The Complete Patents of S.A.Waksman

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ACTINOMYCIN

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9 Claims. (Cl. 260—236.5)

This invention relates to new and useful improvements in bio-chemical substances and more particularly in antimicrobial substances.

In accordance with our invention antimicrobial substances are prepared from cultures of *Actinomyces antibioticus*. *Actinomyces antibioticus* is a micro-organism which occurs inter alia in soils. Specifically, it is a fungus of defined morphology characterized by spore-bearing hyphae produced in the form of straight aerial mycelium; the sporophores are arranged in clusters, no spirals being formed; the spores are nearly spherical to somewhat elliptical. *Actinomyces antibioticus* is fully described and characterized in our publication, Journal of Bacteriology, 42, 231–249, (August, 1941).

The following method was used by us for obtaining cultures of micro-organisms capable of antagonizing various specific bacteria. In accordance with this method suspensions of cultures of the specific bacteria are mixed with an aqueous agar solution and the mixture added to a suitable soil containing the antagonizing micro-organisms. Upon one to 10 days' incubation, colonies of the antagonists will form which may be transferred to fresh bacterial nutrient media and later isolated as pure cultures in accordance with conventional methods.

By means of this method, we have isolated an organism which we have described as *Actinomyces antibioticus*. The method of isolation is illustrated in Example IV.

In the practical application of our invention, a suitable culture of *Actinomyces antibioticus* is extracted with a suitable solvent. Suitable solvents are, for instance, ethyl ether, ethyl alcohol, carbon bisulphide, acetone, and chloroform. Ethyl ether is the preferred solvent. Upon evaporation of the solvent, a solid remains. This substance, hereinafter designated as "actinomycin" possesses marked bacteriostatic and bactericidal properties against a large variety of bacteria, antinomycetes and fungi. In most cases, gram-positive bacteria are much more sensitive against the substance than gram-negative organisms. The antinomycin is completely removable by charcoal from liquid media but is only partly removed therefrom by a Seitz filter. The substance is substantially heat resistant at 100° C. for thirty minutes.

We have found that two different antimicrobial substances may be obtained from the actinomycin. These substances, which will be hereinafter referred to respectively as "actinomycin A" and "actinomycin B" may be obtained by selective extraction with suitable solvents from the actinomycin. Thus, for instance, the actinomycin B being substantially soluble in petroleum ether may be extracted as a solution in this solvent in which the actinomycin A is substantially insoluble. Alternatively, instead of extracting the actinomycin A or actinomycin B from actinomycin, they may be obtained by direct selective solvent extraction from the original cultures.

Actinomycin A is a highly pigmented (red) product substantially soluble in either, ethyl alcohol, water, carbon bisulphide, acetone, chloroform and substantially insoluble in petroleum ether. Its aqueous solutions are of a yellow to orange-red color, depending on concentration. The actinomycin B is substantially soluble in ether, carbon bisulphide, acetone, chloroform, petroleum ether, but difficultly soluble in alcohol, and substantially insoluble in water.

The actinomycin A is highly bacteriostatic, gram-positive bacteria being inhibited by dilutions as low as 1:100,000,000; the A compound is also effective, though not in such degree of sensitivity against gram-negative bacteria which are inhibited by concentration of, for example, 1:5,000 to 1:100,000. The actinomycin A also possesses bactericidal activity, through not as pronounced as its bacteriostatic characteristics. The actinomycin B is markedly bactericidal and to a lesser extent bacteriostatic.

Actinomycin A crystallizes from suitable solutions, such as acetone-benzene or acetone-ether mixtures or ethyl-acetate, as vermillion red platelets which melt at 250° C. with slow decomposition. The actinomycin B crystallizes substantially colorless. The A component exhibits characteristic absorption in the visible and ultraviolet regions. It shows strong absorption at 450 mμ and between 230 and 250 mμ.

The following examples illustrate methods of obtaining actinomycin as well as its A and B components, and it is to be understood that the same is furnished by way of illustration and not of limitation.

