FRACTIONATION AND IDENTIFICATION OF THE NUCLEIC ACID-RELATED SUBSTANCES SECRETED BY *Streptomyces aureofaciens*

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■ ABSTRACT: *S. aureofaciens* growth in a chemically defined medium was associated with the active secretion of nucleic acid-related substances in the medium. High secretion depended on low availability of phosphate, and fractionation showed 7 anionic substances were secreted as major components. When compared to 76 known purine and pyrimidine derivatives only orotic acid was identified. Cationic components are among the minor concentration components secreted which have been identified as cytosine, inosine, cytidine, adenine, guanine and, probably, 1-methyl-adenine.

■ KEYWORDS: *Streptomyces aureofaciens*; nucleic acid-related substances; nucleosides; nitrogenous bases; fractionation and identification.

Introduction

An intensive study was made on the production of nucleotides, nucleosides, nitrogenous bases and related substances by microorganisms.[3-13, 16, 18, 21, 23]

Streptomyces aureofaciens strain nº NRRL-B-1286, which does not produce tetracycline, secrets a number of low molecular weight compounds related to nucleic acids during growth on a chemically defined medium.[3] Simuth & Zelinka[25] observed the accumulation of hypoxanthine, cytosine and guanosine in industrial chlortetracycline

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production and in laboratory experiments using high and low chlortetracycline-producing strains. They associated the production of these substances with chlortetracycline production and assumed that the bases and nucleosides accumulated as a result of nucleic acid decomposition.

They also observed the production of other, unknown UV-absorbing substances and, in some cases, the presence of the nucleotides AMP and CMP. Carvalho & Molinari[3] advanced a hypothesis to explain similar accumulation of UV-absorbing substances. In this work we compare the chemical nature of the substances accumulated by non antibiotic producing strain (NRRL-B-1286) with antibiotic producing strains described by Simuth & Zelinka,[25] and gather data showing that active secretion is dependent on growth in a critically low level of available phosphate.

Materials and methods

Medium

A chemically defined medium was developed for the nucleic acid-related substances accumulation by *Streptomyces aureofaciens*.[3] Maximum production was obtained in a medium containing, per liter, 50 g of sucrose, 8 g of gelatin, 0,26 g of K_2HPO_4, 0,2 g of $MgSO_4 . 7H_2O$, 10 mg of $ZnSO_4 . 7H_2O$, 10 mg of $FeSO_4 . 7H_2O$, 10 mg of $MnSO_4 . H_2O$, 5 mg of $CoSO_4 . 7H_2O$ and pH 7.

Inoculum

A slant sample of the culture (*Streptomyces aureofaciens*, NRRL–B 1286, a non-tetracycline or chlortetracycline producer strain) was inoculated in 250 ml Erlenmeyer flasks containing 40 ml of medium and left to grow for 25 hours at 30°C on a rotary shaker (250 rpm; 3 cm diameter cycles). The culture (2,36 mg/ml biomass) was aseptically homogenized in a sterile aluminum waring blendor and immediately used to prepare large volumes of inoculum cultures (2 ml per 250 ml Erlenmeyer flasks containing 40 ml of medium, grown in a similar manner for 40 hours). The inoculum cultures were also collected and homogenized as described above and aseptically transferred , in 30 ml portions, to 50 ml vials, closed with butyl stoppers, quickly frozen to -30°C and kept frozen until use.

Fermentation

The microorganism was cultivated on a rotary shaker at 250 rpm in revolving cycles of 3 cm diameter and kept in a constant temperature room at 30°C, in 125 ml Erlenmeyer flasks containing 35 ml of fermentation medium inoculated with 1.2 ml of inoculum culture. The flasks were closed with 1 cm thick polyurethane foam covers.

Fermentation time, adjusted to give maximum production yield, was 170 hours.

