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PATENT SPECIFICATION

DRAWINGS ATTACHED

1.166.681



Date of Application (No. 48781/66) and filing Complete Specification: 31 Oct., 1966.

Application made in Canada (No. 945,388) on 15 Nov., 1965.

Application made in Canada (No. 970,545) on 16 Sept., 1966.

Complete Specification Published: 8 Oct., 1969.

Index at acceptance:—C2 A (1B, 2A4)

International Classification:—C 12 d 9/18

COMPLETE SPECIFICATION

Production of 7-Chloro-6-Demethyltetracycline Fermentation

We, AMERICAN CYNAMID COMPANY, a corporation organised under the laws of the State of Maine, United States of America, of Berdan Avenue, Township of Wayne, State of New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an improved process for the production of 7-chloro-6-demethyltetracycline by fermentative biosynthesis using strains of *Streptomyces aureofaciens*.

The antibiotic 7-chloro-6-demethyltetracycline is described and claimed, together with methods of its production, in our British Patent No. 855,169, according to which a 7-chloro-6-demethyltetracycline producing strain of *S. aureofaciens* is cultivated in a fermentation medium. That patent also discloses that other tetracyclines, such as 6-demethyltetracycline, 7-chlorotetracycline and tetracycline are produced at the same time in a fermentation medium when the fermentation conditions, including the micro-organisms therein disclosed, are used. Additionally, that patent discloses that 7-bromo-6-demethyltetracycline may be a concomitant product when bromine is a constituent of the fermentation medium. Further evidence of the production of more than one tetracycline in a fermentation medium in which demethylchlortetracycline may be produced is found in United States Patent Specification No. 3,028,311 and United States Patent Specification No. 3,061,522 wherein so-called 6-methylation inhibitors are present in a fermentation medium in which a 7-chlortetracycline producing strain of *S. aureofaciens* is used.

Additional disclosures of the production of 7-chloro-6-demethyltetracycline by cul-

tivating 7-chlortetracycline producing strains of *S. aureofaciens* in the presence of 6-methylation inhibitors are shown in United States Patent Specification Nos. 3,019,172 and 3,127,328. These processes are also faced with the problem of concomitant formation of other tetracyclines. Thus, in each a mixture of 7-chlortetracycline, tetracycline, 7-chloro-6-demethyltetracycline and 6-demethyltetracycline is produced.

The concomitant production of 6-demethyltetracycline (or other tetracyclines) is objectionable when 7-chloro-6-demethyltetracycline is the principal product being sought. While pharmaceutical quality 7-chloro-6-demethyltetracycline may contain small quantities of 6-demethyltetracycline, the presence of any sizable quantity of 6-demethyltetracycline is objectionable. The presence of 6-demethyltetracycline (or other tetracyclines) in the same fermentation mash in any sizable amounts also involves difficult problems of separation in the refining or extraction procedures when 7-chloro-6-demethyltetracycline is the desired product. It is possible, of course, to extract the antibiotics from the fermentation mash and by selective refining procedures to effect a separation of the antibiotics. However, the refining procedures for effecting separation of the antibiotics are difficult and usually involve some loss in total antibiotic potency. Moreover, in those instances where 7-chloro-6-demethyltetracycline is the principal product of the fermentation, 6-demethyltetracycline (or other tetracyclines) is unwanted and is customarily removed and discarded. Hence, any method that results in the increased production of 7-chloro-6-demethyltetracycline and the lessened concomitant production of 6-demethyltetracycline (or other tetracyclines) constitutes a marked contribution to the art of antibiotic fermentation.

It has now been unexpectedly discovered that

certain novel mutant strains of *Streptomyces aureofaciens* will produce, in a conventional fermentation, 7 - chloro - 6 - demethyltetracycline with no more than 5% of 6-demethyltetracycline as a concomitant product. Furthermore, there is no detectable production of tetracycline or 7-chlorotetracycline with the novel mutant strains as herein described. The discovery of these strains obviates the need for costly and difficult extraction and purification procedures, since the crystals of 7 - chloro - 6 - demethyltetracycline obtained are of pharmaceutical quality.

The novel mutant strains of the present invention are strains of the species *Streptomyces aureofaciens*. The representative *S. aureofaciens* strains described below are direct descendants of the 7 - chlorotetracycline-producing *S. aureofaciens* A-377 soil isolate described in our British Patent Specification No. 690,381 and deposited at the Northern Regional Research Laboratories, Peoria, Illinois, and indexed as NRRL 2209. They have been derived by mutagenic processes known to the art, e.g., ultraviolet irradiation, nicotine, and nitrogen mustard. Typical mutant strains of *S. aureofaciens* which possess the unique properties described herein have been designated by us as IE-1352, IE-1354, IE-1355, IE-2322, IE-2750 and IE-2803. Viable representative cultures of the above novel mutant strains have been deposited at the Northern Regional Research Laboratories, Peoria, Illinois and have been assigned the following accession numbers:

Strain	NRRL No.
IE-1352	3201
IE-1354	3202
IE-1355	3203
IE-2322	3204
IE-2750	3234
IE-2803	3235

It will be understood, of course, that additional mutants within the scope of this invention may be derived from these strains by conventional procedures. These mutants may vary somewhat in general morphological and physiological characteristics as do the various strains of the *S. aureofaciens* species. For example, Strain Nos. IE-2803 and IE-2750 were derived, respectively, from Strain Nos. IE-1352 and IE-2322 using techniques well-known in the art, i.e., of selecting naturally-occurring mutants and of selecting mutants which occurred as the result of deliberate treatment with the chemical mutagen, nitrogen mustard, and with ultra-violet light. Such techniques are, for example, described in the text: "Induced Mutagenesis in Selection of Microorganisms" by S. I. Alikhanian, pp. 1-50, incl., of Progress in Applied Microbiology, Vol. 4 (1962), Academic Press, New

York and London. It is also to be expected that 7 - chloro - 6 - demethyltetracycline-producing strains of *S. aureofaciens* of the invention may be found in nature or derived from presently isolated strains of *S. aureofaciens* by mutagenic methods well known in the art.