Example I

An incubated culture of *Actinomyces antibioticus* was prepared using a medium consisting of 1% tryptone-peptone, 0.5% starch, 0.2% K_2HPO_4, 0.2% NaCl and 0.25% agar in distilled water, grown at a temperature of approximately 25° to 35° C., the incubation being complete after six to ten days. Fifty liters of this incubated culture are extracted approximately six times with ether, using 20 liters of ether for each extraction.

The final extract is faintly pale yellow in color, whereas the previous extracts are orange. The combined ether extracts are concentrated to dryness and about 3 gms. of a reddish-brown residue is obtained. The residue is stirred with approximately 400 cc. of petroleum ether for two to three hours, the solvent decanted and the residue treated again with approximately 400 cc. of petroleum ether. A pale yellow oil constituting crude actinomycin B is recovered by evaporation from the petroleum ether.

The dark petroleum ether insoluble residue is dissolved in one liter of benzene with gentle heating. Usually a small amount of black amorphous material remains undissolved and is filtered off. The benzene solution is permitted to drop through a chromatographic tower (60 x 5 cm.) packed with aluminum oxide (according to Brockman). The pigment is readily adsorbed. The column is washed with about one liter of benzene during which operation very little migration of the color bands occurs.

The column is then washed with benzene acetone solution (15:85) whereby a chromatogram develops. By continued washing, light yellow colored pigments pass out of the column. When the main band (orange-red) reaches the lower end of the column, a solution of 30–70 acetone benzene is passed through the column. The latter solvent elutes the pigment and when the eluate is very pale in color, washing is discontinued.

The eluate is concentrated to dryness under reduced pressure, taken up in 25 cc. of hot acetone, filtered, and diluted with ether. The pigment which crystallizes as red-brick colored platelets is essentially pure but may be recrystallized if desired from hot ethyl acetate. An analysis of the product showed C=59.01; H=6.81; N=13.38.

In accordance with our invention, a variety of derivatives of actinomycin A or actinomycin B may be prepared. Thus, for instance, the following Example II recites, by way of illustration, the preparation of the di-acetate of di-hydro actinomycin A.

Example II

25 mgs. of actinomycin A are dissolved in 1 cc. acetic anhydride and to this is added 3 drops of pyridine (or 3 drops triethylamine) and 100 mg. of zinc dust. On stirring, the red mixture becomes pale yellow in color. After one hour at room temperature, the mixture is filtered from the zinc dust and the filtrate is concentrated to dryness under reduced temperature. The pale yellow residue is dissolved in chloroform and washed with water, with aqueous sodium bicarbonate, and finally with dilute hydrochloric acid. The chloroform solution is concentrated to dryness under reduced pressure and the residue is taken up in 1 cc. acetone. On adding two volumes of ether, the product separates as a pale yellow solid melting at approximately 241° C. Approximate analysis: C 58.32; H 6.36; N 12.48.

Example III

25 mg. of actinomycin A in 1 cc. of pyridine and 0.5 cc. of acetic anhydride are allowed to stand ten hours at room temperature, and then for two hours at 80° C. The red solution upon concentration to dryness under reduced pressure is dissolved in chloroform and the latter solution washed in the order mentioned with dilute hydrochloric acid and aqueous sodium bicarbonate. The chloroform solution is concentrated to dryness and the resulting product recrystallized from ethyl acetate. The same constitutes red-brick prisms melting at approximately 250° C. Approximate analysis: C 58.27; H 6.56; N 12.46.

The method of growing cultures of antagonists to specific bacteria outlined above can be demonstrated by way of the following example.

Example IV

Agar is washed in distilled water and dissolved so as to yield 1.5% concentration. Two gms. of K_2HPO_4 are added per liter. Ten milliliter portions of this agar are distributed in test tubes and sterilized. A washed suspension of *Escherichia coli*, or some other suitable bacterium, obtained by cultivation on solid or in liquid nutrient media is prepared and added to the washed agar which has previously been melted and placed in a water bath at 42° C. One milliliter portions of the still viable bacterial suspension are added to the agar tubes and thoroughly mixed with the agar.

