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DRAWINGS ATTACHED

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COMPLETE SPECIFICATION

Improvements in or relating to Antibiotic Production

We, AMERICAN CYANAMID COMPANY, a corporation organised under the laws of the State of Maine, United States of America, of 30 Rockefeller Plaza, New York 20, New York, 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 the production of tetracycline by fermentation and, more particularly, is concerned with certain novel mutant strains of *Streptomyces aureofaciens* which possess the property of producing tetracycline to the exclusion of chlortetracycline irrespective of the chloride ion content of the fermentation medium.

It has been known for some time that microorganisms of the species *Streptomyces aureofaciens* which produce tetracycline also simultaneously produce chlortetracycline. The concomitant production of chlortetracycline is objectionable when tetracycline is the principal product sought to be produced. Generally, while specification standards permit small quantities of chlortetracycline to be present in specification quality tetracycline, the presence of any sizable quantities of chlortetracycline is objectionable. Moreover, the presence of these two antibiotics in any sizable amounts in the fermentation mash involves difficult problems of separation in the refining or extraction procedures. 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 not without some difficulty and they will usually involve some loss in total antibiotic potency. Moreover, chlortetracycline, which in those instances where tetracycline is the principal product of the fermentation, may be considered a contaminant and is customarily discarded since it is usually not present in

sufficient quantity to warrant the expense of a separate purification procedure to bring it up to specification standards. Heretofore, chloride ion deprivation or the use of chlorination inhibitors have been means available for suppressing the formation of chlortetracycline.

The present invention is based upon the discovery that certain novel mutant strains of *Streptomyces aureofaciens* produce tetracycline, to the total exclusion of chlortetracycline, by fermentation. These novel mutant strains of *Streptomyces aureofaciens* are, in essence, chloride ion-ignoring strains in that they produce tetracycline exclusively regardless of the concentration of chloride ion in the medium.

The novel mutant strains of the present invention are strains of the species *S. aureofaciens* since they are all direct descendants of the chlortetracycline-producing *S. aureofaciens* A—377 soil isolate described in Patent Specification No. 690,381 and deposited at the Northern Regional Research Laboratories, Peoria, Illinois and indexed as NRRL—2209. Derivation of these novel mutant strains of *S. aureofaciens* from the original A—377 strain involved treatment of the A—377 strain with mutagenic agents including ultraviolet irradiation, nicotine, and nitrogen mustard. Typical mutant strains of *S. aureofaciens* which possess the unique property of ignoring chloride ion have been designated by us as T—135, T—140, T—143, T—225, S—2308, and S—2589. Viable cultures representative of some of these novel mutant strains have been deposited with the American Type Culture Collection (ATCC) in Washington, D. C. and have been assigned the following accession numbers.

Strain	ATCC No.
T—135	13,908
T—140	13,909
T—143	13,910
T—225	13,911

[Price 4s. 6d.]

In general, the novel mutant strains of the present invention are characteristic of the species *S. aureofaciens* but differ from previously described strains of *S. aureofaciens*, not only in pigmentation, but also in the colors of the whole harvest meshes obtained therewith. The colors of the whole harvest meshes obtained by fermentation with the novel mutant strains of the present invention all exhibit a value of ΔR at 420 $m\mu$ which is greater than the value of ΔR at 430 $m\mu$.

Figures 1 through 8 of the drawings are spectrophotometric reflectance curves of one-centimeter glass cells filled with the whole harvest meshes obtained with the novel mutant strains of *S. aureofaciens* of the present invention. The spectrophotometric reflectance curve of a material constitutes a permanent record that does not require the maintenance of a sample. Furthermore, the units in which the curve is expressed are understood and accepted in every civilized country. In Figures 1 through 8, the wavelength of light in millimicrons is plotted as the abscissa against the reflectance as the ordinate. The wavelength of light has been adopted inter-

nationally as the fundamental standard of length to which all other standards of length are referred. These spectrophotometric reflectance curves were determined with a standard spectrophotometer using magnesium carbonate as a reference.

The letter R denotes the reflectance value at some particular wavelength, for example, R_{500} is the reflectance value at a wavelength of 500 $m\mu$. The symbol ΔR denotes the vertical distance between the reflectance curve (when both per cent reflectance and wavelength are plotted on linear scales) and a straight line drawn through the reflectance curve intercepts at 400 $m\mu$ and 550 $m\mu$. This graphical determination of the values of ΔR at 420 $m\mu$ and at 430 $m\mu$ may be readily carried out, and in every case the novel mutant strains of the present invention impart a color to the whole harvest meshes such that ΔR at 420 $m\mu$ is greater than ΔR at 430 $m\mu$.

The mathematical determination of the values of ΔR at 420 $m\mu$ and at 430 $m\mu$ may also be accomplished by means of the following equations:

$$\Delta R \text{ at } 420 \text{ } m\mu = 20(R_{550}) + 130(R_{400}) - 150(R_{420})$$

$$\Delta R \text{ at } 430 \text{ } m\mu = 30(R_{550}) + 120(R_{400}) - 150(R_{430})$$

wherein R_{400} , R_{420} , R_{430} and R_{550} are the reflectance values at wavelengths of 400 $m\mu$, 420 $m\mu$, 430 $m\mu$, and 550 $m\mu$, respectively. This mathematical determination is not dependent upon the scale used for either per cent reflectance or wavelength.