The strains of the present invention display the taxonomic characteristics of the species *S. aureofaciens* and may be readily identified as members of this species although differing from previously described strains of *S. aureofaciens*, not only in pigmentation on solid media, but also in the colors of the whole harvest mash obtained therewith.

In general, these new strains and the strains which are presently known in the art which produce 7 - chloro - 6 - demethyltetracycline in conventional media may be divided into three categories: 1) *S. aureofaciens* strains ATCC 12551, 12552, 12553 and 12554 which are described in British Patent No. 855,169 and which produce varying amounts of 7 - chloro - 6 - demethyltetracycline, 6 - demethyltetracycline, tetracycline and 7 - chlorotetracycline; 2) *S. aureofaciens* strains V-138, E-475, E-1311, ED-1723, ED-2047 and ED-2314 which are disclosed in the following patent specifications: V-138 in U.S. Patent Specification No. 3,037,916, E-475, E-1311, ED-1723 and ED-2047 in U.S. Patent Specification No. 3,050,446 (Goodman), ED-2314 in U.S. Patent Specification No. 3,145,154; and which produce varying amounts of 7 - chloro - 6 - demethyltetracycline and 6 - demethyltetracycline to the virtual exclusion of methylated tetracyclines; and 3) the new strains of the present invention, for example, the exemplified strains IE-1352, IE-1354, IE-1355, IE-2803, IE-2322 and IE-2750, which produce 7 - chloro - 6 - demethyltetracycline to the substantial exclusion of all other tetracyclines.

The novel strains employed according to this invention fall into one of two classes, viz. Class I or Class II. The Class I strains of the present invention, represented by Strain Nos. IE-1352, IE-1354, IE-1355 and IE-2803, may be distinguished from the two groups of prior art strains by a mathematical interpretation of the spectrophotometric reflectance curves of their respective whole harvest mash. More precisely, when the percent reflectance of diluted samples (1:200 aqueous) of the whole harvest mash as hereinafter defined, of good yield (such as examples set forth hereinafter) of the novel strains of the present invention is measured at specific wavelengths (460 mμ, 540 mμ, 560 mμ and 660 mμ) and the percentage reflectances in these specific wavelengths are inserted in the formula:

$$\Delta R = R_{540} + R_{560} - 1.1 R_{460} - 0.9 R_{660} - 2.0$$

a definite pattern appears. Thus, the strains of Class I of the present invention, e.g. the four mutant strains IE-1352, IE-1354, IE-1355 and IE-2803, each produce a positive ΔR value (greater than 0) while each of representative strains of the prior art produce a negative ΔR value (less than 0). The letter R denotes the reflectance value at some particular wavelength, for example R_{560} is the reflectance value at a wavelength of 560 m μ .

The Class II novel strains of the present invention, represented by Strain Nos. IE-2322 and IE-2750, may be distinguished from the two groups of prior art strains by the fact that the color reflectance scan of a 1 to 200 aqueous dilution of the whole harvest mash, made under the same spectrophotometric conditions and with the same apparatus as the curves of the other four strains, has the property that when the percent reflectance is plotted linearly against the wavelength, the resulting curve between 460 m μ and 530 m μ will have one point of inflection and will have at least one point of zero slope and will be convex upward to the left of the point of inflection and concave upward to the right of the point of inflection. For example, the resulting curve for representative Strain IE-2322, shown as Figure 14 of the accompanying drawings, exhibits a maximum between 460 m μ and 520 m μ (at approximately 500 m μ), this maximum being a point of zero slope; exhibits a point of inflection at approximately 510 m μ ; and exhibits a minimum at approximately 520 m μ , this minimum being a point of zero slope. As a further example, the resulting curve for representative strain IE-2750, shown as Figure 16 of the accompanying drawing, does not exhibit a maximum between 460 m μ and 520 m μ , i.e. it is plateau-like between 460 m μ and 520 m μ . The curve further exhibits a point of zero slope in the vicinity of 500 m μ and a point of inflection at approximately 517 m μ . Furthermore, strains of this class produce 7-chloro-6-demethyltetracycline to the substantial exclusion of 6-demethyltetracycline.

Figures 1 to 16 of the accompanying

drawings are spectrophotometric reflectance curves of one-centimeter glass cells filled with a diluted (1:200) whole harvest mash sample which is taken from the harvest fermentation broth of the novel mutant strains representative of the present invention as well as of the ten previously described prior art strains listed in Table I. The spectrophotometric reflectance curve of a material constitutes a permanent record that does not require maintenance of a sample. Furthermore, the units in which the curve is expressed are internationally understood and accepted as a means for measuring light. In Figures 1 to 16, the wavelength of light in millimicrons is plotted as the abscissa against the reflectance as the ordinate. The wavelength of light has been adopted internationally as the fundamental standard of length to which all other standards of length are referred. These spectrophotometric reflectance curves were determined with a recording spectrophotometer known as the General Electric Hardy Recording Spectrophotometer (General Electric Company, Schenectady, N.Y., U.S.A.) with the sample backed with magnesium oxide, the reference standard being magnesium oxide, and with the specular component excluded. The curves are recorded on General Electric Co. Chart paper NP 62581.