A suitable soil is suspended in sterile tap water using a series of dilutions from 1:10 to 1:10,000. One milliliter portions of these dilutions are placed in sterile Petri dishes and the bacterial agar, prepared as above, is added. The plates are well shaken to distribute the soil suspension thoroughly and are incubated at 28° or 37° C. After one to ten days' incubation of the plates, colonies of the antagonists will appear being surrounded by clear zones. These colonies are transferred to fresh bacterial agar plates and are later isolated in pure culture by the use of convenient media. *Actinomyces antibioticus* is one of the antagonists isolated by this method.

We have further discovered a method and culture medium for increasing the production of actinomycin or its components by the cultures of *Actinomyces antibioticus*. The culture medium in accordance with this embodiment essentially comprises 1 to 10 and preferably 5 parts by weight of starch, 1 to 20 and preferably 10 parts by weight of a suitable nitrogen containing material of an organic or an inorganic nature, a small amount of buffering agent sufficient to maintain the pH of the culture substantially between pH 5.6 and pH 8 and preferably at pH7, 1 to 5 and preferably 2 parts by weight of a mineral salt, preferably sodium chloride, and 2 to 20, preferably 15 parts by weight agar, and 1000 parts by weight of water. The buffering agent may be any one of the known buffering agents and preferably K_2HPO_4; amounts of 1 to 3 and preferably 2 parts by weight of the buffering agent will yield the desired pH or pH range. As a source of nitrogen a variety of substances may be used including organic and inorganic nitrogen containing materials such as asparagine, alanine, phenylalanine, peptone, tryptone and sodium nitrate. We prefer, however, for best results, to use tryptone as the nitrogen yielding substance. Representative compositions of preferred formulae are, for instance, the following:

1.5% agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5 to 2% starch, 0.1 to 0.2% asparagine;

1.5% agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5% starch, 1% peptone;

1.5% agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5% starch, 1.0% tryptone;

0.25% agar, 0.2% K_2HPO_4, 0.2% NaCl, 1.5% starch, 0.2% $NaNO_3$.

Cultures are preferably incubated at 25 to 35° C., substantially maintaining the pH at the indicated point or range. Growth proceeds rapidly and is accompanied by the formation of a soluble dark-brown pigment on organic media. It be-

comes rapidly covered with a white mycelium having a faint yellowish-green tinge. After 6 to 10 days' incubation, growth and production of actinomycin are usually complete.

We have found that the actinomycin in accordance with our invention possesses a selective bacteriostatic action with respect to different varieties of bacteria and that the markedly greater sensitivity of some bacteria to actinomycin than of others may be utilized for the purpose of isolating specific organisms from a mixed population. Actinomycin has a bacteriostatic effect in relatively small concentrations on gram-positive bacteria, while it requires much higher concentrations to be effective bacteriostatically with respect to gram-negative bacteria. Thus, for instance, when adding 0.1 mg. actinomycin to 10 cc. agar and incubating thereon a substrate such as milk or sewage containing both gram-positive and gram-negative bacteria, the growth of the gram-positive bacteria will be inhibited by the actinomycin. Generally, concentrations of 0.1 to 1.0 mg. per 10 cc. agar permit the selective growth of various bacteria by reason of the differentiation in sensitivity of varying amounts of the active actinomycin with respect to specific bacterial growth.

Both the actinomycin A and actinomycin B possess a relatively high molecular weight. Actinomycin A has the general empirical composition of 58.7 to 59.3% C, 6.5 to 7.5% H, 13.05 to 13.65% N, and a molecular weight in excess of 700.

Modifications may be made in carrying out the present invention without departing from the spirit and scope thereof, and we are to be limited only by the appended claims.