A "whole harvest mash" is the untreated mash obtained after fermentation has proceeded to the point where biosynthesis of the primary product has stopped for all practical

purposes. Generally, the antibiotic potency of the fermentation mash ceases to rise appreciably after the fermentation has proceeded for from 140 to 160 hours.

To illustrate the visual color variations among the novel mutant strains of *S. aureofaciens* which produce tetracycline exclusively, these strains were grown on cornsteep agar and the following observations were made.

Color Observations¹: *S. aureofaciens*:

AP4 Cornsteep Agar:

Six day incubation at 26.5° C.

Strain	Single Colonies	Mass Growth
T-135	Dark luggage tan	Maple
T-140	Orange rust	Maple
T-143	Oak brown	Light brown
T-225	Dark luggage tan	Yellow maple

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

Color Observations¹: *S. aureofaciens*

AP6 Cornsteep Agar:

Six day incubation at 26.5° C.

Strain	Single Colonies	Mass Growth
T-135	Luggage tan	Light spice brown
T-140	Orange rust	Maple
T-143	Dark luggage tan	Maple
T-225	Luggage tan	Maple

¹ Colors according to the Color Harmony Manual, Third, Edition, Container Corporation of America.

Formulation of AP4 Agar

Sucrose	10 grams
MgSO ₄ ·7H ₂ O	0.25 grams
KH ₂ PO ₄	2 grams
(NH ₄) ₃ PO ₄	2 grams
Cornsteep	4 grams
Bacto Agar	20 grams
Water, q.s.	1000 milliliters

Formulation of AP6 Agar

Same as AP4 Agar, except cornsteep level is 6 grams per liter.

- 5 The novel mutant strains of *S. aureofaciens* of the present invention which produce tetracycline exclusively possess the same general characteristics as do the strains which produce both chlortetracycline and tetracycline, and differ among themselves in the same general manner that the tetracycline-producing and chlortetracycline-producing 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 novel mutant strains of *S. aureofaciens* of the present invention from previously known strains of *S. aureofaciens* such as the original A-377 strain available as NRRL-2209.
- 10
- 15

The novel *Streptomyces aureofaciens* mutant strains were differentiated from *S. aureofaciens* A-377 (NRRL-2209) by observation of growth characteristics on various media incubated at 26.5°C.

1. Glycerol Asparagine Beef Extract Agar

Glycerol	1.0	per cent
L-Asparagine	0.05	„ „
Beef Extract	0.2	„ „
KH ₂ PO ₄	0.05	„ „
Bacto Agar	1.5	„ „
Distilled water, q.s.	100.0	„ „
pH adjustment with 50% KOH	7.0	„ „
Post sterilization pH	7.2	

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	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Growth	Good, hyaline; apigmentous to topaz ¹	Good, hyaline; apigmentous to topaz ¹
Aerial hyphae	Slight becoming moderate; white	Slight becoming moderate; white
Sporulation	None	None
Diffusible pigment	None	None
Reverse	Hyaline; topaz becoming oak brown	Hyaline; topaz becoming oak brown
	Strain T—143	Strain T—225
Growth	Good, hyaline; dark luggage tan ¹	Excellent
Aerial Hyphae	Sparse to fair; white becoming silver gray	Abundant to profuse; white becoming light fawn ¹ to beaver
Sporulation	Fair	Profuse
Diffusible pigment	Light amber	Yellow
Reverse	Hyaline; deep brown	Dark brown mahogany
	Strain A—377	
Growth	Good	
Aerial Hyphae	Slight to fair; white to light gray ¹	
Sporulation	Fair	
Diffusible pigment	Light yellow	
Reverse	Yellow to light orange-yellow	

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

2. Dextrin Czapek-Dox Agar

Dextrin	1.0	per cent
NaNO ₃	0.2	„ „
K ₂ HPO ₄	0.1	„ „
MgSO ₄ ·7H ₂ O	0.05	„ „
KCl	0.05	„ „
FeSO ₄ ·7H ₂ O	0.001	„ „
Bacto Agar	1.5	„ „
Distilled water, q.s.	100.0	„ „
Post sterilization pH	7.2	

	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Growth	Slight, thin, semi-translucent; white ¹	Slight, thin, semi-translucent; white ¹
Aerial Hyphae	Slight; white	None
Sporulation	None	None
Diffusible pigment	None	None
Reverse	White, translucent	White, translucent
	Strain T—143	Strain T—225
	Growth	Slight, thin, semi-opaque; white ¹
Aerial Hyphae	Slight; white	Slight, white
Sporulation	None	None
Diffusible pigment	None	None
Reverse	White; translucent	White; translucent
	Strain A—377	
	Growth	Good
Aerial Hyphae	Abundant, mouse gray ¹ to lead gray; water-white surface globules	
Sporulation	Profuse	
Diffusible Pigment	Trace; pale yellow	
Reverse	Apigmentous; pink trace	

¹ Colours according to the Color Harmony Manual, Third Edition, Container Corporation of America.