With regard to the aforementioned spectrophotometric determination it should be noted that dilution of the whole harvest mash is necessary to produce the mathematical results as stated. If the whole harvest mash is used undiluted, the difference in the ΔR value between the new and prior art strains will not be evident. It is only when the whole harvest mash is diluted with water at the rate of 1 part of whole harvest mash to 200 parts of water that the unusual results of a positive ΔR value for the Class I new strains and a negative ΔR value for the prior art strains becomes evident.

A "whole harvest mash" is the untreated mash obtained after a fermentation employing the following medium:

Starch	45	grams
CaCO ₃	10.5	grams
NH ₄ Cl	1.5	grams
Cottonseed flour	45	grams
Yeast	1.5	grams
Water	qs. to 1000	ml.
Lard Oil	3%	(Vol./vol. of medium)

has proceeded to the point where biosynthesis of the primary antibiotic product has stopped for all practical purposes. Generally, under the conditions set forth hereinbelow, the antibiotic potency of the fermentation mash, as may be conventionally determined, ceases to rise appreciably after the fermentation has proceeded for from about 140 to about 160 hours. It is conceivable, however, that under slightly different conditions maximum antibiotic potency may be reached in somewhat longer or shorter periods of time.

The novel strains of *S. aureofaciens* of the present invention which produce substantially no other tetracycline except 7-chloro-6-demethyltetracycline possess the same morphological characteristics as do the strains which concomitantly produce 7-chlortetracycline, tetracycline, 7-chloro-6-demethyltetracycline and 6-demethyltetracycline and those strains which produce 7-chloro-6-

demethyltetracycline and a substantial proportion of 6-demethyltetracycline, and differ among themselves in the same general manner that these prior art strains differ from each other as has been described in a number of scientific papers which have been published. The data appearing below will serve to further distinguish the hereindescribed Class I and II novel mutant strains of *S. aureofaciens* from the original A-377 strain available as NRRL 2209 and the strain, ATCC No. 12551 (S-604). In the following color observations on a variety of media, the color data of strain S-604 (ATCC 12551) have been reproduced from British Patent No. 855,169.

To illustrate the visual color variations among the novel mutant strains of *S. aureofaciens* of the present invention, these strains were grown on AP4 agar and the following observations were made.

Color Observations: ¹*S. aureofaciens*: **AP4 Agar: Six Day
Incubation at 26.5°C.

Strain	Single Colonies		Mass Growth
	Major Colony Type I	Minor Colony Type II	
1E—1352	5pn (Chocolate brown) ¹ to 6 pl (deep brown mahogany) ¹	8pn (Ebony brown) ¹	5pn (Chocolate brown) ¹ to 6pn (dark brown mahogany) ¹
1E—1354	4pi (Oak brown) ¹ to 5pl (deep brown) ¹	6pn (Dark brown mahogany) ¹	5pi (Copper brown) ¹ to 5nl (chocolate) ¹
1E—1355	5pl (Deep brown) ¹ to 5pn (chocolate brown) ¹	6pn (Dark brown mahogany) ¹	6pn (Dark brown mahogany) ¹
1E—2322	7pl (Burgundy) ¹ to 7nl (dark rose brown) ¹	8pn (Ebony brown) ¹	7pl (Burgundy) ¹ to 7nl (dark rose brown) ¹
1E—2750	5pn (Chocolate brown) ¹	8pn (Ebony brown) ¹	6 1/2 ni (Rose brown) ¹
1E—2803	5pl (Deep brown) ¹ to 5pn (chocolate brown) ¹	6pn (Dark brown mahogany) ¹ to 7po*	5lg (Cocoa brown) ¹

¹Colors here and elsewhere throughout this specification (*when designated*¹) are according to the Color Harmony Manual, Third Edition, Container Corporation of America (Publisher).

*Chip code is the only designation, there being no assigned color name listed in the Color Harmony Manual.

**Formulation of AP4 Agar

Sucrose	1.0	grams
MgSO ₄ ·7H ₂ O	0.025	grams
KH ₂ PO ₄	0.2	grams
(NH ₄) ₂ HPO ₄	0.2	grams
Cornsteep	0.4	grams
Bacto agar	2.0	grams
Water, q.s.	100.0	milliliters
Post Sterilization pH is 6.3		

The novel *S. aureofaciens* exemplary strains of the present invention as herein described were differentiated from *S. aureofaciens* A—377 (NRRL 2209) by observation of growth characteristics on various media incubated at 26.5°C. until maturity.

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(1) Glycerol Asparagine Beef Extract Agar

Glycerol	1.0 grams
L-asparagine	0.05 grams
Beef extract	0.2 grams
KH ₂ PO ₄	0.05 grams
Bacto agar	1.5 grams
Distilled water, q.s.	100 millilitres
Adjust to pH 7.0 with 50% KOH Post sterilization pH is 7.1	

	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1352	Strain 1E—1354
Growth	Scanty to thin, 2ie (light mustard tan) ¹ to 2 lg (mustard tan) ¹	Moderate to abundant, light amber
Aerial hyphae	Thin becoming fair to moderate; 5cb*	Fair to moderate, 5dc (pussywillow grey) ¹ to 5fe (ashes) ¹
Sporulation	Fair to moderate	Fair to moderate
Diffusible pigment	Yellow	Yellow
Reverse	Light grey becoming olive green	Light amber
	Strain 1E—1355	Strain 1E—2322
Growth	Moderate, 2ie (light mustard tan) ¹ to 2lg (mustard tan) ¹	Moderate, 5nl (chocolate) ¹ to 6 1/2 ni (rose brown) ¹ brown ¹
Aerial hyphae	Thin, white becoming 5dc (pussywillow grey) ¹ to 5fe (ashes) ¹	Abundant, white becoming 5dc (pussywillow grey) ¹
Sporulation	Incomplete	Incomplete
Diffusible pigment	Yellow	Deep amber
Reverse	Light grey	5pl (Deep brown) ¹ to 6 1/2 ni (rose brown)

*Color chip code according to the Color Harmony Manual, Third Edition, Container Corp. of America; no assigned color name.