Analytical methods

Fractionation of the solube, low molecular weight substances was carried out by a series of separation on ion-exchange columns and the purified fractions were analyzed by both ascending paper chromatography and ultraviolet spectral analysis, as used by Simuth et al.[24] except that the ascending paper chromatography was carried out using only two distinct solvent systems: S_1, as described by Wyatt[27] and S_2 as described by Kirby,[17] and following essentially the technique used by Wyatt.[27] Following localization of spots by UV light and calculation of the corresponding Rf values, each spot was cut from the paper, cut into 2x2 mm pieces and eluted with 3 ml of HCl 0.1N for 5 hours with occasional stirring. The UV spectrum of each component was determined in the 210-320 nm range. Blanks were cut at equivalent heights from a clean chromatographic paper developed with the same chromatographic solvent as described by Wyatt.[27] Identification of the components eluted from the spots was made by comparing Rf values in both the S_1 and S_2 solvent systems and the UV absorption spectra with the Rf's and spectra of 76 natural and non-natural nucleic acid-related substances similarly treated. Following a preliminary identification, an unknown substance and the known reference compound to which it appeared to correspond were placed on the same paper chromatogram and developed in parallel. This chromatography was performed in both S_1 and S_2 solvent systems to confirm both Rf's and spectral superposition. The spectral identifications were carried out as described by Ayres[1] and the corresponding chemical component quantification was calculated using simple comparative calculations with a known amount of reference standard. From these results and a knowledge of volumes employed, the concentrations in unextracted broth could be calculated.

To aid in partial characterization of some unknown compounds the hydrolysis procedure of Marshak & Vogel[19] and the open tube hydrolysis procedure of Vischer & Chargaff[26] (which only hydrolyzes purine derivatives) were used. To differentiate between the two procedures the former was called "total" and the later "partial" hydrolysis.

Phosphorus was determined by the Fiske & Subbarow[15] method, pentose by the orcinol procedure of Mejbaum[20] and deoxypentose by the diphenylamine method of Dische.[14]

References substances (sigma chemical company)

Purine standards

- adenine; guanine; 2,6-diaminopurine (hemisulfate 1 ½ hydrate); 8-azaguanine; 7-methylguanine; 6-methylaminopurine; 2,6-dithiopurine; xanthine; 2-thioadenine sulfate; 6-methyl-mercaptopurine; hypoxanthine; 1-methyladenine; uric acid; 6-dimethylaminopurine; 6-(γ, γ-dimethylallylamino)-purine; 2-acetylamino-6-hydroxypurine; 2-aminopurine; adenosine; guanosine; 1-methyladenosine; inosine; 1-methyl inosine; 6-methyl-aminopurine-9-ribofuranoside; 6-dimethylaminopurine-9-riboside; 6-(γ, γ-dimethylallylamino)-purine riboside; adenosine-5'-monophosphoric acid (AMP); guanosine-5'-monophosphoric-acid (GMP); inosine-5'monophosphoric acid (IMP) (sodium salt); adenosine-5'-diphosphoric acid (ADP); or Adenosine 5'-diphosphate (ADP) ; guanosine 5'-diphosphate (GDP); Adenosine 5'-triphosphate (ATP); 2'-deoxyadenosine; 2'-deoxyguanosine; 2'-deoxyadenosine-5'-monophosphoric acid (d-AMP) (disodium salt); 2'-deoxyguanosine-5'-monophosphoric acid (d-GMP) (disodium salt); β-nicotinamide adenine dinucleotide (β-NAD); nicotinamide adenine dinucleotide phosphate (NADP); 2-dimethylamino-6-hydroxypurine.

Pyrimidine standards

- cytosine; uracil; thymine; 5-methylcytosine. HCl; 5-nitrouracil; 5-bromouracil, 5-iodouracil; 5-fluorouracil; 2-thiouracil; 2-amino-4-hydroxy-6-methylpyrimidine; dithiouracil; 2-thiocytosine; 6-methyluracil; 6-azauracil; 5-methyl pyrimidine; 5-methyl-2-thiocytosine; 2-amino-4,6-dimethylpyrimidine; 5-hydroxyuracil; 5-hydroxymethylcytosine; 6-methyl-2-thiouracil; orotic acid; 6-methylmercaptouracil; 5-methyl orotic acid; cytidine; uridine; thymidine; pseudouridine (5-ribofuranosyluracil); 3-methylcytidine methosulfate; uridine-4-disulfide (4,4'-dithiouridine); 5-methylcytidine;3-methyluridine; cytidine-5'monophosphoric acid (CMP) (free acid); uridine-5'-monophosphoric acid (UMP) (disodium salt); thymidine-5'-monophosphoric acid (TMP) (disodium salt); 2'-deoxycytidine; 2'-deoxyuridine; 2'-deoxycytidine-5'-monophosphoric acid (d-CMP) (disodium salt); 5-methyl deoxycytidine-5'-monophosphoric acid.

Results

Fractionation by ion exchange chromatography

Table 1 shows the anionic exchange fractionation of the deproteinized concentrated filtrate into "one non-anionic" fraction (A_0) and 5 anionic fractions (A_1 to A_5).

