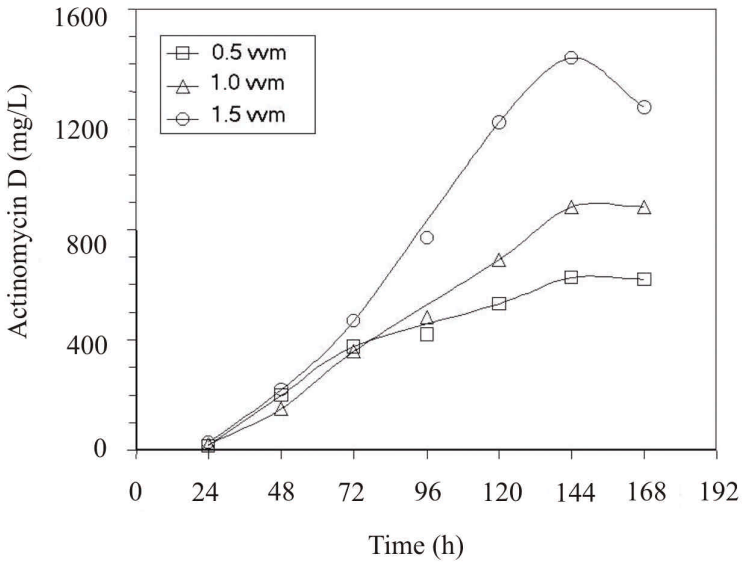

Figure 4: Concentrations of actinomycin-D in the bioreactor, using a stirring speed of 300 rpm and different degrees of aeration.

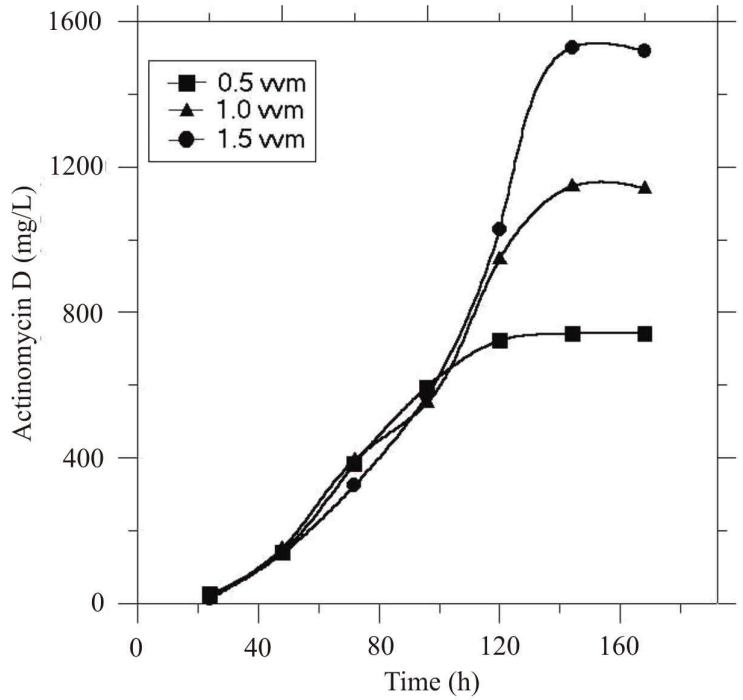

Figure 5: Concentrations of actinomycin-D in the bioreactor at 500 rpm and different degrees of aeration.

Table 2: Values for productivity (Q_{act-D}) and product yield on substrate consumed ($Y_{P/S}$) after 144 h fermentation for the assays carried out in a bioreactor.

Assay Nº	Aeration (vvm)	Stirring (rpm)	$Y_{P/S}$ (mg/g) (vol.)	(mass)	Q_{act-D} (mg/L.h)	(mg/h)
1	0.5	300	21.6	13.8	4.3	23.0
2	1.0	300	34.4	18.4	6.1	28.7
3	1.5	300	49.7	18.7	9.9	31.7
4	0.5	500	23.8	16.1	5.1	28.0
5	1.0	500	38.0	20.4	7.9	36.5
6	1.5	500	53.7	19.8	10.6	36.1

