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A N I N T E R N A T I O N A L J O U R N A L

A Chemically Defined Medium for Production of Actinomycin D by *Streptomyces parvulus*

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ABSTRACT

A chemically defined medium consisting of D(+)fructose, L(-)threonine, K_2HPO_4, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ and deionized water, was developed to maximize the synthesis of actinomycin D by the Streptomyces parvulus *DAUFPE 3124 strain. This medium resulted in the maximum antibiotic concentration of 133mg/L while using the original medium the production of actinomycin D was poor not surpassing 43mg/L.*

Key words : Actinomycin D, production, *Streptomyces. parvulus*, synthetic medium

INTRODUCTION

The actinomycins are a family of chromopeptide antibiotics that present antitumoral properties, being employed in the treatment of several human neoplasies (Waksman & Furness, 1960). Structurally, they have a chromophorous group, identical in all actinomycins, and two pentapeptide chains with a variable composition of amino acids (Brockmann, 1960). They are synthesized by *Streptomyces* as mixtures of different actinomycins, however, the *S. parvulus* species produces actinomycin D almost exclusively (>95%) (Meienhofer & Atherton, 1973). Over the last ten years, research into actinomycin D has been directed mainly towards clinical applications and no work related to production was found. In a previous study the authors optimized a complex medium for the production of actinomycin D by *S. parvulus* DAUFPE 3124 strain, increasing the final antibiotic concentration from 245 mg/L to 530 mg/L (Sousa, *et al.*, 1997). Few papers have been found that focus on the nutritional requirements of *Streptomyces* species for achieving a high actinomycin yield (Katz *et al.*,1958; Williams & Katz, 1977). Katz *et al.* (1958) showed that the maximum antibiotic synthesis ocurred in a chemically defined medium containing L(-) galactose (10 g/L), L(-) glutamic acid (2 g/L), phosphate (1 g/L) and mineral salts. Although this composition proved to be satisfactory for production of actinomycin mixtures by *S. antibioticus*, the synthesis of actinomycin D by *S. parvulus* was rather poor and extremely variable. Williams & Katz (1977) proposed a chemically defined medium for the synthesis of high yields (500 to 600 mg/L) of actinomycin D by *S. parvulus* ATCC 12434. Dalili & Chau, (1988) working with the same medium and the same strain immobilized in calcium alginate, using an air lift column operating under discontinuous, fed batch and continuous conditions, obtained 50, 73 and 80 mg/L, respectively. However, since nutritional requirements for growth and product formation vary from strain to strain, the medium suggested by Williams & Katz (1977) resulted in a low antibiotic production by the *S. parvulus* DAUFPE

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3124 strain. This work presents a set of experiments that culminated in the development of a chemically defined medium in which higher concentration of actinomycin D was achieved using the *S. parvulus* DAUFPE 3124 strain.

MATERIALS AND METHODS

Microorganism: *Streptomyces parvulus* DAUFPE 3124 strain was used in all assays. This strain was maintained on slants of 4 g/L glucose, 4 g/L yeast extract and 10 g/L malt extract agar medium. Incubation was carried out for 3 to 5 days at 30°C until gray spores developed and the slants were stored at 4°C.

Preparation of Spore Suspension: The spores were scraped from the surface of heavily sporulated slants with 3 ml of 9 g/L NaCl solution containing 1 ml/L of Tween 80, following the Hopwood *et al.*, (1985) technique. The spore concentration was quantified in the suspension and stored in the freezer at -4°C.

Growth and Production Media: The medium used during the biomass growth had the following composition: 5 g/L tryptone and 3 g/L yeast extract. In the antibiotic production several media were used with pH adjusted to 7.0 and in all of them, the following basal mineral salts medium K_2HPO_4 1.0 g/L; $MgSO_4.7H_2O$ 25 mg/L; $ZnSO_4.7H_2O$ 25 mg/L; $CaCl_2.2H_2O$ 25 mg/L; $FeSO_4.7H_2O$ 25 mg/L was used. A number of amino acids and carbohydrates were tested as carbon and nitrogen sources so that the C/N ratio was held around 41.7, as recommended by Williams & Katz (1977), and several C/N ratios were employed when the best carbon and nitrogen sources were selected. The amino acids, carbohydrates and salts were sterilized separately and added just prior to inoculation.