Table 1 – Dowex-1 crude fractions*

Fraction	S_{260} units	Percent of deproteinized filtrate abdorption
Column load	2432	100
A_0	447	18.4
A_1	111	4.6
A_2	143	5.9
A_3	202	8.3
A_4	181	7.4
A_5	359	14.8
(Discarded tubes)	429	17.6
Total Recovery	1872	77.0

* Dowex 1 x 8, 200-400 mesh (Cl^-).

Fraction A_0 is a complex misture of at least 3 fractions eluted with water. Further fractionation of this mixture into 7 distinct cationic fractions is shown in Table 2.

Table 2 – Dowex-50 crude fractions*

Fraction	S_{260} units	Percent distribution	Percent of deproteinized filtrate abdorption
Column load	3204	100	18.4
C_0	598	18.7	3.4
C_1	202	6.3	1.2
C_2	98	3.1	0.6
C_3	122	3.8	0.7
C_4	331	10.3	1.9
C_5	228	7.1	1.3
C_6	223	7.0	1.3
C_7	124	3.9	0.7
(Discarded tubes)	684	21.3	3.9
Total Recovery	2610	81.5	15.0

* Dowex 50W x 8, 200 -400 mesh (H^+).

Analysis of the anionic fractions

Table 3 contains the results of the paper chromatographic analysis of the anionic fractions and the supposed number of independent chemical components in each fraction.

Table 3 – Paper chromatography analysis of the anionic fractions

Fraction	Rf of the spots						Proposed componentes
	System S_1			System S_2			
A_{11}		0.51	0.60		0.53		1 & 2
A_{21}		0.60	0.72		0.61	0.77	3 & 4
A_{31}	0.33		0.74	0.37		0.75	5 & 6
A_{41}	no detected spots			no detected spots			–
A_{42}			0.72			0.80	7
A_{43}	0.37	0.60	0.71	0.37	0.56	0.76	8, 9 & 10
A_{51}	0.37	0.66		0.37	0.63	0.74	11, 12 & 13
A_{52}		0.68			0.65		14

The spots were eluted as described and the corresponding spectral characteristics along with their Rf values are shown in Table 4.

Table 4 – Spectral characteristics of the anionic fractions components

Fraction	Component	Rf		Spectral maxima (nm)			
		S_1	S_2	From S_1	Spot	From S_2	Spot
A_{11}	1	0.51	0.53	240	288	–	
A_{11}	2	0.60	0.53		280	–	
A_{21}	3	0.60	0.61	260		260	
A_{21}	4	0.72	0.77	250	295	250	296
A_{31}	5	0.33	0.37	inaccurate		inaccurate	
A_{31}	6	0.74	0.75	250	295	249	294
A_{42}	7	0.72	0.80	inaccurate		inaccurate	
A_{43}	8	0.37	0.35	inaccurate		inaccurate	
A_{43}	9	0.60	0.56	inaccurate		inaccurate	
A_{43}	10	0.71	0.76	248	298	249	298
A_{51}	11	0.37	0.37	inaccurate		inaccurate	
A_{51}	12	0.66	0.63	–		inaccurate	
A_{51}	13	0.66	0.74	–		248	290
A_{52}	14	0.68	0.65			(268)	

The components 5, 7, 8, 9, 11 and 12 eluted from the corresponding chromatographic paper spots were not concentrated enough to give accurate spectra having precisely defined maxima for analytical comparison.

From the remaining 8 components only component 2 could be identified with one of the 76 standard reference substances. It seems to be identical to orotic acid as shown by parallel chromatography and spectra superposition, as summarized in Table 5.

Table 5 – Identification of component 2

Substance	$Rf(S_1)$	$Rf(S_2)$	λ maximum (nm)
Component 2	0.62	0.52	280
Orotic acid	0.60	0.53	280

Component 2, assumed to be orotic acid, is found in the crude filtrate broth in the minimal concentration of 11 mg per liter.

The other 7 anionic components (1, 3, 4, 6, 10, 13 and 14) could not be identified with any of the 76 reference substances.

Some characteristics of these substances are the following:

1 They do not contain phosphorus. This is consistent with the fact that phosphorus starvation causes the production of these substances.

2 Probably they do not contain either ribose or deoxyribose because the same above A fractions gave negative or inconclusive results when submitted to the orcinol and to the diphenylamine assays.

3 Components 1, 10, 13 and 14 have different Rf and spectra before and after the "partial" hydrolysis procedure, which suggests for them a hydrolyzable structure like purine derivatives.