We claim:

1. An antimicrobial substance predominantly comprising a substantially ether-soluble concentrate, extracted from an *Actinomyces antibioticus* culture, and composed of a high molecular, substantially water-soluble, petroleum ether insoluble component and a substantially high molecular, water-insoluble, petroleum ether-soluble component.

2. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with ethyl ether to thereby extract actinomycin and recovering actinomycin from said ether extract.

3. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with petroleum ether to thereby extract actinomycin B.

4. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with a solvent comprising at least one member selected from the group consisting of ethyl ether, ethyl alcohol, carbon bisulphide, acetone, and separating by selective solvent extraction actinomycin A and actinomycin B.

5. Crude actinomycin A, an antimicrobial substance predominantly comprising a high molecular, substantially ether- and water-soluble, petroleum ether-insoluble concentrate, extracted from an *Actinomyces antibioticus* culture, and recovered as a dark reddish solid exhibiting strong absorption at 450 mμ and between 230 and 250 mμ.

6. Crude actinomycin B, an antimicrobial substance predominantly comprising a high molecular, substantially water-insoluble, ether- and petroleum ether-soluble concentrate, extracted from an *Actinomyces antibioticus* culture, and recovered as a pale yellow oil.

7. The process that comprises treating a culture of *Actinomyces antibioticus* with an organic solvent to thereby extract from said culture a concentrate comprising at least one antimicrobial substance of the class consisting of actinomycin A and actinomycin B.

8. The process that comprises treating a culture of *Actinomyces antibioticus* with a solvent comprising at least one member of the class consisting of ethyl ether, ethyl alcohol, ethyl acetate, carbon disulfide, acetone and chloroform to thereby extract actinomycin from said culture.

9. As a new product, pure actinomycin A, which is identical with the actinomycin A extracted from cultures of *Actinomyces antibioticus*, which is a brick-red colored, crystalline, thermostable solid melting at approximately 250° C., and exhibiting strong absorption at 450 mμ and between 230 and 250 mμ, and which has the approximate composition of 59.01% C, 6.81% H, 13.38% N, and a molecular weight in excess of 700.

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CERTIFICATE OF CORRECTION.

Patent No. 2,378,876. June 19, 1945.

SELMAN A. WAKSMAN, ET AL.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction as follows: Page 1, first column, line 42, for "antinomycetes" read --actinomycetes--; line 46, for "antinomycin" read --actinomycin--; and second column, line 11, for "in either" read --in ether--; and that the said Letters Patent should be read with this correction therein that the same may conform to the record of the case in the Patent Office.

Signed and sealed this 20th day of November, A. D. 1945.

Leslie Frazer

(Seal) First Assistant Commissioner of Patents.

comes rapidly covered with a white mycelium having a faint yellowish-green tinge. After 6 to 10 days' incubation, growth and production of actinomycin are usually complete.

We have found that the actinomycin in accordance with our invention possesses a selective bacteriostatic action with respect to different varieties of bacteria and that the markedly greater sensitivity of some bacteria to actinomycin than of others may be utilized for the purpose of isolating specific organisms from a mixed population. Actinomycin has a bacteriostatic effect in relatively small concentrations on gram-positive bacteria, while it requires much higher concentrations to be effective bacteriostatically with respect to gram-negative bacteria. Thus, for instance, when adding 0.1 mg. actinomycin to 10 cc. agar and incubating thereon a substrate such as milk or sewage containing both gram-positive and gram-negative bacteria, the growth of the gram-positive bacteria will be inhibited by the actinomycin. Generally, concentrations of 0.1 to 1.0 mg. per 10 cc. agar permit the selective growth of various bacteria by reason of the differentiation in sensitivity of varying amounts of the active actinomycin with respect to specific bacterial growth.

Both the actinomycin A and actinomycin B possess a relatively high molecular weight. Actinomycin A has the general empirical composition of 58.7 to 59.3% C, 6.5 to 7.5% H, 13.05 to 13.65% N, and a molecular weight in excess of 700.