3. AP4 Cornsteep Agar

Cornsteep	0.4 per cent
Sucrose	1.0 „ „
MgSO ₄ ·7H ₂ O	0.025 „ „
KH ₂ PO ₄	0.2 „ „
(NH ₄) ₂ HPO ₄	0.2 „ „
Bacto Agar	2.0 „ „
Tap water, q.s.	100.0 „ „
Post sterilization pH	6.5

	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Growth	Moderate	Moderate
Aerial hyphae	Scarce becoming abundant; beaver ¹	Moderate; beaver ¹ to chocolate
Sporulation	Moderate to abundant	Moderate
Soluble pigment	Orange-brown	Orange-brown
Reverse	Light brown to chocolate brown	Chestnut brown to deep brown
	Strain T—143	Strain T—225
Growth	Abundant	Abundant
Aerial Hyphae	Abundant; fawn ¹ to beaver	Profuse; chocolate ¹
Sporulation	Moderate to abundant	Profuse
Soluble pigment	Light orange-brown	Light orange-brown
Reverse	Deep brown to dark brown	Dark rose brown to ebony brown
	Strain A—377	
Growth	Excellent	
Aerial Hyphae	Abundant; fawn ¹	
Sporulation	Profuse	
Soluble pigment	Light yellow to amber	
Reverse	Light tan	

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

4. AP6 Cornsteep Agar

Cornsteep	6 grams
Sucrose	10 grams
MgSO ₄ ·7H ₂ O	0.25 grams
KH ₂ PO ₄	2 grams
(NH ₄) ₂ HPO ₄	2 grams
Bacto Agar	20 grams
Tap Water, q.s.	1000 ml.
Post sterilization pH	6.5

	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Growth	Abundant	Abundant
Aerial hyphae	Moderate to abundant; fawn ¹ to mist brown	Moderate to abundant; beaver ¹ to fawn
Sporulation	Moderate to abundant	Profuse
Soluble pigment	Orange to orange-brown	Orange to orange-brown
Reverse	Chestnut brown to deep brown	Chestnut brown to deep brown
	Strain T—143	Strain T—225
Growth	Abundant	Abundant
Aerial Hyphae	Slight to moderate; beige ¹ to mist brown	Profuse; chocolate ¹
Sporulation	Fair	Profuse
Soluble pigment	Orange-brown	Orange-brown
Reverse	Chocolate brown	Ebony brown
	Strain A—377	
Growth	Excellent	
Aerial Hyphae	Moderate to abundant; fawn ¹	
Sporulation	Profuse	
Soluble pigment	Orange-brown	
Reverse	Orange to orange-yellow	

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

5. Q4 Cornsteep Agar

Cornsteep	9 grams
Sucrose	10 grams
MgSO ₄ ·7H ₂ O	0.25 grams
(NH ₄) ₂ HPO ₄	2 grams
Crude Agar	30 grams
Water, q.s.	1000 ml.
Post sterilization pH	6.5

	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Growth	Abundant	Profuse
Aerial hyphae	Abundant; fawn ¹ to beaver	Profuse; fawn ¹ to beaver
Sporulation	Abundant	Abundant
Soluble pigment	Deep orange-brown	Deep orange-brown
Reverse	Copper brown to deep brown	Copper brown to deep brown
	Strain T—143	Strain T—225
Growth	Profuse	Profuse
Aerial Hyphae	Profuse; beige ¹ to mist brown	Profuse; chocolate ¹
Sporulation	Profuse	Profuse
Soluble pigment	Deep orange-brown	Deep amber
Reverse	Deep brown to chocolate brown	Ebony brown
	Strain A—377	
Growth	Excellent; pale yellow ¹	
Aerial Hyphae	Profuse; dark brown	
Sporulation	Profuse	
Soluble pigment	Orange-brown	
Reverse	Orange to orange-yellow	

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

6. Other Agar Media

Medium	<i>Streptomyces aureofaciens</i>	
	Strain T—135	Strain T—140
Nutrient Agar	Poor to fair growth; white to pale yellow ¹ . No aerial hyphae. Reverse: white to pale yellow	Poor growth; white to pale yellow ¹ . No aerial hyphae. Reverse: white to pale yellow
Glucose Asparagine Meat Extract Agar	Good growth; hyaline, apigmentous to pale orange ¹ . No aerial hyphae. Reverse: apigmentous to pale orange	Good growth; hyaline, apigmentous to pale orange ¹ . Slight aerial hyphae formation; white. Reverse: apigmentous to pale orange
Waksman's Agar	Good growth, semi-opaque; white to pale orange ¹ . Aerial hyphae: sparse becoming abundant; white to fawn. Sporulation: sparse becoming abundant. Reverse: dark luggage tan to dark spice brown. Light amber soluble pigment	Good growth, semi-opaque; white to pale orange ¹ becoming orange with age. Aerial hyphae: sparse becoming abundant; white to beaver. Sporulation: sparse becoming profuse. Reverse: beige to copper brown. Trace of light amber soluble pigment
Purple Milk	Pale yellow ¹ growth collar. Partial peptonization, pH 7.1	Pale yellow growth collar ¹ . Partial peptonization, pH 7.0
Potato Slants	Profuse, moist, nodulated growth; chestnut brown ¹ to dark spice brown. Rust brown soluble pigment	Profuse, moist, nodulated growth; light spice brown ¹ . Barn red soluble pigment. Slight white aerial hyphae formation