4 Components 3, 4 and 6 show no differences in paper chromatography and spectral analysis before and after "partial" hydrolysis. This behaviour can be taken either as a indication of anionic compounds having a pyrimidine portion or of anionic simple nitrogen bases.

The characteristics of components 4 and 6 are so similar that they can be taken as the same component appearing in both A_{21} and A_{31} fractions.

5 The unidentified compounds 14, 4 (and/or 6), and 10, seem to occur in the culture filtrate in significantly high concentration, when compared with orotic acid concentration, based on the relative A_{260} values.

6 Component 14 could not be extracted from its corresponding S_1 and S_2 paper chromatograms with 0.1 N HCl or with 1.0N ammonia. Since fraction A_{52} clearly showed one single component (14) its spectrum was taken as component 14 spectrum (Figure 7).

Figures 1-7 show respectively the absorption spectra of the unidentified anionic components 1, 3, 4, 6, 10, 13 and 14.

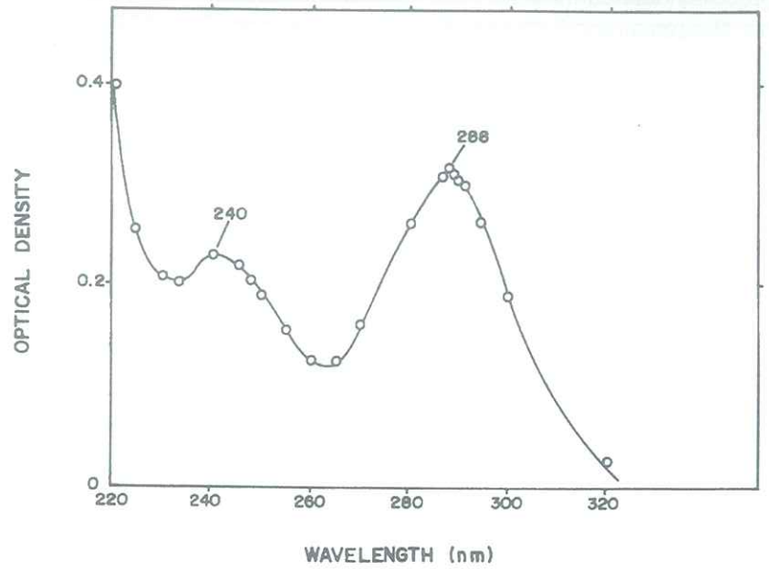

FIGURE 1 – Absorption spectrum of the unidentified component 1 evoluted from solvente S_1 paper chromatogram.

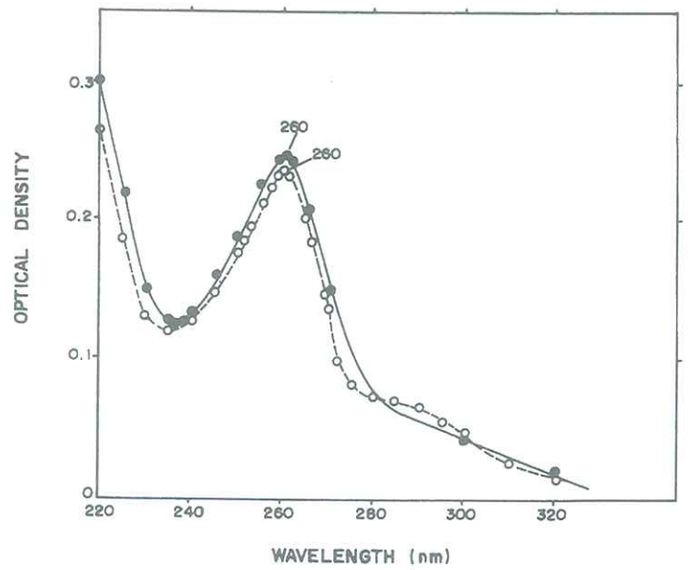

FIGURE 2 – Absorption spectra of the unidentified component 3 (o-eluted from solvent S_1 paper chromatogram; ●-eluted from solvent S_2 paper chromatogram).