	<i>Streptomyces aureofaciens</i>	
	Strain 1E—2750	Strain 1E—2803
Growth	Moderate, 3pn (Sepia brown) ¹ to 6pl (Deep brown mahogany) ¹	Moderate to abundant, 3ni (Clove brown) ¹ to 3pl (Deep brown) ¹
Aerial hyphae	Thin to fair, white	Moderate to abundant, 5dc (pussywillow grey) ¹
Sporulation	None	Moderate to abundant
Diffusible pigment	Tawny becoming amber	Light tawny becoming amber
Reverse	3pn (Sepia brown) ¹ to 6pl (Deep brown mahogany) ¹	5pi (Copper brown) ¹ to 5pl Deep brown) ¹
	Strain S—604	Strain A—377
Growth	Abundant, 6pi (venetian red) ¹	Fair
Aerial hyphae	Abundant, white to rose grey	White, uniform
Sporulation	Slight becoming abundant	None
Diffusible pigment	Reddish-brown	Yellow
Reverse	6pi (Brown-mahogany) ¹	Yellow to light orange-yellow

(2) Dextrin Czapek-Dox Agar

Dextrin	1.0 grams
NaNO ₃	0.2 grams
K ₂ HPO ₄	0.1 grams
MgSO ₄ ·7H ₂ O	0.05 grams
KCl	0.05 grams
FeSO ₄ ·7H ₂ O	0.001 grams
Bacto agar	1.5 grams
Distilled water, q.s.	100 milliliters
Post Sterilization pH is 7.2	

	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1352	Strain 1E—1354
Growth	Confluent, thin translucent, colorless	Confluent, thin, translucent, colorless
Aerial hyphae	None	None
Sporulation	None	None
Diffusible pigment	None	None
Reverse	Translucent	Translucent

	Strain 1E—1355	Strain 1E—2322
	Strain 1E—2750	Strain 1E—2803
Growth	Confluent, thin translucent, colorless	Confluent, thin, translucent, colorless
Aerial hyphae	None	None
Sporulation	None	None
Diffusible pigment	None	None
Reverse	Translucent	Translucent
Growth	Confluent, thin, transparent, colorless	Confluent, thin, transparent, colorless
Aerial hyphae	None	None
Sporulation	None	None
Diffusible pigment	None	None
Reverse	Translucent	Translucent

	<i>Streptomyces aureofaciens</i>	
	Strain S—604	Strain A—377
Growth	Sparse, hyaline	Profuse
Aerial hyphae	None	Abundant, lead grey water white surface globules
Sporulation	None	Abundant
Diffusible pigment	None	Slight, pale yellow
Reverse	Apigmentous	Apigmentous

(3) AP4 AGAR (as described above)

	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1352	Strain 1E—1354
Growth	Profuse, 5pn (chocolate brown) ¹ to 7nl (dark rose brown) ¹	Profuse, 5pi (copper brown) ¹ to 7nl (dark rose brown) ¹
Aerial hyphae	Fair to abundant, white becoming 5dc (pussywillow grey) ¹	Thin, 5dc (pussywillow grey) ¹
Sporulation	Fair, complete	Thin, complete
Diffusible pigment	Reddish-brown	Amber to light red brown
Reverse	5pn (Chocolate brown) ¹ to 7nl (dark rose brown) ¹	5pi (copper brown) ¹ to 7nl (dark rose brown) ¹

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	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1355	Strain 1E—2322
Growth	Profuse, 6pn (dark brown mahogany) ¹	Profuse, 7pl (burgundy) ¹ to 7nl (dark rose brown)
Aerial hyphae	Moderate, 5fe (ashes) ¹	Moderate, 5dc (pussywillow grey) ¹ to 5fe (ashes) ¹
Sporulation	Fair	Fair
Diffusible pigment	Red-brown	Deep red-brown
Reverse	6pn (Dark brown mahogany) ¹	7pl (Burgundy) ¹ to 7nl (dark rose brown) ¹
	Strain 1E—2750	Strain 1E—2803
	Growth	Abundant to profuse, 8pl (Cordovan) ¹ to 8pn (Ebony brown) ¹
	Aerial hyphae	Thin to moderate 5dc (Pussywillow grey) ¹
	Sporulation	Thin to moderate
	Diffusible pigment	Reddish brown to Deep reddish brown
	Reverse	8pl (Cordovan) ¹ to 8pn (Ebony brown) ¹
	Strain S—604	Strain A—377
	Growth	Profuse
	Aerial hyphae	Abundant, 7li (dark rose taupe) ¹
	Sporulation	Very abundant, uniform
	Diffusible pigment	Very concentrated, 5pl (Deep brown) ¹ to 6pl (deep brown mahogany) ¹
	Reverse	6pn (Dark brown mahogany) ¹
		2nl (Covert brown) ¹

