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Bacitracin Production by a New Strain of *Bacillus subtilis*

Extraction, Purification, and Characterization

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ABSTRACT

A new strain of *Bacillus subtilis* C 126 was isolated from sugar cane fermentation and produced an antibiotic that inhibited the growth of *Micrococcus flavus*. The production of the antibiotic in culture medium followed to extraction with *n*-butanol, thin layer chromatography, and microbiological tests indicated that a polypeptide antibiotic was produced. The fraction obtained by Sephadex G-25 column and analyzed by HPLC indicated that bacitracin complex was produced.

Index Entries: *Bacillus subtilis*; bacitracin production; *Micrococcus flavus*.

INTRODUCTION

Bacitracin is a peptide antibiotic produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* (*1–3*).

Otherwise, the peptide antibiotic's production as function in the microorganisms is widely unknown (*4,5*). The production of peptide antibiotics

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by several microorganisms suggests a function of the cells or mycelium in the stationary phase, e.g. penicillin, gramicidin S, cephalosporin C, tyrocidin, actinomycin, mycobacillin, and polymyxins (*6–11*).

The bacitracin antibiotic is produced by the bacteria at this stage of the life cycle only, after the growth and before the sporulation (*12,13*). Snoke (*14,15*) reported that glucose markedly stimulated the synthesis of *Bacillus licheniformis* in defined medium. In addition, Haavik (*15*) reported that glucose inhibited bacitracin production during the first hours of the growth, whereas growth was not affected. The catabolite repression by glucose in the medium was prevented by neutralizing the culture fluid with $CaCO_3$. Here we describe experiments of the screening, the strain *Bacillus subtilis* C 126 by bacitracin production, the kinetics of bacitracin formation, and the extraction and purification of the antibiotic produced.

MATERIALS AND METHODS

Organisms

The strain of *Bacillus subtilis* C 126 isolated from alcoolich fermentation of sugar cane was selected by test of bacitracin production and kept at 4°C.

Detection of Bacitracin Production and Microbiological Assay

The screening was carried out by diffusion method (discs) of ten strains of *Bacillus subtilis* and detected by the growth of *Micrococcus flavus*.

The bacitracin production in the fermentation was accompanied by pH determination, agar diffusion method, and detected by growth of *Micrococcus flavus* after thin layer chromatography (TLC), as described previously (*16,18*).

Medium and Growth Conditions

The chemically defined medium (*20*) for growth and bacitracin production had the following composition (g/L deionized and distilled water): L glutamic acid, 10.0; citric acid, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7\ H_2O$, 0.2; $MnSO_4 \cdot 4\ H_2O$, 0.01; $FeSO_4 \cdot 7\ H_2O$, 0.01.

The pH was adjusted to 7.0 with NaOH before autoclaving at 121°C for 20 min. Glucose was sterilized separately before addition. The inoculum consisted of spores. The growth and bacitracin production occurred in 500 mL of Erlenmeyer flask incubated at 37°C at 200 rev/min on a New Brunswick rotatory shaker (model G-52).

Growth

The bacterial growth was measured as extinction at 600 nm (E = 600) in a spectrophotometer and biomass was determined.

Table 1
Bacitracin Production During the Growth of *B. subtilis* in Hendlin Medium

Time of incubation	Growth, E_{600}/mg	Culture fluid, pH	Antibiotic Waksman, U/mL	Activity, IU/mL	Proteins, mg/mL
12	0.3	7.2	0	0	0
24	1.0	6.5	0	0	0
36	1.5	7.4	10	32	0.0236
48	2.5	7.6	10	33	0.0436
60	2.0	8.1	100	38	0.0272
72	1.6	8.3	100	28	0.0254

Preparation of Inoculum

The bacteria were spread on the surface of agar plates (Nutritive agar) and incubated at 37°C, for 6 d. The spores produced were washed with sterile saline and glass beads, in order to destroy all the bacteria and debris. The spores were washed three times with sterile saline and finally diluted to obtain 40% of transmitance, at E_{600}.

Protein Determination

Protein determination used the method described by Lowry et al. (*21*) using serum albumin as the standard.

Extraction, Purification, and Analysis of Bacitracin

The bacitracin was extracted by the use of *n*-butanol (2:1 v/v) and acidified to pH 5.1 (*21*). The purification was done in Sephadex G-25 column using phosphate buffer (pH=6.0) as eluting, blue dextran 2000, and Chloride Cobalt as standard of mol wt (*22*).

Antibiotic activity (diffusion method), V.V absorption at 252 nm, and high performance liquid chromatography were carried out with the fractions obtained (*23,24*).

Results

The results indicated the extracellular antibiotic production by *Bacillus subtilis* 126 after 36 h of incubation, and reached the maximum titers 100 U/mL waksman and 38 IU/mL, at 60 h (Table 1). Bacitracin was produced by *Bacillus subtilis* in the culture fluid at about the same time, which may be sufficient for sporulation to occur at the stationary phase, at 8.8 pH. During all stages of growth, amounts of bacitracin were detected by thin layer chromatography and identified Rf 0.52 and 0.46, similar to bacitracin standard (Table 2).