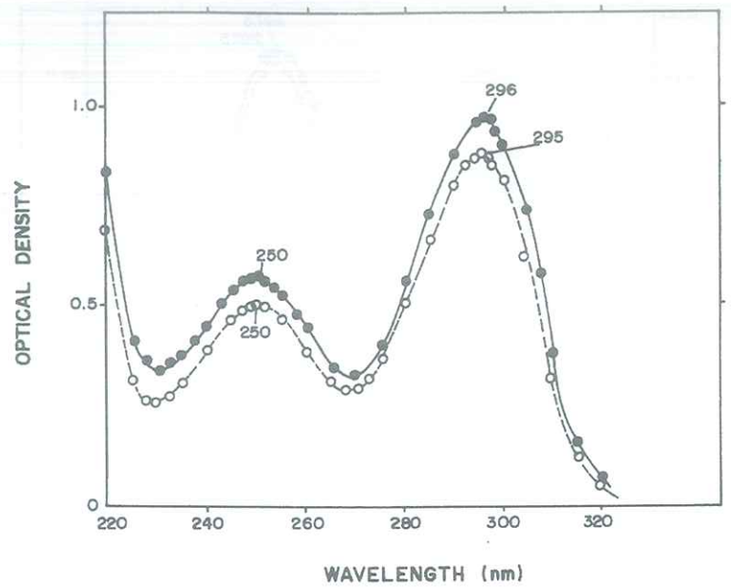

FIGURE 3 – Absorption spectra of the unidentified component 4 (o-eluted from solvent S_1 paper chromatogram; ●-eluted from solvent S_2 paper chromatogram).

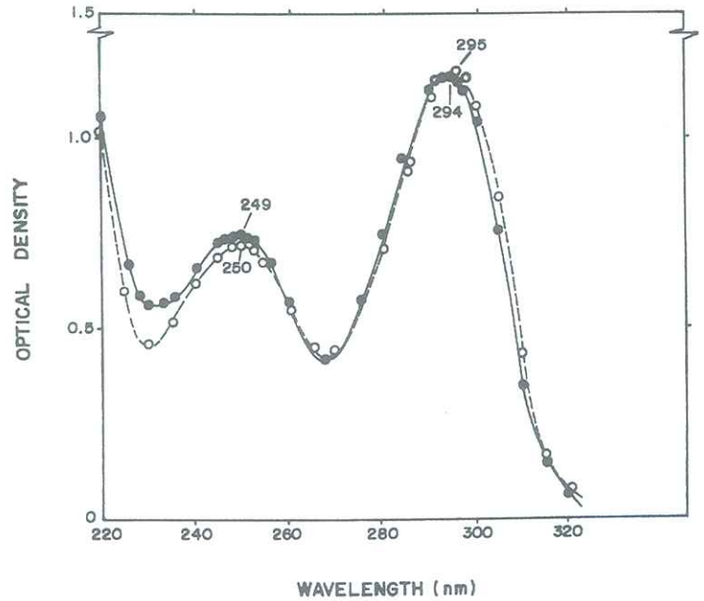

FIGURE 4 – Absorption spectra of the unidentified component 6 (o-eluted from solvent S_1 paper chromatogram; ●-eluted from solvent S_2 paper chromatogram).

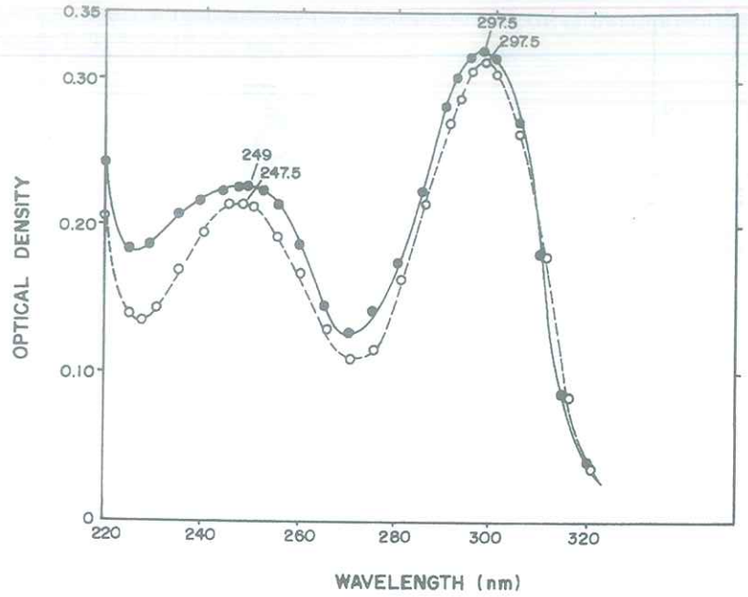

FIGURE 5 – Absorption spectra of the unidentified component 10 (o-eluted from solvent S_1 paper chromatogram; ●-eluted from solvent S_2 paper chromatogram).