(4) Q4 Cornsteep Agar

Cornsteep	9 grams
Sucrose	10 grams
MgSO ₄ ·7H ₂ O	0.25 grams
(NH ₄) ₂ HPO ₄	2 grams
KH ₂ PO ₄	4 grams
Crude agar	30 grams
Water q.s.	1000 milliliters
Post sterilization pH is 6.5	

	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1352	Strain 1E—1354
Growth	Abundant, 7pl (burgundy) ¹	Abundant, 7ng (brick red) ¹
Aerial hyphae	Thin to fair, white becoming 5dc (pussywillow grey) ¹	Thin to moderate, white becoming 5fe (ashes) ¹
Sporulation	Thin to fair	Thin to moderate
Diffusible pigment	Red-brown	Red-brown
Reverse	7pl (Burgundy) ¹	7ng (Brick red) ¹
	Strain 1E—1355	Strain 1E—2322
Growth	Abundant, 7pl (burgundy) ¹	Abundant, 7pl (burgundy) ¹ to 7nl (dark rose brown) ¹
Aerial hyphae	Fair to moderate, white becoming 5dc (pussywillow grey) ¹	Thin, 5fe (ashes) ¹
Sporulation	Thin to fair	Scant
Diffusible pigment	Amber	7pl (Burgundy) ¹ to 7nl (dark rose brown) ¹
Reverse	7pl (Burgundy) ¹	7pl (Burgundy) ¹ to 7nl (dark rose brown) ¹

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	<i>Streptomyces aureofaciens</i>	
	Strain 1E-2750	Strain 1E-2803
Growth	Abundant, 8pl (Cordovan) ¹ to 8pn (Ebony brown) ¹	Abundant, 5pi (Copper brown) ¹ becoming 7pn (Dark rose brown) ¹
Aerial hyphae	Fair to moderate, 5dc (Pussywillow grey) ¹ to 5fe (Ashes) ¹	Fair to moderate, 5fe (Ashes) ¹
Sporulation	Thin to fair	Thin to fair
Diffusible pigment	8pl (Cordovan) ¹	Dark red brown
Reverse	8pn (Ebony brown) ¹	7pl (Burgundy) ¹
	Strain S-604	Strain A-377
Growth	Profuse, 7pl (burgundy) ¹	Excellent, pale yellow
Aerial hyphae	Profuse, 5nl (chocolate) ¹	Profuse, 2pn (dark brown) ¹
Sporulation	Profuse	Profuse
Diffusible pigment	7pl (Burgundy) ¹	Orange-brown
Reverse	7pl (Burgundy)	Orange to orange-yellow

(5) Other Agar Media

Medium	<i>Streptomyces aureofaciens</i>	
	Strain 1E—1352	Strain 1E—1354
Nutrient Agar	Poor to fair growth; 4pl (dark spice brown) ¹ to 5pn (chocolate brown) ¹ . No aerial hyphae. Light amber soluble pigment. Reverse: 5pn (chocolate brown) ¹ .	Thin growth; 5pl (deep brown) ¹ to 6pn (dark brown mahogany) ¹ . No aerial hyphae. Faint red-brown soluble pigment. Reverse; 5 pl. (Deep brown) ¹ to 6pn (dark brown mahogany) ¹ .
Glucose Asparagine-Meat Extract Agar	Abundant growth; 5pi (copper brown) ¹ . Abundant aerial hyphae; 5dc (pussywillow grey) ¹ becoming 5fe (ashes) ¹ . Sporulation: abundant. Deep amber soluble pigment. Reverse: 5pi (Copper brown) ¹ .	Abundant growth; 4le (maple) ¹ to 4ng (light brown) ¹ . Abundant aerial hyphae 5fe (ashes) ¹ . Moderate to abundant sporulation. Deep yellow soluble pigment. Reverse: 4le (Maple) ¹ to 4ng (light brown) ¹ .
Purple milk	Pronounced growth collar; 5li (taupe brown) ¹ to 6 1/2 ni (rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.55 at 2 weeks.	Pronounced growth collar; 5li (taupe brown) ¹ to 6 1/2 ni (rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.8 at 2 weeks.
Potato slants	Profuse, moist crenulated growth; 4 ni (chestnut brown) ¹ to 5pn (chocolate brown) ¹ becoming 3po (ebony) ¹ . 6ig (Rose taupe) ¹ soluble pigment. No aerial hyphae.	Profuse, moist crenulated growth; 2nl (covert brown) ¹ to 4ne (luggage tan) ¹ becoming 5pn (chocolate brown) ¹ . 6ig (Rose taupe) ¹ soluble pigment. No aerial hyphae.

Medium	<i>Streptomyces aureofaciens</i>	
	Strain 1E-1355	Strain 1E-2322
Nutrient Agar	Thin growth; 4pl (Dark spice brown) ¹ to 5pn (chocolate brown) ¹ . No aerial hyphae. Very light amber soluble pigment. Reverse: 5pn (chocolate brown) ¹	Thin growth; 5pn (chocolate brown) ¹ . No aerial hyphae. Light purplish soluble pigment. Reverse: 5pn (chocolate brown) ¹ .
Glucose Asparagine-Meat Extract Agar	Abundant growth; 5ni (cocoa brown) ¹ to 5pi (copper brown ¹ .) Abundant aerial hyphae; 5fe (ashes) ¹ . Abundant sporulation. Deep yellow soluble pigment. Reverse: 5ni (cocoa brown) ¹ to 5pi (copper brown) ¹	Abundant growth; 5pl (deep brown) ¹ . Abundant aerial hyphae; white becoming 5fe (ashes) ¹ . Abundant sporulation. Deep yellow soluble pigment. Reverse: 5pl (deep brown) ¹ .
Purple milk	Pronounced growth collar; 5li (taupe brown) ¹ to 6 1/2 ni (rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.75 at 2 weeks.	Pronounced growth collar; 5li (taupe brown) ¹ to 6 1/2 ni (rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.75 at 2 weeks.
Potato slants	Profuse, moist crenulated growth; 5pi (copper brown) ¹ to 5pl (deep brown) ¹ becoming 5pn (chocolate brown) ¹ . 6ig (Rose taupe) ¹ soluble pigment. No aerial hyphae.	Profuse, moist crenulated growth; 4pg (dark luggage tan) ¹ becoming *7po to 10 po (black plum) ¹ . 6ig (Rose taupe) ¹ soluble pigment. No aerial hyphae