Experimental Procedure: The growth was performed in two steps. In the first one, the inoculum was prepared in a 250 ml flask containing 25 ml of growth medium inoculated with 50 µl of spore suspension (8.0×10^7 cfu/ml).The flasks were placed on a rotary shaker at 250 rpm at 30ºC for 48 h. In the second step, 20 ml of the mycelial suspension was used to inoculate a 2000 ml flask containing 200 ml of growth medium which was subjected to the same conditions as described above for 24 h. The antibiotic production was conducted in 500 ml shaken flasks, containing 50 ml of the fermentation medium. These flasks were inoculated with 3 ml of mycelial suspensions and subjected to the same conditions as cited above. Samples were taken at 24 hour intervals and analysed in terms of actinomycin D concentration by spectrophotometric method (Katz & Weissbach, 1963). The data reported in all experiments represent the yield of actinomycin D for 144 hours of cultivation except for the last one.

RESULTS AND DISCUSSION

As observed by Williams & Katz (1977), the association of L(-)glutamic acid with another amino acid potentialized the synthesis of actinomycin D by *S. parvulus* ATCC 12434. This fact was tested using the *S. parvulus* DAUFPE 3124 and the results are shown in the Table 1. This table shows that a maximum concentration of actinomycin D was obtained when the L(-) glutamic acid was combined with L(-)histidine, L(-)ornithine or L(-)threonine (70.8 mg/L).

Table 1 - Effect of L(-) amino acids provided in combination with L(-) glutamic acid for actinomycin production by *S. parvulus* DAUFPE 3124 grown in a 40 g/L D(+) fructose, 2.2 g/L L(-) glutamic acid basal mineral salts medium for 144 h at 30ºC (C/N ratios between 41.2 and 42.8).

L(-) glutamic acid + L (-) amino acids (g/L)	Actinomycin D (mg/L)
Histidine (0.775)	70.8
Ornithine (0.99)	70.8
Threonine (1.787)	70.8
Alanine (1.336)	65.0
Leucine (1.967)	62.7
Glutamine (1.096)	38.6
Glicine (1.126)	47.3
Valine (1.757)	53.7
Proline (1.727)	68.5
Serine (1.576)	22.1
Asparagine (0.99)	53.7
Tryptophan (1.53)	42.1
Methionine (2.387)	18.4

Assays were carried out with several L(-) amino acids to determine the one more

appropriated for antibiotic synthesis (Table 2). The best result (87.7 mg/L) was achieved when L(-) threonine was the sole nitrogen source. Surprisingly, this result was higher than the one obtained (70.8 mg/L) by the combination of L(-) glutamic acid and L(-) threonine. In the literature there is no explanation for this effect and, furthermore, it results in a cheaper chemically defined medium composition.

Table 2 - Effect of L(-) amino acids in actinomycin D production by *S. parvulus* DAUFPE 3124 grown in a 20 g/L D(+) fructose basal mineral salts medium for 144 h at 30ºC (C/N ratios between 38.9 and 43.3)

L(-) amino acids (g/L)	Actinomycin D (mg/L)
Threonine (1.785)	87.7
Ornithine (0.99)	34.6
Histidine (0.775)	77.1
Alanine (0.445)	49.8
Leucine (1.96)	16.6
Glutamine (1.1)	70.2
Glicine (1.125)	82.0
Valine (1.75)	74.0
Proline (1.725)	77.1
Aspartic acid (1.1)	35.7
Asparagine (1.0)	84.5
Glutamic acid (2.2)	54.7
Methionine (2.2)	7.7

L(-) threonine is one of the amino acids constituents of the actinomycin molecules. Katz *et al.* (1965) employed labeled L(-) threonine - ^{14}C in experiments of short incubation and verified that *S. antibioticus* strains metabolizes this amino acid in both protein and actinomycin synthesis. Due to this evidence, these authors came to the conclusion that there is an intracellular pool of threonine commom to the two synthesizer systems although these systems were differents.

A number of carbohydrates were investigated as carbon sources for actinomycin-D production employing the basal mineral salts medium with 1.8 g/L of threonine and the results can be seen in Table 3. This table shows that D(+) fructose was superior to the others carbohydrates tested in the same concentration. This result agrees with the findings of Williams & Katz (1977), who affirmed that D fructose was the most effective carbon source for antibiotic synthesis by *S. parvulus*. No actinomycin-D production was detected when glucose and galactose were employed as sole carbon sources. Gallo & Katz (1972) demonstrated that glucose supports a rapid growth rate of *S. antibioticus*, but it exerts severe catabolite repression in antibiotic production, particulary in the synthesis of phenoxazinone synthase, an essential enzyme for the formation of the actinomycin chromophorous group. Williams & Katz (1977) working with *S. parvulus* ATCC 12434, also showed the repression caused by glucose additions to a chemically defined medium containing fructose (40g/L) as the main substrate. Marshall *et al.* (1968) verified the inhibitory effect of galactose on phenoxazinone synthetase specific activity in cells of *S. antibioticus* harvested after six hours of incubation.