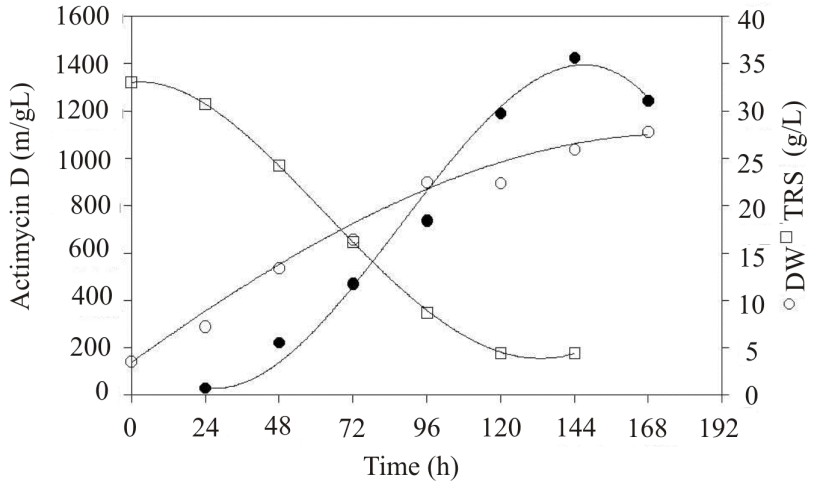

Figure 6: Concentration profiles for actinomycin-D (•), dry weight (○) and total reducing sugars (ÿ) in a typical experiment held at 300 rpm and 1.5 vvm.

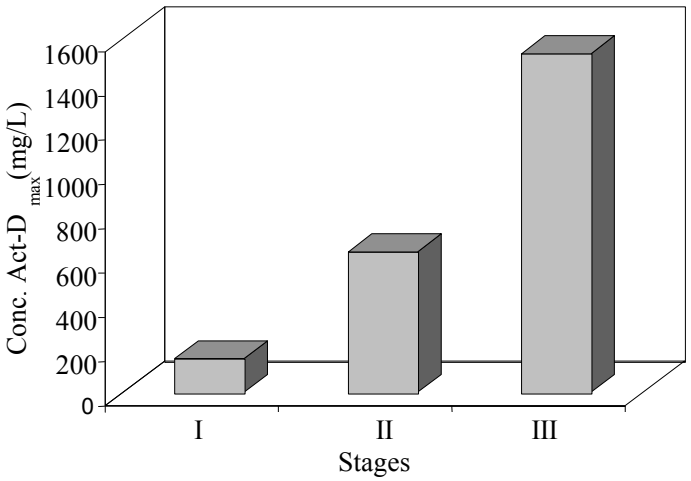

Figure 7: Maximum concentrations of actinomycin-D obtained by *S. parvulus* in the following stages: (I) flasks with nonoptimized medium; (II) flasks with an optimized medium; and (III) bioreactor with the optimized medium.

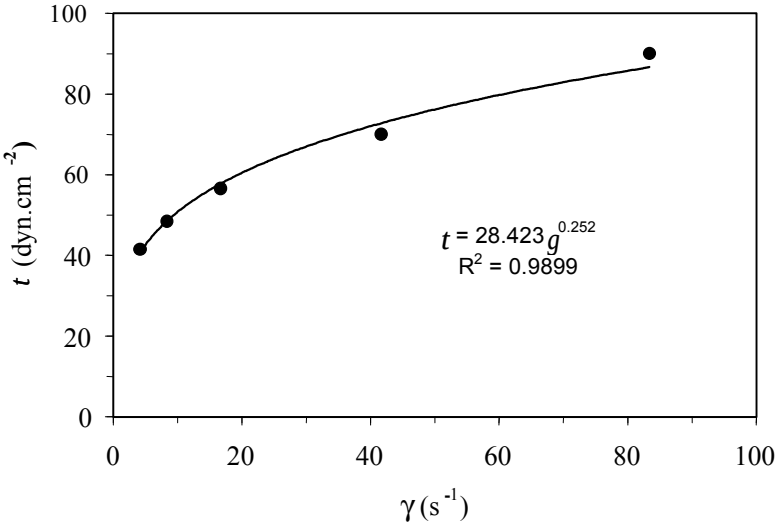

Figure 8: Shear stress (t) versus shear rate (g).