Table 2
Thin Layer Chromatography of Crude Extracts of Bacitracin During Growth

Time of Incubation, h	Bands, no.	Color produced	Ref
30	2	Pink	0
		Violet	0
36	2	Pink	0.48
		Violet	0
42	2	Pink	0.46
		Violet	0
48	2	Pink	0.53
		Violet	0.20
54	3	Pink	0.59
		Violet	0.46
		—	0
60	3	Pink	0.57
		Violet	0.46
		—	0
72	3	Pink	0.55
		Violet	0.42
Bacitracin standard	2	Brown	0.42
		Pink	0.51

The fraction obtained using *n*-butanol was purified by Sephadex G-25 and indicated a fraction as mol wt 1400–1500, 52 mL of elution, and 38.1 IU/mL (Fig. 1).

The analysis by liquid high performance chromatography indicated retention time, 1.9, 7.22, 9.54 and 10.7 min probably A, B, C, and F bacitracin (Table 3).

DISCUSSION

The release of bacitracin in Hendlin medium (*19*), from *Bacillus subtilis* 126 is greatly affected by the pH of the culture fluid (Table 1). Demain (*26*) described the synthesis of secondary metabolites found in the culture fluid at the same time of stationary phase. Bernlohr and Novelli (*26*), Snoke (*14*), and Haavik (*27*) suggested that bacitracin synthesis was inhibited by both high and low pH values.

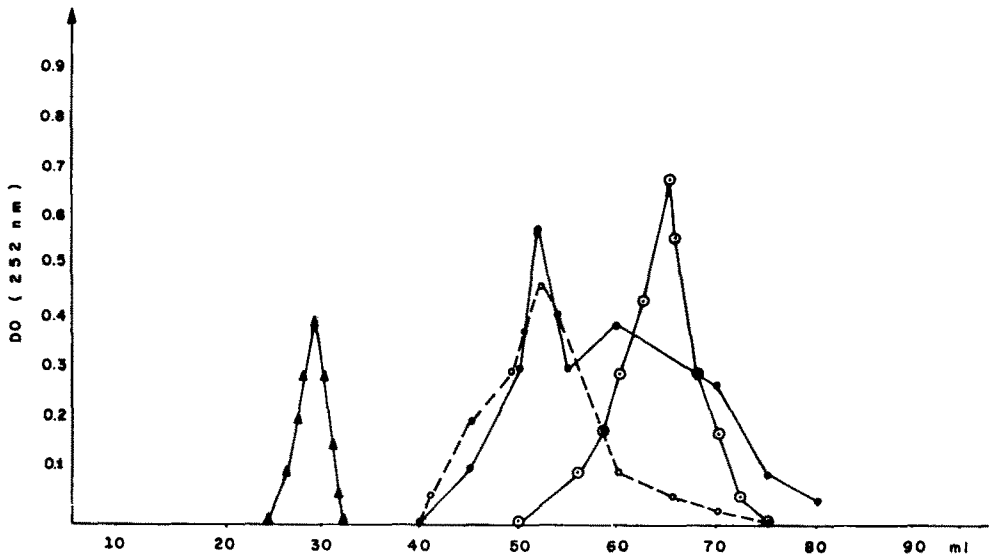

Figure 1

Table 3
UV Spectrum Thin Layer Chromatography and Antibiotic Activity of the Fractions Obtained by Crude Extracts of *Bacillus subtilis*

Fraction	UV, E_{252mm}	Color	Ref	IU/mL
24	0.34	Pink	0.53	
		Brown	0.45	31.8
		Violet	0.21	
25	0.37	Pink	0.59	
		Brown	0.48	36.5
		Violet	0.10	
26	0.61	Pink	0.52	
		Brown	0.46	38.1
		Violet	0.19	
27	0.34	Pink	0.47	
		Brown	0.19	31.8
Bacitracin standard	0.53	Pink	0.52	77.0
		Brown	0.47	

The medium using glucose as the carbon source stimulates bacitracin production, by reducing pH during later stages of growth (Table 1). Snoke (*14*) observed that optimal pH 8.0 value promotes the bacitracin synthesis. Otherwise, Handlin (*19*) suggested the bacitracin reached a high level at pH 8.4.

Demain (*25*) pointed out that many fermentations require glucose as the carbon source, and that production may be controlled by catabolite repression. In *Bacillus subtilis*, bacitracin produced an initial pH fall, but the production of peptide induced alkaline pH (Table 1).

Hickey (*2*), Rabinovitch (*28*), and Rabinovitch and Filho (*29*) suggested the presence of bacitracin by TLC identification. Similar Rf was observed by TLC from culture fluid in *B. subtilis* and bacitracin standard (Table 2).

The purification of the bacitracin water extracts by Sephadex G-25, indicated a fraction 52 mL of eluting, 38.1 IU/mL, and at E_{252} nm (Table 3 and Fig. 1).

The results are corroborated by Haavik (*30*). Haavik and Froyshov (*31*) contributed information that correlated bands (brown and pink) to bacitracin.

The HPLC analysis suggested the purpose of A, B, and F bacitracin components produced by *B. subtilis* 126 with retention time 1.3, 7.2, and 9.4 min. The results were compared with bacitracin standard constituted by B, C, and F components with time 7.2, 10.7, and 9.5 min. Retention time also has been reported by Hickey (*2*), and Tsuji, Robertson, and Bach (*32*).

Therefore, new experiments should be performed to explain the bacitracin production in *Bacillus subtilis*.

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