¹Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

Medium	<i>Streptomyces aureofaciens</i>	
	Strain T—143	Strain T—225
Nutrient Agar	Poor growth: white to light orange-yellow ¹ . No aerial hyphae. Reverse: white to light orange-yellow	Poor to fair growth: white to golden brown ¹ . No aerial hyphae. Reverse: translucent, white to adobe brown. No soluble pigment
Glucose Asparagine Meat Extract Agar	Good growth: hyaline, pale yellow to orange ¹ . Aerial hyphae: slight, white. Reverse: hyaline; pale yellow to copper brown to deep brown	Abundant growth. Profuse aerial hyphae; beaver ¹ . Sporulation: profuse. Reverse: dark eggplant. Very light amber soluble pigment
Waksman's Agar	Good growth; light tan to topaz ¹ . Aerial hyphae: fair to abundant, beige brown to fawn. Sporulation: poor becoming profuse. Reverse: dark luggage tan to deep brown. Light amber to amber soluble pigment	Abundant growth. Abundant aerial hyphae; taupe brown ¹ to chocolate. Reverse: ebony. Light amber soluble pigment
Potato Slants	Profuse, moist, nodulated growth; beaver ¹ to light spice brown. Dense white aerial hyphae at isolated foci. Rust brown soluble pigment	Profuse, moist, smooth nodulated growth; orange rust ¹ to oak brown becoming chocolate with age. Orange brown soluble pigment
Purple Milk	Pale yellow ¹ growth collar. Little significant pH change or apparent peptonization, pH 6.7	Heavy pale yellow ¹ growth collar. Partial peptonization, pH 6.78. No soluble pigment

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

Medium	<i>Streptomyces aureofaciens</i>
	Strain A—377
Nutrient Agar	Good growth. No aerial hyphae. Pale yellow ¹ soluble pigment. Reverse: pale yellow
Glucose Asparagine Meat Extract Agar	Good growth. Aerial hyphae white becoming increasingly gray ¹ with increase in spore formation. Trace: yellow-orange soluble pigment. Reverse: light yellow to pink-orange
Waksman's Agar	Good growth. Aerial hyphae fair becoming abundant: white to taupe brown ¹ . Light yellow soluble pigment. Reverse: camel to adobe brown
Potato Slants	Profuse, moist, smooth, nodulated growth; light brown-yellow ¹ to beige to cedar. No soluble pigment
Purple Milk	Slight yellow ¹ to pale yellow growth collar. Little significant pH change or apparent peptonization in 14 days pH 6.45

¹ Colors according to the Color Harmony Manual, Third Edition, Container Corporation of America.

7. Microscopic Observations

Medium	<i>Streptomyces aureofaciens</i>			
	Strain T—135		Strain T—140	
	Mycelium	Spores	Mycelium	Spores
AP4 Corn-steep Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 0.8 to 1.5 μ .	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 0.8 to 1.5 μ .
Q4 Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.2 μ .	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.2 μ .
Waksman's Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.2 to 1.5 μ .	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.2 μ .
Medium	<i>Streptomyces aureofaciens</i>			
	Strain T—143		Strain T—225	
	Mycelium	Spores	Mycelium	Spores
Glycerol Asparagine Meat Extract Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.2 μ .	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.2 to 1.5 μ .
AP4 Corn-steep Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.2 μ .	Spheroidal to ovoidal. Diam. 0.8 to 1.5 μ .	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.2 μ .

Medium	<i>Streptomyces aureofaciens</i>	
	Strain A—377	
	Mycelium	Spores
Glycerol Asparagine Meat Extract Agar	Flexuous, continuous, branched. Diam. 0.8 to 1.0 μ .	Spheroidal to ovoidal. Diam. 1.0 to 1.5 μ .
AP4 Corn-steep Agar	Flexuous, continuous, branched. Diam. 1.0 to 1.2 μ .	Spheroidal to ovoidal. Diam. 1.2 to 1.5 μ .

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 nutrients and mineral substances. Suitable nutrient substances include starch, dextrose, cane sugar, glucose, molasses, soybean meal, milk solids, yeast, meat extracts, peptone, urea, cornsteep liquor, distillers' solubles and fish meal. The inorganic salts include such things as calcium carbonate, ammonium sulfate, ammonium chloride, and salts of the various trace elements such as manganese, cobalt, zinc, copper and iron.

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 chlortetracycline shown in Patent Specification No. 690,381.

The recovery of the tetracycline from the fermentation liquor is conventional and need not be described as numerous methods for recovering tetracycline from fermentation liquors have already been published.

The invention will be described in greater detail in conjunction with the following specific examples.

EXAMPLE 1

Inoculum Preparation of *S. aureofaciens* Strain S-2308

Spores of *S. aureofaciens* strain S-2308 were washed from a streaked agar slant with sterile distilled water to form a suspension containing $60-80 \times 10^6$ spores/ml. A 0.33-ml. portion of this suspension was used to inoculate an eight-inch shaker tube containing 8 ml. of a medium prepared according to the following formulation:

Sucrose	30	grams
Ammonium sulfate	2	grams
Calcium carbonate	7	grams
Cornsteep	16.5	ml.
Tap water, q.s.	1000	ml.