Modifications may be made in carrying out the present invention without departing from the spirit and scope thereof, and we are to be limited only by the appended claims.

We claim:

1. An antimicrobial substance predominantly comprising a substantially ether-soluble concentrate, extracted from an *Actinomyces antibioticus* culture, and composed of a high molecular, substantially water-soluble, petroleum ether insoluble component and a substantially high molecular, water-insoluble, petroleum ether-soluble component.

2. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with ethyl ether to thereby extract actinomycin and recovering actinomycin from said ether extract.

3. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with petroleum ether to thereby extract actinomycin B.

4. In the method of producing an antimicrobial substance the steps comprising treating a culture of *Actinomyces antibioticus* with a solvent comprising at least one member selected from the group consisting of ethyl ether, ethyl alcohol, carbon bisulphide, acetone, and separating by selective solvent extraction actinomycin A and actinomycin B.

5. Crude actinomycin A, an antimicrobial substance predominantly comprising a high molecular, substantially ether- and water-soluble, petroleum ether-insoluble concentrate, extracted from an *Actinomyces antibioticus* culture, and recovered as a dark reddish solid exhibiting strong absorption at 450 mμ and between 230 and 250 mμ.

6. Crude actinomycin B, an antimicrobial substance predominantly comprising a high molecular, substantially water-insoluble, ether- and petroleum ether-soluble concentrate, extracted from an *Actinomyces antibioticus* culture, and recovered as a pale yellow oil.

7. The process that comprises treating a culture of *Actinomyces antibioticus* with an organic solvent to thereby extract from said culture a concentrate comprising at least one antimicrobial substance of the class consisting of actinomycin A and actinomycin B.

8. The process that comprises treating a culture of *Actinomyces antibioticus* with a solvent comprising at least one member of the class consisting of ethyl ether, ethyl alcohol, ethyl acetate, carbon disulfide, acetone and chloroform to thereby extract actinomycin from said culture.

9. As a new product, pure actinomycin A, which is identical with the actinomycin A extracted from cultures of *Actinomyces antibioticus*, which is a brick-red colored, crystalline, thermostable solid melting at approximately 250° C., and exhibiting strong absorption at 450 mμ and between 230 and 250 mμ, and which has the approximate composition of 59.01% C, 6.81% H, 13.38% N, and a molecular weight in excess of 700.

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Signed and sealed this 20th day of November, A. D. 1945.

Leslie Frazer

(Seal) First Assistant Commissioner of Patents.

(19) Canadian Intellectual Property Office / Office de la Propriété Intellectuelle du Canada
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(54) **ACTINOMYCIN**
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(57) **Abstract:**

This invention relates to new and useful improvements in bio-chemical substances and more particularly in anti-microbial substances.

In accordance with our invention antimicrobial substances are prepared from cultures of Actinomyces antibioticus. Actinomyces antibioticus is a micro-organism which occurs inter alia in soils. Specifically, it is a fungus of defined morphology characterized by spore-bearing hyphae produced in the form of straight aerial mycelium; the sporophores are arranged in clusters, no spirals being formed; the spores are nearly spherical to somewhat elliptical.

The following method was used by us for obtaining cultures of micro-organisms capable of antagonizing various specific bacteria. In accordance with this method suspensions of cultures of the specific bacteria are mixed with an

aqueous agar solution and the mixture added to a suitable soil containing the antagonizing micro-organisms. Upon one to 10 days' incubation, colonies of the antagonists will form which may be transferred to fresh bacterial nutrient media and later isolated as pure cultures in accordance with conventional methods.

By means of this method, we have isolated an organism which we have described as Actinomyces antibioticus. The method of isolation is illustrated in Example IV.