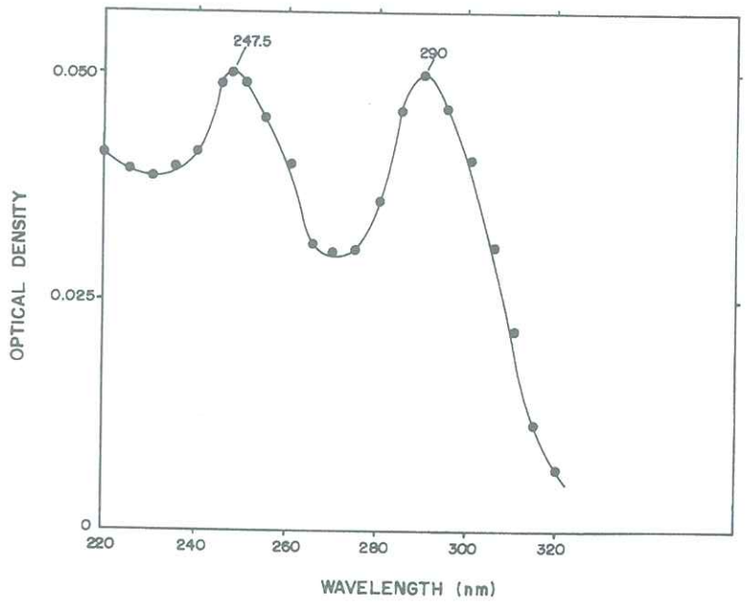

FIGURE 6 – Absorption spectrum of unidentified component 13 eluted from solvent S_2 paper chromatogram.

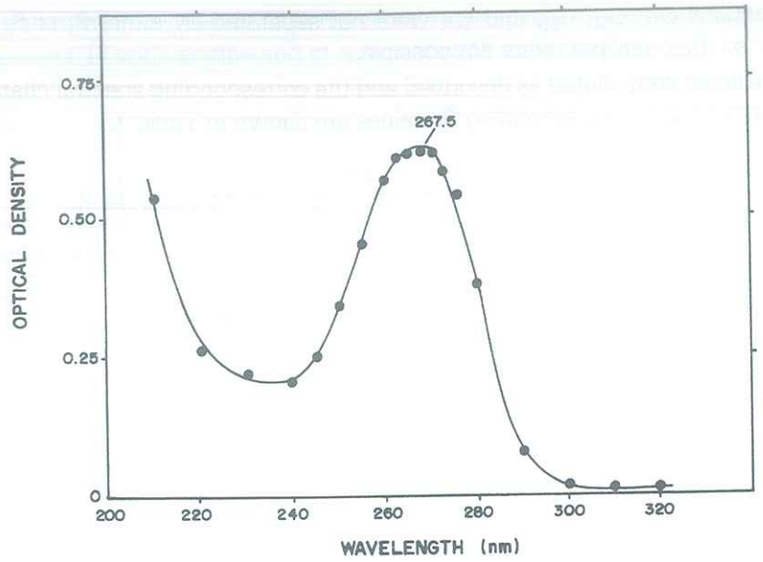

FIGURE 7 – Absorption spectrum of fraction A_{52} (unidentified component 14).

Analysis of the cationic fractions

Table 6 contains the results of the paper chromatographic analysis of the cationic fractions and the supposed number of independent chemical components in each fraction.

Table 6 – Paper chromatography analysis of the cationic fractions

Fraction	Rf of the spots		Proposed components
	System S_1	System S_2	
C_{11}	non-detected spots	non-detected spots	–
C_{12}	0.29	0.30	15
C_{21}	0.49	0.54	16
C_{31}	0.34	0.36	17
C_{41}	0.48	0.45	18
C_{51}	non-detected spots	non-detected spots	–
C_{52}	0.31	0.38	19
C_{53}	0.26	0.18	20
C_{54}	0.32	0.20 (?)	21
C_{61}	non-detected spots	non-detected spots	–
C_{62}	0.35	0.44	22
C_{71}	non-detected spots	non-detected spots	–

Fractions C_{11}, C_{51}, C_{61}, and C_{71} were not separated by either S_1 or S_2 solvent systems, so their analyses were not possible.

The spots were eluted as described and the corresponding spectral characteristics along with their corresponding Rf values are shown in Table 7.