Table 3 - Influence of several carbon sources on actinomycin production by *S. parvulus* DAUFPE 3124 grown in a 1.8 g/L L(-) threonine basal mineral salts medium for 144 h at 30ºC (C/N=41.5)

Carbon sources (20g/L)	Actinomycin D (mg/L)
D(-) arabinose	5.3
D(+) fructose	55.8
D(-) galactose	ND*
D(+) glucose	ND*
Myo-inositol	43.2
D(-) manytol	36.2
D(+) manose	18.2
Sucrose	21.6
D(+) xylose	44.8

*ND – not detected

To evaluate the quantitative influence of C/N ratios upon antibiotic synthesis by *S. parvulus* DAUFPE 3124, the concentration of D(+) fructose (20 g/L) was held constant while the amount of L(-) threonine was varied to provide different C/N ratios in the medium. The results of these experiments are shown in Table 4.

Higher production of actinomycin-D (67.9mg/L) was achieved at C/N ratio of 22.5 which corresponded to 20 g/L D(+) fructose and 3.57 g/L L(-) threonine.

Comparative study of the medium reported by Williams & Katz (1977) and the one developed in this work was done employing *S. parvulus* DAUFPE 3124 strain. The concentration curves of actinomycin D production are shown in Figure 1 where can see that the antibiotic concentration reached the maximum value of 133 mg/L in the proposed medium and in the medium suggested by cited authors the maximum value was 43 mg/L.

Table 4 - Effect of several C/N ratios on actinomycin D production by *S. parvulus* DAUFPE 3124 grown in a basal mineral salts medium for 144 h at 30ºC.

D(+) Fructose (g/L)	L(-) Threonine (g/L)	C/N	Actinomycin D (mg/L)
20	3.57	22.5	67.9
20	2.38	32.0	57.5
20	1.78	41.6	58.9
20	1.19	60.6	53.8

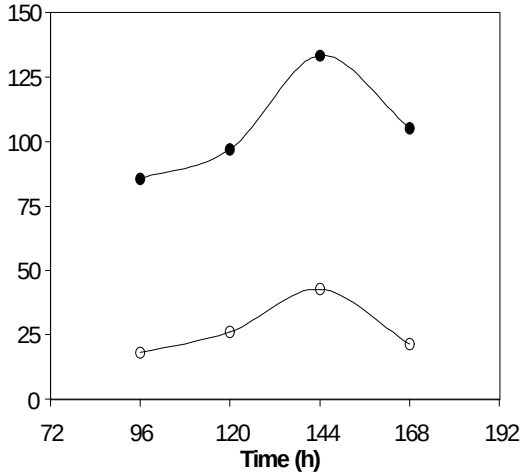

Figure 1 – Actinomycin D concentrations obtained in shaken flasks by *S. parvulus* DAUFPE 3124, using chemically defined media (○)reported by Williams & Katz (1977) and (●) suggested by present work.

The results clearly show the dependence of the antibiotic synthesis on medium constituents. The medium developed in this study to obtain high yield of actinomycin D by *S. parvulus* DAUFPE 3124 has the following composition: 20 g/L D(+) fructose; 3.57 g/L L(-) threonine; 1.0 g/L K_2HPO_4; 25 mg/L $MgSO_4.7H_2O$, 25 mg/L $ZnSO_4.7H_2O$, 25 mg/L $CaCl_2.2H_2O$ and 25 mg/L $FeSO_4.7H_2O$. This composition allowed an improvement of over 200 % in the concentration of actinomycin D.

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RESUMO

Um meio quimicamente definido composto de D (+) frutose, L (-) treonina, K_2HPO_4, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ e água deionizada, foi desenvolvido para maximizar a síntese de actinomicina D pelo *Streptomyces parvulus* DAUFPE 3124. O meio proposto resultou numa concentração antibiótica máxima de 133 mg/L enquanto que no meio inicial a produção antibiótica foi baixa, não ultrapassando 43 mg/L.

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