In the practical application of our invention, a suitable culture of Actinomyces antibioticus is extracted with a suitable solvent. Suitable solvents are, for instance, ethyl ether, ethyl alcohol, carbon bisulphide, acetone, and chloroform. Ethyl ether is the preferred solvent. Upon evaporation of the solvent, a solid remains. This substance, hereinafter designated as "actinomycin" possesses marked bacteriostatic and bactericidal properties against a large variety of bacteria, actinomycetes and fungi. In most cases, gram-positive bacteria are much more sensitive against the substance than gram-negative organisms. The actinomycin is completely removable by charcoal from liquid media but is only partly removed therefrom by a Seitz filter. The substance is substantially heat resistant at 100°C. for thirty minutes.

We have found that two different antimicrobial substances may be obtained from the actinomycin. These substances, which will be hereinafter referred to respectively as "actinomycin A" and "actinomycin B" may be obtained by selective extraction with suitable solvents from the actinomycin. Thus, for instance, the actinomycin B being substantially soluble in petroleum ether may be extracted as a

solution in this solvent in which the actinomycin A is substantially insoluble. Alternatively, instead of extracting the actinomycin A or actinomycin B from actinomycin, they may be obtained by direct selective solvent extraction from the original cultures.

Actinomycin A is a highly pigmented (red) product substantially soluble in ether, ethyl alcohol, water, carbon bisulphide, acetone, chloroform and substantially insoluble in petroleum ether. Its aqueous solutions are of a yellow to orange-red color, depending on concentration. The actinomycin B is substantially soluble in ether, carbon bisulphide, acetone, chloroform, petroleum ether, but difficultly soluble in alcohol, and substantially insoluble in water.

The actinomycin A is highly bacteriostatic, gram-positive bacteria being inhibited by dilutions as low as 1:100,000,000; the A compound is also effective, though not in such degree of sensitivity against gram-negative bacteria which are inhibited by concentration of, for example, 1:5,000 to 1:100,000. The actinomycin A also possesses bactericidal activity, though not as pronounced as its bacteriostatic characteristics. The actinomycin B is markedly bactericidal and to a lesser extent bacteriostatic.

Actinomycin A crystallizes from suitable solutions, such as acetone-benzene or acetone-ether mixtures or ethyl-acetate, as vermillion red platelets which melt at 250°C. with slow decomposition. The actinomycin B crystallizes substantially colorless. The A component exhibits characteristic absorption in the visible and ultra-violet regions. It shows strong absorption at 450 mμ and between 230 and 250 mμ.

The following examples illustrate methods of obtain-

ing actinomycin as well as its A and B components, and it is to be understood that the same is furnished by way of illustration and not of limitation.

Example I

An incubated culture of Actinomyces antibioticus was prepared using a medium consisting of 1% tryptone-peptone, 0.5% starch, 0.2% K_2HPO_4, 0.2% NaCl and 0.25% agar in distilled water, grown at a temperature of approximately 25° to 35°C., the incubation being complete after six to ten days. Fifty liters of this incubated culture are extracted approximately six times with ether, using 20 liters of ether for each extraction. The final extract is faintly pale yellow in color, whereas the previous extracts are orange. The combined ether extracts are concentrated to dryness and about 3 gms. of a reddish-brown residue is obtained. The residue is stirred with approximately 400 cc. of petroleum ether for two to three hours, the solvent decanted and the residue treated again with approximately 400 cc. of petroleum ether. A pale yellow oil constituting crude actinomycin B is recovered by evaporation from the petroleum ether.

The dark petroleum ether insoluble residue is dissolved in one liter of benzene with gentle heating. Usually a small amount of black amorphous material remains undissolved and is filtered off. The benzene solution is permitted to drop through a chromatographic tower (60 x 5 cm.) packed with aluminum oxide (according to Brockman). The pigment is readily adsorbed. The column is washed with about one liter of benzene during which operation very little migration of the color bands occurs.

The column is then washed with benzene acetone

solution (15:85) whereby a chromatogram develops. By continued washing, light yellow colored pigments pass out of the column. When the main band (orange-red) reaches the lower end of the column, a solution of 30-70 acetone benzene is passed through the column. The latter solvent elutes the pigment and when the eluate is very pale in color, washing is discontinued.