Table 7 – Spectral characteristics of the cationic fractions components

Fraction	Component	Rf		Spectral maxima (nm)	
		S_1	S_2	From S_1 spot	From S_2 spot
C_{12}	15	0.29	0.30	265	264
C_{21}	16	0.49	0.54	276.5	277.5
C_{31}	17	0.34	0.36	247.5	247.5
C_{41}	18	0.48	0.45	275	275
C_{52}	19	0.31	0.38	inaccurate	inaccurate
C_{53}	20	0.26	0.18	248.5	245 (?)
C_{54}	21	0.32	0.20 (?)	inaccurate	inaccurate
C_{62}	22	0.35	0.43	indeterminate	260

All the 6 cationic components (19 and 21 gave inaccurate spectra) could be identified, despite their low concentration in the deproteinized filtrate.

Components of the cationic fractions seems to be identical with adenine, cytidine, inosine, cytosine, guanine and 1-methyl adenine, judging by the parallel chromatography and spectra superposition criteria described (Table 8).

Table 8 – Identification of the cationic components

Substance	Rf(S_1)	Rf(S_2)	Spectra λ máxima (nm)	Filtrate broth component concentration (mg/1)
Component 15	0.29	0.30	265	0.2
Adenine	0.34	0.34	263	
Component 16	0.49	0.53	277	0.3
Cytidine	0.49	0.50	278	
Component 17	0.34	0.34	247	0.5
Inosine	0.29	0.34	247	
Component 18	0.48	0.44	275	1.9
Cytosine	0.48	0.46	275	
Component 20	0.26	0.18	249	0.1
Guanine	0.24	0.18	249	
Component 22	0.35	0.43	260	0.1
1-methyl adenine	0.35	0.43	260	

As an illustration, Figures 8 and 9 show, respectively, the spectra superposition of component 17 with inosine and of component 18 with cytosine.

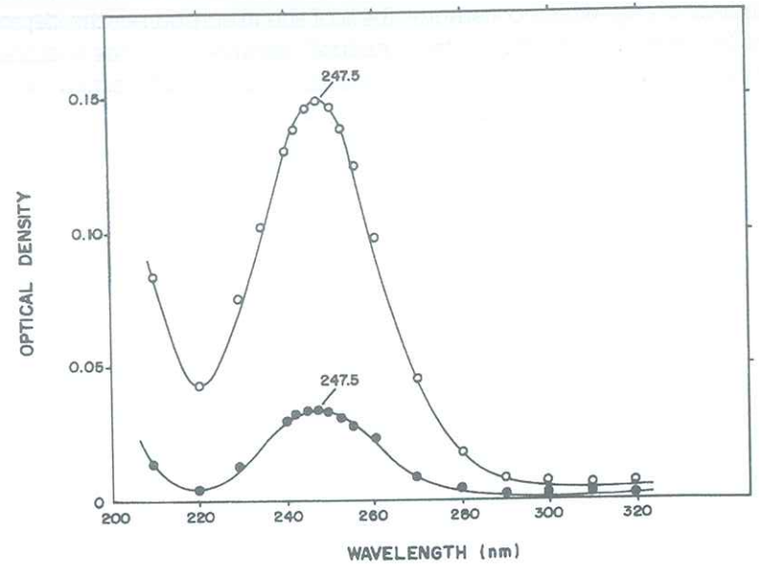

FIGURE 8 – Spectral comparison of component 17 (●) with inosine (○).

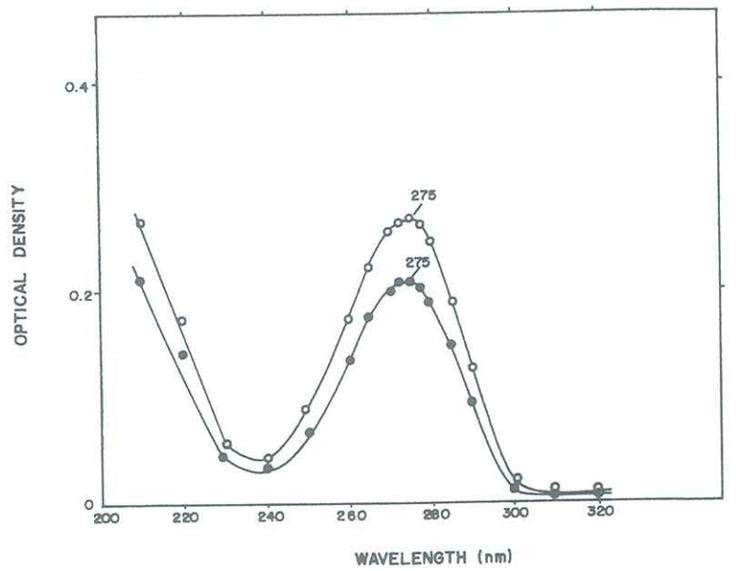

FIGURE 9 – Spectral comparison of component 18 (●) with cytosine (○).