Prior to inoculation, the medium was sterilized by autoclaving for 20 minutes under a pressure of 15 pounds per square inch, during which the pH changed from 6.3 to 6.9. The inoculated shaker tube was then incubated for 24 hours at 28°C. on a reciprocating shaker operating at 116 oscillations per minute. Inocula of the other novel mutant strains of the present invention are prepared in an identical fashion.

EXAMPLE 2

Chloramine-T Test for Tetracycline-Chlortetracycline Differentiation

Stock solution A was prepared by dissolving 100 grams of Chloramine-T in 900 ml. of

0.1M Na_3PO_4 (38 grams of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ per 1,000 ml. of distilled water). A working solution B was prepared by mixing 180 ml. of stock solution A with 820 ml. of an aqueous solution of ethylenediaminetetraacetic acid sodium salt (3 grams per 1,000 ml. of distilled water). In performing the actual test, 0.5 ml. of the fermentation mash to be tested is mixed in a test tube with 20 ml. of working solution B. The tube is shaken intermittently for a period varying from 30 to 60 minutes. At the termination of this period of shaking, the test tube is examined for color. Mash containing high concentrations of tetracycline turn wine red (a positive reaction), while mash containing high concentrations of chlortetracycline develop a deep amber color. This Chloramine-T test is a further means of differentiating the novel mutant strains of the present invention from previously known strains of *S. aureofaciens*.

EXAMPLE 3

Silver Nitrate Test for Chloride Ion

To a 10-ml. portion of whole fermentation mash, there is added 10 ml. of 0.2M HNO_3 and 0.5 gram of a chloride free, diatomaceous earth filter aid. The combined materials are mixed thoroughly and then filtered. The filtrate thus obtained is re-filtered as often as necessary to obtain a clear filtrate. A 2-ml. portion of 0.01M AgNO_3 is next added to a 2-ml. aliquot of the clear filtrate in a test tube. The mixture is observed for a period of 1 minute. The formation of AgCl , evidenced either as turbidity or as a white precipitate, indicates the presence of free unbound chloride ion in the whole mash samples.

EXAMPLE 4

Tetracycline Production in Shaker Flask Fermentations

A. Full Chloride Medium

A full chloride fermentation medium for tetracycline production was prepared as follows:

$(\text{NH}_4)_2\text{SO}_4$	5	grams	105
CaCO_3	7-9	grams	
NH_4Cl	1.5	grams	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.4-0.6	grams	
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.05	grams	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1	grams	110
Cornsteep liquor	20-30	grams	
Cottonseed meal	0-2	grams	
Starch	55	grams	
Lard oil	20	ml.	
Tap water, q.s.	1000	ml.	115

B. Chloride-Limited Medium

A chloride-limited fermentation medium for tetracycline production was prepared as follows:

	(NH ₄) ₂ SO ₄	6.83 grams	placed in ten 250-ml. Erlenmeyer flasks, sealed in each case with a cotton plug, and sterilized in an autoclave for 20 minutes at a pressure of 15 pounds per square inch. After sterilization, each pair of 25-ml. aliquots were variously inoculated with 1.0 ml. of inocula prepared as in Example 1. The fermentation was carried out at 28°C. from 0 to 18 hours and then at 24°C. from 18 to 142 hours, at pH 6—7, on a rotary shaker operating at 180 revolutions per minute. Under these conditions, the results set forth in Table I below were obtained.	
	CaCO ₃	7—9 grams		
	FeSO ₄ ·7H ₂ O	0.4—0.6 grams		
	MnSO ₄ ·4H ₂ O	0.05 grams		15
5	ZnSO ₄ ·7H ₂ O	0.1 grams		
	Cornsteep liquor	20—30 grams		
	Cottonseed meal	0—2 grams		
	Starch	55 grams		
	Lard oil	20 ml.		20
10	Tap water, q.s.	1000 ml.		

Five, 25-ml. aliquots of both media were

TABLE I

Strain No.	Medium	Assays		Chloramine-T Test ¹	Silver Nitrate Test ²
		Chlortetracycline mcg./ml.	Tetracycline mcg./ml.		
T-135	A	<50 ³	2460	+	+
	B	<50	2510	+	+
T-140	A	<50	2210	+	+
	B	<50	2200	+	+
T-143	A	<50	3820	+	+
	B	<50	3470	+	+
T-225	A	<50	3890	+	+
	B	<50	3990	+	+
S-2308	A	<50	6580	+	+
	B	<50	6520	+	+

¹ Chloramine-T Test as per Example 2.

² Silver Nitrate Test as per Example 3.

³ The chlortetracycline assay method is not sensitive to less than 50 mcg./ml. However, the cultures showed no chlortetracycline by paper strip chromatography.

EXAMPLE 5

Tetracycline Production in Shaker Flask Fermentations Effect of Ammonium Chloride Concentration

25 Fermentation medium A of Example 4 was

prepared with levels of 0.0, 0.1, 0.2, 0.5, and 1.5 gL. of NH₄Cl and a constant 5.0 gL. level of (NH₄)₂SO₄. Fermentation was carried out as described in Example 4, with the results set forth in Table II below.