*Chip Code is the only designation, there being no assigned color name listed in the Color Harmony Manual.

	<i>Streptomyces aureofaciens</i>	
	Strain 1E-2750	Strain 1E-2803
Nutrient Agar	Poor to fair confluent growth, 6pn (Dark brown mahogany) ¹ . No aerial hyphae. Tawny diffusible pigment tinted reddish brown. Reverse: 6pn (Dark brown mahogany) ¹ .	Poor to fair confluent growth, 3pl (clove brown) ¹ to 3pn (Sepia brown) ¹ . No aerial hyphae. Tawny diffusible pigment tinted reddish brown. Reverse: 3 pl (Clove brown) ¹ to 3pn (Sepia brown) ¹ becoming 3pn (Sepia brown) ¹ .
Glucose Asparagine- Meat Extract Agar	Good to abundant growth, 5pi (copper brown) ¹ to 6pi (Brown mahogany) ¹ . Abundant aerial hyphae uniformly 5dc (Pussywillow grey) ¹ . Abundant sporulation. Diffusible pigment light Tawny-yellow becoming Amber. Reverse: 5pi (Copper brown) ¹ to 6pi (Brown mahogany) ¹ becoming 6pn (Dark brown mahogany) ¹ .	Abundant growth, (5pl Deep brown) ¹ . Abundant compact aerial hyphae, 5fe (ashes) ¹ . Abundant sporulation. Light to moderate Reddish brown diffusible pigment becoming amber. Reverse: 5pl (Deep brown) ¹ becoming 6pn (Dark brown mahogany) ¹ .
Purple Milk	Pronounced growth collar, 5li (Taupe brown) ¹ to 7nl (Dark rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.75 at 2 weeks.	Pronounced growth collar, 5li (Taupe brown) ¹ to 7nl (Dark rose brown) ¹ . Little significant pH change. False alkaline color reaction due to pigment diffusion. pH 6.75 at 2 weeks.
Potato slants	Profuse, moist, crenulated growth, Light 4gl (fawn) ¹ to 5pi (Copper brown) ¹ becoming 10po Black plum ¹ . Tendency to form white dense areas of aerial hyphae at separated foci. Diffusible pigment: 5ig (Rose taupe) ¹ to 5li (Taupe brown) ¹ .	Profuse, moist, crenulated growth, 6pi (Brown mahogany) ¹ becoming 3po (Ebony) ¹ to 5po (chocolate brown) ¹ . Occasional areas of dense white aerial hyphae at separated foci. Diffusible pigment: 5ig (Rose taupe) ¹ to 5li (Taupe brown) ¹ .

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Medium	<i>Streptomyces aureofaciens</i>	
	Strain S—604	Strain A—377
Nutrient Agar	Poor growth. 51i (Taupe brown) ¹ to 2pn (dark brown) ¹ . No aerial hyphae. Reverse: 2pn (Taupe brown) ¹ . Reddish brown soluble pigment.	Fair growth. No aerial hyphae. Reverse: Pale yellow. Yellow to light brownish yellow soluble pigment.
Glucose Asparagine-Meat Extract Agar	Abundant growth. Heavy aerial hyphae mottled. 5ig (Rose taupe) ¹ to 4ig (fawn) ¹ to 3ge (camel) ¹ . 2pn (taupe brown) ¹ . Reddish brown Sporulation: abundant. Reverse soluble pigment.	Fair growth. Aerial hyphae white becoming increasingly grey with increase in spore formation. Reverse: light yellow. Light yellow soluble pigment.
Purple milk	Slight 6 1/2 pl (deep red mahogany) ¹ growth collar. Little significant pH change nor appararent peptonization. Slight false alkaline color reaction due to diffusion of soluble pigment.	Slight white to pale yellow growth collar. Little significant pH change nor apparent peptonization in 15 days.
Potato slants	Profuse, moist smooth nodulated growth; 6pn (dark brown mahogany) ¹ with 5gc (peach tan) ¹ trace. Aerial hyphae; none to abundant becoming white to 3ge (camel) ¹ . Sporulation abundant in areas of heavy aerial hyphae. 5nl (Chocolate) ¹ to 5pn (chocolate brown) ¹ soluble pigment.	Profuse, moist smooth nodulated growth; 3ea (light melon yellow) ¹ to 71e (antique rose) ¹ . Aerial hyphae trace. No soluble pigment.