The identification of component 16 with cytidine was confirmed by the "total" hydrolysis and hydrolyzate analysis of both component 16 and cytidine, whose respective properties which were consistent with cytosine characteristics.

The fraction C_o, which constitutes 3.4% of the absorption of the deproteinized filtrate at 260 nm, and should contain "neutral" components, has a characteristic spectrum with a single peak at 264 nm. However, it could not be separated by either solvents S_1 and S_2 even after active carbon adsorption and ethanol 50 per cent-ammonia 0.1 N elution for concentration and purification.

Discussion

The *S. aureofaciens* nucleic acid-related substances were previously presumed to be secondary metabolites actively secreted by the microorganism in all growth phases as a response to low phopshate level available in chemically defined medium specifically developed for such secretion.[3] Whoever, Simuth & Zelinka[25] have proposed RNA degradation by an active ribonuclease as the explanation for accumulation nucleic acid-related substances by a chlortetracycline producer *S. aureofaciens*. The absence of phosphorus in our studied compounds could be either explained as a consequence of nucleotide degradation, as supposed by Simuth & Zelinka,[25] or could be explained by the lack of enough phosphate for normal nucleotide synthesis, as we have presumed (Carvalho & Molinari[3]). This agrees with our observation that orotic acid, a pyrimidine precursor, was the most abundant identified component of our S_{260} complex mixture. Another observation which argues against the RNA decomposition hypothesis is the absence of uracil or uracil derivatives in all the fractions studied.

Simuth & Zelinka[25] have found that the accumulation of the nitrogenous bases and chlortetracycline production are related processes. This observation can also be explained by our hypothesis because both phenomena are linked to low phosphate concentrations in fermentation broth, as shown by Biffi et al.[2] for chlortetracycline production and by Molinari[22] for tetracycline production. It is difficult to explain Simuth & Zelinka's, finding of the occasional presence AMP and CMP in their fermented media.

It was relatively easy to identify the cationic components except for two compounds present in low concentrations. The other 6 substances were identified as common nucleic acid derivatives, with the exception of 1-methyl adenine. These 6 components together occur in a total minimum concentration of about 3.1 mg per liter of fermented broth, which represents about 28 per cent of the orotic acid concentration found.

Of the anionic components, which are more abundant, both qualitatively as quantitatively, only orotic acid was identified. This component contributes only about 2 per cent of the 260 nm absorption of the deproteinized extract. Of the 8 most abundant anionic components, 7 were not identified with any of the 76 reference substances selected as standards. Six of these unidentified components may be described as anionic, phosphate free, purine or pyrimidine-like derivatives or related substances contributing to, at least, 22 per cent of the light absorption.

Since they have been produced in unfavorable circumstances of growth, i.e in low phosphate medium, they probably belong to the secondary metabolite category and, may not be related to normal purine or pyrimidine nucleic acid intermediates. Orotic acid would be an exception as, despite not having phosphorus, it is a normal pyrimidine precursor.

CARVALHO, A. de et al. Fracionamento e identificação de substâncias relacionadas a ácidos nucléicos, produzidas por *Streptomyces aureofaciens*. *Ecl. Quím. (São Paulo)*, v.21, p.71-87, 1996.

■ *RESUMO: O crescimento do* S. aureofaciens, *em meio de cultura quimicamente definido e especialmente desenvolvido, está associado a produção e acúmulo no meio de substâncias relacionadas a ácidos nucléicos. Baixa concentração de fosfato disponível no meio resulta em elevada secreção das substâncias em estudo. A análise do material por cromatografia de troca iônica, espectrofotometria ultravioleta e cromatografia em papel permitiu a identificação e quantificação das substâncias: ácido orótico, citosina, inosina, citidina, adenina, guanina e, provavelmente, 1-metiladenina, quando comparadas a 76 derivados purínicos e pirimidínicos utilizados como padrões cromatográficos de referência.*

■ *PALAVRAS-CHAVE:* Streptomyces aureofaciens; *substâncias relacionadas a ácidos nucléicos; nucleosídeos; bases nitrogenadas; fracionamento e identificação.*

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