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TABLE II

Strain No.	NH ₄ Cl Level g./L.	Assays		Silver Nitrate Test ¹
		Chlortetracycline mcg./ml.	Tetracycline mcg./ml.	
T-225	0.0	<50 ²	3430	±
	0.1	<50	2770	+
	0.2	<50	2900	+
	0.5	<50	2940	+
	1.5	<50	3390	+
S-2308	0.0	<50	5760	±
	0.1	<50	4580	+
	0.2	<50	5080	+
	0.5	<50	5440	+
	1.5	<50	6080	+

¹ Silver Nitrate Test as per Example 3.

² The chlortetracycline assay method is not sensitive to less than 50 mcg./ml. However, the cultures showed no chlortetracycline by paper strip chromatography.

EXAMPLE 6 Response of Tetracycline-Producing Strains to Ultraviolet Light

5 The novel mutant strains of the present invention may be readily differentiated from previously known chlortetracycline-producing strains of *S. aureofaciens* (as well as from established strains producing primarily other known tetracyclines such as 6-demethyl-tetracycline, 7-chloro-6-demethyltetracycline, and 10 7-chloro-5a(11a)-dehydrotetracycline) by comparison of the fluorescence produced under ultraviolet light of longer wave length, primarily at 3660Å. A medium which lends 15 itself particularly well to growth and fluorescence observations is Q_s sporulation agar, prepared as follows:

20	MgSO ₄ ·7H ₂ O	0.25	grams
	KH ₂ PO ₄	0.4	grams
	(NH ₄) ₂ HPO ₄	0.4	grams
	Sucrose	15	grams
	Staley's Nutrient #22	9	grams
25	Crude agar	30	grams
	Tap water, q.s.	1000	ml.

The pH of this medium was adjusted to 6.6 by means of a 50% solution of potassium hydroxide, then 17.5-ml. aliquots of the medium were dispensed into 25 × 150 mm. glass culture tubes and the tubes closed with cotton plugs. The culture tubes containing the medium were sterilized at 120°C. for 20 minutes, allowed to solidify in slanted position to provide maximum surface area, and refrigerated until needed. Spores of *S. aureofaciens* strain A-377 were washed from an agar slant with sterile distilled water to form a suspension containing 60—80 × 10⁸ spores/ml. A 0.1 ml. portion of this suspension was used to inoculate a portion of the Q_s sporulation agar slants. After inoculation, the Q_s agar slants were incubated at 26.5°—27.5°C. Sporulation was generally complete after 14 days. The same procedure was followed in the case of all the other microorganisms tested which are listed in Table III below.

When observed under ultraviolet light (3660Å) during the 0—14 days incubation period, vegetative growth of tetracycline-pro-

15 flasks, sealed and sterilized at a pressure of 15 lb. per square inch for 15 minutes. After sterilization, the flasks were cooled to 18 hours at 12 hours, at 20 operating at these conditions below

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- ducing strains became brilliant golden-orange while the butt became increasingly brilliant orange-yellow to yellow. During this phase of growth, chlortetracycline-producing strains may also exhibit a slight amount of fluorescence of a similar type. Simultaneously, the upper portion of the agar slant, where the medium thickness is minimal, fluoresces green-yellow for tetracycline-producing strains and golden-yellow for chlortetracycline-producing strains. Further, as the slants mature, the tetracycline-producing strains retain the yellow fluorescent response in the agar butt to ultraviolet light at 3660Å whereas the chlortetracycline-producing strains become pale blue.
- A fermentation medium for tetracycline production was prepared according to the following formulation:
- | | | |
|---|-------------|----|
| (NH ₄) ₂ SO ₄ | 5.58 grams | |
| CaCO ₃ | 7—10 grams | 20 |
| NH ₄ Cl | 1.66 grams | |
| MnSO ₄ (70% tech. grade) | 0.08 grams | |
| Cornsteep liquor | 20—30 grams | 5 |
| Starch | 47.5 grams | |
| Corn flour | 14.5 grams | 25 |
| Lard oil | 20—32 ml. | |
| Tap water, q.s. | 1000 ml. | 10 |

The fermentation procedures were carried out according to the directions given in Example 4, using the microorganisms listed in Table III below, with the results set forth in Table III below.

TABLE III

<i>S. aureofaciens</i> Strain No.	Assays		Q ₈ Agar Slant Fluorescence at 3660Å
	Chlortetracycline mcg./ml.	Tetracycline mcg./ml.	
A-377	50	0	Pale blue
T-135	<50 ¹	620	Brilliant yellow
T-140	<50	730	Brilliant yellow
T-143	<50	5670	Brilliant yellow
T-225	<50	4140	Brilliant yellow
S-2308	<50	6640	Brilliant yellow
S-2589	<50	8770	Brilliant yellow

¹ The chlortetracycline assay method is not sensitive to less than 50 mcg./ml. However, the cultures showed no chlortetracycline by paper strip chromatography.