(6) Microscopic Observations

<i>S. aureofaciens</i>		Glucose Asparagine Beef Extract Agar	Q4 Agar
Strain 1E-1352	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain 1E-1354	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .	Flexuous, continuous, branched. Diameter. 0.8 to 1.2 μ .
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain 1E-1355	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain 1E-2322	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain 1E-2750	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain 1E-2803	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .	Spherical to ovoidal. Diameter 0.8 to 1.5 μ .
Strain S-604	Mycelium	Flexuous, continuous, branched. Diameter 0.8 to 1.0 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spheroidal to ovoidal. Diameter 1.5 to 2.0 μ .	Spheroidal to ovoidal. Diameter 1.5 to 2.0 μ .

(6) Microscopic Observations

<i>S. aureofaciens</i>		Glucose Asparagine Beef Extract Agar	Q4 Agar
Strain A-377	Mycelium	Flexuous, continuous, branched. Diameter 0.7 to 1.0 μ .	Flexuous, continuous, branched. Diameter 0.8 to 1.2 μ .
	Spores	Spheroidal to ovoidal. Diameter 1.5 to 2.0 μ .	Spheroidal to ovoidal. Diameter 1.5 to 2.0 μ .

Mycelial and spore morphology, as viewed through the light microscope, is apparently similar for strain 1E-1352, 1E-1354, 1E-1355, 1E-2322, 1E-2750 and 1E-2803 as for strains A-377 and S-604. However, the diameters for typical mycelium and for spores of the former strains appear to be characteristically smaller than for the latter. All strains demonstrate continuous, flexuous, branched mycelium with an occasional tendency of aerial hyphae to develop hooks or loops or to coil into primitive loose spirals as is typical of the species *S. aureofaciens*.

The conditions of the fermentation are generally the same as for the presently known methods of producing chlortetracycline by fermentation. That is, the fermentation medium contains the usual assimilable sources of carbon, nitrogen and inorganic salts. Suitable nutrient substances include starch, dextrose, cane sugar, glucose, molasses, soy bean meal, milk solids, yeast, meat extracts, peptone, urea, cornsteep liquor, cottonseed meal, distillers' solubles, glyceride oils, fish meal and other conventional substances. The inorganic salts include calcium carbonate, ammonium sulfate, ammonium chloride, and salts of the various trace elements such as manganese, cobalt, zinc, copper, iron and the like.

The other general conditions of the fermentation such as hydrogen ion concentration,

temperature, time, rate of aeration, preparation of the inoculum, sterilization, inoculation and the like are conventional and may be similar to those for the production of 7-chloro-6-demethyltetracycline shown in British Patent No. 855,169.

The recovery of the 7-chloro-6-demethyltetracycline from the fermentation liquor is conventional and need not be described, as numerous methods for the recovery of this product from fermentation liquors have already been published.

This invention is illustrated by the following specific example.

EXAMPLE

A fermentation medium was prepared which contained the following ingredients:

Starch	45	grams
CaCO ₃	10.5	grams
NH ₄ Cl	1.5	grams
Cottonseed flour	45	grams
Yeast	1.5	grams
Water	qs. to 1000	milliliters

This medium was dispensed in appropriate amounts into flasks containing 3% (vol./vol. of medium) of lard oil and then sterilized. These flasks were then divided into sixteen

approximately equal groups. Each group was then inoculated with a vegetative inoculum of one of the following strains of *S. aureofaciens*.

	S-604	(ATCC 12551)	the following methods: Assay No. 1: This	
	S-1071	(ATCC 12552)	assay is based upon the difference in the rates	
	V-62	(ATCC 12553)	of conversion of 7-chloro-6-demethyltetra-	
	B-740	(ATCC 12554)	cycline and 6-demethyltetracycline to their	
5	V-138		respective anhydro forms by an acid catalyzed,	30
	E-475		first order reaction. The degree of conversion	
	B-1311		to the anhydro form depends upon time, tem-	
	ED-1723		perature and acid strength involving a reaction	
	ED-2047		which results in a decrease in absorbance at	
10	ED-2314		368 m μ and an increase in absorbance at	35
	IE-1352		440 m μ . Assay No. 2: This assay is based	
	IE-1354		upon the same basic principle as Assay No. 1	
	IE-1355		except that in this case the sample is treated	
	IE-2322		with acid to degrade all tetracyclines present	
15	IE-2803		to the anhydro form in one instance and com-	40
	IE-2750		pared to a sample treated with water in the	
			second instance.	

All of the flasks were then incubated on a rotary shaker with a 2 $\frac{1}{2}$ " circular motion at 185—200 r.p.m. at 25° C. for 180 hours. Samples were withdrawn, diluted with 199 parts of water, and the reflectance curves determined as set forth hereinabove. The results are shown by Figures 1 to 16. The harvest mash assays are recorded in Table I below. These assays were performed using

Assay No. 3: The assay figures in this column were determined by a conventional paper chromatographic procedure using the system 0.3 M sodium phosphate *n*-butyl acetate, giving an *R_f* of 0.30 for 6-demethyltetracycline. Three similar systems, published in the Journal of the American Chemical Society, Vol. 79, pp. 4561—4563 (1957), (McCormick et al.) are listed below.

System	6-Demethyltetracycline <i>R_f</i>
0.3 M Sodium Phosphate/ <i>n</i> -butanol (pH 3.0)	.30
Mac Ilvaine's Buffer/ethyl acetate (pH 4.7)	.27
0.3N H ₃ PO ₄ , 0.1% CCL ₃ COOH 9:1 CHCl ₃ - <i>n</i> -Butanol	.22

In this Table I the percent 6-demethyltetracycline (Column 4) of the first four strains (S-604, S-1071, V-62 and B-740) was determined by finding the amount of 6-demethyltetracycline by paper chromatography (Column 3) and dividing this figure by the total amount of demethyltetracyclines determined by spectrophotometric analysis (Column 1) and by chromatographic analysis (Column 3). The percent 6-demethyltetracycline (Column 4) produced by the balance of the strains is determined by dividing the chromatographic analysis (Column 3) by the spectrophotometric analysis of the total tetracyclines (Column 2).