CONCLUSIONS

S. parvulus was the species that had the highest level of antibiotic activity and produced only actinomycin-D. Fructose showed to be a better substrate than glucose for the antibiotic production, obtaining a maximum actinomycin-D concentration of 635mg/L for an initial fructose concentration of 30 g/L. The highest yield of actinomycin-D (1530 mg/L) in the bioreactor was obtained with 500 rpm and 1.5 vvm. The production of actinomycin-D did not follow a typical trophophase–idiophase dynamic, but rather a standard kinetic whereby growth is partially associated with product formation. The fermented medium showed rheological behavior typical of a pseudoplastic fluid.

ACKNOWLEDGEMENTS

This study received financial support from the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE.

NOMENCLATURE

Latin Letters

CFU	colony-forming units
K	consistency index (dyn.cm^{-2}.s^{n})
n	power law index
$Q_{act\text{-}D}$	actinomycin-D productivity (mg/L.h)
R_f	reference factor = distance moved by substance/distance moved by solvent front
rpm	rotation per minute
vvm	volume of air per volume of medium per minute
$Y_{P/S}$	product yield on substrate consumed (mg/g)

Greek Letters

t	shear stress (dyn.cm^{-2})
g	shear rate (s^{-1})

REFERENCES

Atkinson, B. and Mavituna, F., Biochemical Engineering and Biotechnology Handbook. The Nature Press, London (1983).

Banks, G.T., Aeration of Mold and *Streptomyces* Culture Fluids. *In:* Wiseman, A. (ed.) Topics in Enzyme and Fermentation Biotechnology. Ellis Horwood, Chichester (1977).

Bu'Lock, J.D., Intermediary Metabolism and Antibiotic Synthesis. Adv. Appl. Microbiol., 3, 293-342 (1961).

Dalili, M. and Chau, P.C., Production of Actinomycin-D with Immobilized *Streptomyces parvulus* under Nitrogen and Carbon Starvation Conditions. Biotechnol. Lett., 10, No. 5, 331-336 (1988).

Farmacopéia Brasileira, 4th ed., Atheneu, São Paulo (1988).

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward J.M. and Schrempf, H., Genetic Manipulation of *Streptomyces*: A Laboratory Manual. The John Innes Foundation, Norwich (1985).

Isaacson, D.M. and Kirschbaum, J., Assays of Antimicrobial Substances. *In*: Demain, A.L. and Solomon, N.A., Manual of Industrial Microbiology and Biotechnology. ASM, Washington (1986).

Katz, E. and Weissbach, H., Incorporation of ^{14}C-labeled Aminoacids into Actinomycin and Protein by *S. antibioticus*. J. Biol. Chem., 238, 666-675 (1963).

Korzybski, T., Kowszyk-Gindifer, Z. and Kurylowicz, W., Antibiotics: Origin, Nature and Properties. Polish Scientific Publishers, Warzawa (1967).

Martin, J.F. and Demain, A.L., Control of Antibiotics Biosynthesis. Microbiol. Rev., 44, 230-251 (1980).

Martin, J.F., Luengo J.M., Revilla, G. and Villanueva J.R., Biochemical Genetics of the β-lactam Antibiotic Biosynthesis. In: Sebek, O. and Lskin, A.I. (eds.) Genetics of Industrial Microorganisms, American Society for Microbiology, Washington (1979).

Meienhofer, J. and Atherton E., Structure-Activity Relationship in the Actinomycins. Adv. Appl. Microbiol.,16, 203-300 (1973).

Miller, G.L., Use of Dinitrosalicyclic Acid Reagent for Determination of Reducing Sugar. Anal. Chem., 31, 3, 426-428 (1959).

Sousa, M.F.V.Q., Lopes C.E. and Pereira Jr., N., Medium Optimization for the Production of Actinomycin-D by *Streptomyces parvulus*. Arq. Biol. Tecnol., 40, 2, 405-411 (1997).

Warren, S.J., Moore, E.K., Shamlou, P.A., Lilly, M.D., Thomas, C.R. and Dixon, K., Rheologies and Morphologies of Three Actinomycetes in Submerged Culture. Biotechnol. Bioeng., 45, 80-85 (1995).

Williams, W.K. and Katz, E., Development of a Chemically Defined Medium for the Synthesis of Actinomycin D by *Streptomyces parvulus*. Antimicrob. Agents Chemother., 11, 281-290 (1977).