EXAMPLE 7

- Characterization of Tetracycline - Producing Strains of *S. aureofaciens* by Spectrophotometric Reflectance of the Whole Harvest Mash in Cornsteep Medium
- Spores of *S. aureofaciens* strain T-135 were washed from an agar slant with sterile distilled water to form a suspension containing 60—80 × 10⁶ spores/ml. A 0.33 ml. portion of this suspension was used to inoculate an eight-inch shaker tube containing 8 ml. of a medium prepared according to the formulation disclosed in Example 1. Subsequent preparation of inocula of the novel mutant strains of *S. aureofaciens* employed followed the method of Example 1.
- A fermentation medium of the following composition was prepared:
- | | | |
|---|-----------------|----|
| (NH ₄) ₂ SO ₄ | 5.5—7.0 grams | |
| CaCO ₃ | 7—10 grams | |
| NH ₄ Cl | 1.5—2.0 grams | |
| MnSO ₄ (70% tech. grade) | 0.08—0.15 grams | 55 |
| Cornsteep liquor | 20—30 grams | |
| Cottonseed meal | 0—5 grams | 35 |
| Starch | 45—55 grams | |
| Corn flour | 14.5 grams | |
| Lard oil | 20—35 ml. | 60 |
| Tap water, q.s. | 1000 ml. | |
- After sterilization of this medium in an autoclave for 20 minutes at a pressure of 15 pounds per square inch, 25 ml. aliquots in 250-ml. Erlenmeyer flasks were inoculated with 1.0 ml. portions of the inoculum prepared as described above. The fermentation was carried out at 28°C. from 0 to 18 hours

8 grams
10 grams 20
6 grams
8 grams
10 grams
5 grams
5 grams 25
32 ml.
10 ml.

and then at 24°C. from 18 to 142 hours, on a rotary shaker operating at 180 revolutions per minute. The same procedure was followed in the case of the other microorganisms employed which are listed in Table IV below. Assays for tetracycline were performed according to the standard Hiscox method while chlortetracycline assays were obtained by a fluorimetric technique. The results are set forth in Table IV below. Reflectance curves were obtained for the whole harvest mash by the use of a General Electric Co. type

spectrophotometer, and are reproduced in Figures 1 through 6 of the drawings. The samples were run in a one-centimeter glass cell placed in the reflectance position at the back of the sphere; magnesium carbonate was used as the reference. The specular reflectance of the cell was included and a range of 400 mμ to 700 mμ was scanned. The values of ΔR at 420 mμ and at 430 mμ were determined and are set forth in Table IV below.

TABLE IV

<i>S. aureofaciens</i> Strain No.	Assays		ΔR** at	
	Chlortetracycline % by weight	Tetracycline % by weight	420 mμ	430 mμ
T-135	None*	100	27	18
T-140	None*	100	32	18
T-143	None*	100	59	-9
T-225	None*	100	72	3
S-2308	None*	100	63	12
S-2589	None*	100	94	36

* The cultures showed no chlortetracycline by paper strip chromatography.

** The scales of reflectance and wavelength in Figures 1 through 8 are not linear with the quantities plotted. This is due to the particular plotting mechanism of the spectrophotometer used and in no way effects the calculation of the ΔR values.

EXAMPLE 8

Characterization of a Tetracycline-Producing Strain of *S. aureofaciens* by Spectrophotometric Reflectance of the Whole Harvest Mash in Synthetic Medium

30 Spores of *S. aureofaciens* strain S-2589 were washed from an agar slant with sterile distilled water to form a suspension containing 60-80 × 10⁶ spores per ml. A 0.33 ml. portion of this suspension was used to inoculate an eight-inch shaker tube containing 8 ml. of a medium prepared according to the formulation disclosed in Example 1.

A fermentation medium of the following composition was prepared:

40	CaCO ₃	10.0	grams
	(NH ₄) ₂ SO ₄	8.0	grams
	NH ₄ Cl	1.5	grams
	Cornstarch	55.0	grams
	MgCl ₂ ·6H ₂ O	2.0	grams
45	FeSO ₄ ·7H ₂ O	0.06	grams
	ZnSO ₄ ·7H ₂ O	0.10	grams
	MnSO ₄ ·H ₂ O	0.05	grams

CoCl ₂ ·6H ₂ O	0.005	grams
KCl	1.28	grams
H ₃ PO ₄	0.36	grams
L-Methionine	0.60	grams
L-Histidine	0.80	grams
Distilled H ₂ O, q.s.	1000	ml.

To this medium was added a lard oil-mineral oil combination, consisting of 3 parts of lard oil to 1 part of U.S.P. grade mineral oil, at the rate of 0.63 ml. per 25 ml. of medium. After sterilization of this medium in an autoclave for 20 minutes at a pressure of 15 pounds per square inch, 25 ml. aliquots in 250-ml. Erlenmeyer flasks were inoculated with 1.0 ml. portions of the inoculum prepared as described above. The fermentation was carried out at 28°C. from 0 to 18 hours and then at 24°C. from 18 to 142 hours, on a rotary shaker operating at 180 revolutions per minute.