TABLE I

	Assay			
	No. 1	No. 2	No. 3	
<i>S. aureofaciens</i> Strain No.	DMCTC v/ml.	Total Tetracyclines v/ml.	DMTC v/ml. Quantitative Paper Chromatogram	% DMTC
S-604	1193	2037	110	8.4
S-1071	876	2428	138	13.1
V-62	1177	2255	573	32.7
B-740	1042	2553	380	26.7
V-138	960	1817	645	35.5
E-475	2348	3601	847	23.5
E-1311	3186	4263	820	19.2
ED-1723	1961	3304	1320	39.9
ED-2047	4726	6316	1775	28.1
ED-2314	4946	6650	1100	16.5
1E-1352	5780	5990	247	4.1
1E-1354	4420	4565	145	3.9
1E-1355	6060	6390	230	4.2
1E-2803	6034	6220	186	2.8
1E-2322	3281	3197	none* detected	0
1E-2750	3451	3566	none* detected	0

*No 6-demethyltetracycline was found at the lowest detectable level of 1 v/ml.

WHAT WE CLAIM IS:—

1. A process of producing 7-chloro-6-demethyltetracycline which comprises cultivating a strain of *Streptomyces aureofaciens* which produces 7-chloro-6-demethyltetracycline selectively under submerged aerobic conditions in a aqueous nutrient medium containing assimilable sources of carbon, nitrogen and inorganic salts until substantial quantities of 7-chloro-6-demethyltetracycline are produced, said 7-chloro-6-demethyltetracycline producing strain of *Streptomyces aureofaciens* being characterized by its ability to impart to a 200 fold aqueous dilu-

tion of the whole harvest mash, as defined herein, a color characterized by either

- (a) a reflectance curve, when plotted linearly, wherein the values of the percent reflectance at 460 m μ , 540 m μ , 560 m μ and 660 m μ when substituted into the formula:

$$\Delta R = R_{540} + R_{560} - 1.1 R_{460} - 0.9 R_{660} - 2.0$$

gives a positive value for ΔR , or

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25

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TABLE I

	Assay			
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WHAT WE CLAIM IS:—

1. A process of producing 7-chloro-6-demethyltetracycline which comprises culti-
- 5 vating a strain of *Streptomyces aureofaciens* which produces 7-chloro-6-demethyltetracycline selectively under submerged aerobic conditions in a aqueous nutrient medium containing assimilable sources of carbon, nitro-
- 10 gen and inorganic salts until substantial quantities of 7-chloro-6-demethyltetracycline are produced, said 7-chloro-6-demethyltetracycline producing strain of *Streptomyces aureofaciens* being characterized by its ability
- 15 to impart to a 200 fold aqueous dilu-

tion of the whole harvest mash, as defined herein, a color characterized by either

- (a) a reflectance curve, when plotted linearly, wherein the values of the percent reflectance at 460 mμ, 540 mμ, 560 mμ and 660 mμ when substituted into the formula:

$$\Delta R = R_{540} + R_{560} - 1.1 R_{460} - 0.9 R_{660} - 2.0$$

gives a positive value for ΔR, or 25

- (b) a reflectance curve such that when the percent reflectance is plotted linearly against the wave length, the resulting curve between 460 mμ and 530 mμ will have one point of inflection and will have at least one point of zero slope and will be convex upward to the left of the point of inflection and concave upward to the right of the point of inflection.
2. A process according to Claim 1 in which the 7-chloro-6-demethyltetracycline-producing strain of *Streptomyces aureofaciens* is characterized by its ability to impart to a 200 fold aqueous dilution of the whole harvest mash, as defined herein, a color characterized by a reflectance curve, when plotted linearly, wherein the values of the percent reflectance at 460 mμ, 540 mμ, 560 mμ and 660 mμ when substituted into the formula:
- $$\Delta R = R_{540} + R_{560} - 1.1 R_{460} - 0.9 R_{660} - 2.0$$
- give a positive value for ΔR.
3. A process according to Claim 1 in which the 7-chloro-6-demethyltetracycline-producing strain of *Streptomyces aureofaciens* is characterized by its ability to impart to a 200 fold aqueous dilution of the whole harvest mash, as defined herein, a color characterized by a reflectance curve such that when the percent reflectance is plotted linearly against the wavelength, the resulting curve between 460 mμ and 530 mμ will have one point of inflection and will have at least one point of zero slope and will be convex upward to the left of the point of inflection and concave upward to the right of the point of inflection.
4. A process according to Claim 3 in which said resulting curve will have a maximum between 460 mμ and 520 mμ.
5. A process according to Claim 3 in which said resulting curve will not have a maximum between 460 mμ and 520 mμ.
6. A process for producing 7-chloro-6-demethyltetracycline according to Claim 2 and substantially as hereinbefore described.
7. A process for producing 7-chloro-6-demethyltetracycline according to Claim 3 and substantially as hereinbefore described.
8. 7-chloro-6-demethyltetracycline whenever produced by a process according to Claim 2 or Claim 6.
9. 7-chloro-6-demethyltetracycline whenever produced by a process according to any one of Claims 3-5 or Claim 7.
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Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1969.
Published by the Patent Office, 25 Southampton Buildings, London, W.C.2, from which copies may be obtained.



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