The whole harvest mash assayed 8060 micrograms of tetracycline per ml. according to the standard Hiscox assay method whereas

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micrograms of tetracycline per ml. according to the standard Hiscox assay method whereas the culture showed no chlortetracycline by paper strip chromatography. A reflectance curve was obtained for the whole harvest mash by the use of a General Electric Co. type spectrophotometer, and is reproduced in Figure 8 of the drawings. The sample was run in a one-centimeter glass cell placed in the reflectance position at the back of the sphere; magnesium carbonate was used as the reference. The specular reflectance of the cell was included and a range of 400 $m\mu$ to 700 $m\mu$ was scanned. The value of ΔR at 420 $m\mu$ was 129 whereas the value of ΔR at 430 $m\mu$ was 66.

WHAT WE CLAIM IS:—

1. The process of producing tetracycline which comprises cultivating a strain of *Streptomyces aureofaciens* which produces tetracycline to the substantial exclusion of chlortetracycline under submerged aerobic conditions in an aqueous nutrient medium containing assimilable sources of carbohydrate, nitrogen, and inorganic salts until substantial quantities of tetracycline are produced, said tetracycline-producing strain of *Streptomyces aureofaciens* being also characterized by its ability to impart to the whole harvest mash a color characterized by a reflectance curve, when plotted linearly, wherein the vertical distance between the reflectance curve and a straight line drawn through the reflectance curve intercepts at 400 $m\mu$ and 550 $m\mu$ is greater at 420 $m\mu$ than at 430 $m\mu$.

2. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,908.

3. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,909.

4. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,910.

5. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,911.

6. A process of producing tetracycline substantially as hereinbefore described with reference to any of the accompanying Examples.

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the culture showed no chlortetracycline by paper strip chromatography. A reflectance curve was obtained for the whole harvest mash by the use of a General Electric Co. type spectrophotometer, and is reproduced in Figure 7 of the drawings. The sample was run in a one-centimeter glass cell placed in the reflectance position at the back of the sphere; magnesium carbonate was used as the reference. The specular reflectance of the cell was included and a range of 400 m μ to 700 m μ was scanned. The value of ΔR at 420 m μ was 118 whereas the value of ΔR at 430 m μ was 87.

EXAMPLE 9

Characterization of a Tetracycline-Producing Strain of *S. aureofaciens* by Spectrophotometric Reflectance of the Whole Harvest Mash in Dried Whey Medium

Spores of *S. aureofaciens* strain S—2589 were washed from an agar slant with sterile distilled water to form a suspension containing 60—80 $\times 10^6$ spores per ml. A 0.33 ml. portion of this suspension was used to inoculate an eight-inch shaker tube containing 8 ml. of a medium prepared according to the formulation disclosed in Example 1.

A fermentation medium of the following composition was prepared:

Dried Whey	15.5	grams
Cornflour	42.7	grams
Cornstarch	30.8	grams
CaCO ₃	11.5	grams
(NH ₄) ₂ SO ₄	8.5	grams
NH ₄ Cl	2.0	grams
MnSO ₄ (70% Technical Grade)	0.140	grams
CoCl ₂ ·6H ₂ O	0.005	grams
Lactic Acid	0.650	grams
Tap Water, q.s.	1000	ml.

To this medium was added 0.75 ml. of lard oil per 25 ml. of medium. After sterilization of this medium in an autoclave for 20 minutes at a pressure of 15 pounds per square inch, 25 ml. aliquots in 250 ml.-Erlenmeyer flasks were inoculated with 1.0 ml. portions of the inoculum prepared as described above. The fermentation was carried out at 28°C. from 0 to 18 hours and then at 24°C. from 18 to 142 hours, on a rotary shaker operating at 180 revolutions per minute.

The whole harvest mash assayed 6070

micrograms of tetracycline per ml. according to the standard Hiscox assay method whereas the culture showed no chlortetracycline by paper strip chromatography. A reflectance curve was obtained for the whole harvest mash by the use of a General Electric Co. type spectrophotometer, and is reproduced in Figure 8 of the drawings. The sample was run in a one-centimeter glass cell placed in the reflectance position at the back of the sphere; magnesium carbonate was used as the reference. The specular reflectance of the cell was included and a range of 400 m μ to 700 m μ was scanned. The value of ΔR at 420 m μ was 129 whereas the value of ΔR at 430 m μ was 66.

WHAT WE CLAIM IS:—

1. The process of producing tetracycline which comprises cultivating a strain of *Streptomyces aureofaciens* which produces tetracycline to the substantial exclusion of chlortetracycline under submerged aerobic conditions in an aqueous nutrient medium containing assimilable sources of carbohydrate, nitrogen, and inorganic salts until substantial quantities of tetracycline are produced, said tetracycline-producing strain of *Streptomyces aureofaciens* being also characterized by its ability to impart to the whole harvest mash a color characterized by a reflectance curve, when plotted linearly, wherein the vertical distance between the reflectance curve and a straight line drawn through the reflectance curve intercepts at 400 m μ and 550 m μ is greater at 420 m μ than at 430 m μ .

2. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,908.

3. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,909.

4. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,910.

5. A process as claimed in claim 1, wherein the strain of *Streptomyces aureofaciens* is ATCC No. 13,911.

6. A process of producing tetracycline substantially as hereinbefore described with reference to any of the accompanying Examples.

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