The eluate is concentrated to dryness under reduced pressure, taken up in 25 cc. of hot acetone, filtered, and diluted with ether. The pigment which crystallizes as red-brick colored platelets is essentially pure but may be recrystallized if desired from hot ethyl acetate. An analysis of the product showed C = 59.01; H = 6.81; N = 13.38.

In accordance with our invention, a variety of derivatives of actinomycin A or actinomycin B may be prepared. Thus, for instance, the following example II recites, by way of illustration, the preparation of the di-acetate of di-hydro actinomycin A.

Example II.

25 mgs. of actinomycin A are dissolved in 1 cc. acetic anhydride and to this is added 3 drops of pyridine (or 3 drops triethylamine) and 100 mg. of zinc dust. On stirring, the red mixture becomes pale yellow in color. After one hour at room temperature, the mixture is filtered from the zinc dust and the filtrate is concentrated to dryness under reduced temperature. The pale yellow residue is dissolved in chloroform and washed with water, with aqueous sodium bicarbonate, and finally with dilute hydrochloric acid. The chloroform solution is concentrated to dryness under reduced pressure and the residue is taken up in 1 cc. acetone. On adding

two volumes of ether, the product separates as a pale yellow solid melting at approximately 241°C. Approximate analysis: C 58.32; H 6.56; N 12.48.

Example III

25 mg. of actinomycin A in 1 cc. of pyridine and 0.5 cc. of acetic anhydride are allowed to stand ten hours at room temperature, and then for two hours at 80°C. The red solution upon concentration to dryness under reduced pressure is dissolved in chloroform and the latter solution washed in the order mentioned with dilute hydrochloric acid and aqueous sodium bicarbonate. The chloroform solution is concentrated to dryness and the resulting product recrystallized from ethyl acetate. The same constitutes red-brick prisms melting at approximately 250°C. Approximate analysis: C 58.27; H 6.56; N 12.46.

The method of growing cultures of antagonists to specific bacteria outlined above can be demonstrated by way of the following example.

Example IV

Agar is washed in distilled water and dissolved so as to yield 1.5% concentration. Two gms. of K_2HPO_4 are added per liter. Ten milliliter portions of this agar are distributed in test tubes and sterilized. A washed suspension of Escherichia coli, or some other suitable bacterium, obtained by cultivation on solid or in liquid nutrient media is prepared and added to the washed agar which has previously been melted and placed in a water bath at 42°C. One milliliter portions of the still viable bacterial suspension are added to the agar tubes and thoroughly mixed with the agar.

A suitable soil is suspended in sterile tap water

using a series of dilutions from 1:10 to 1:10,000. One milliliter portions of these dilutions are placed in sterile Petri dishes and the bacterial agar, prepared as above, is added. The plates are well shaken to distribute the soil suspension thoroughly and are incubated at 28° or 37°C. After one to ten days' incubation of the plates, colonies of the antagonists will appear being surrounded by clear zones. These colonies are transferred to fresh bacterial agar plates and are later isolated in pure culture by the use of convenient media. Actinomyces antibioticus is one of the antagonists isolated by this method.

We have further discovered a method and culture medium for increasing the production of actinomycin or its components by the cultures of Actinomyces antibioticus. The culture medium in accordance with this embodiment essentially comprises 1 to 10 and preferably 5 parts by weight of starch, 1 to 20 and preferably 10 parts by weight of a suitable nitrogen containing material of an organic or an inorganic nature, a small amount of buffering agent sufficient to maintain the pH of the culture substantially between pH 5.6 and pH 8 and preferably at pH 7, 1 to 5 and preferably 2 parts by weight of a mineral salt, preferably sodium chloride, and 2 to 20, preferably 15 parts by weight agar, and 1000 parts by weight of water. The buffering agent may be any one of the known buffering agents and preferably K_2HPO_4; amounts of 1 to 3 and preferably 2 parts by weight of the buffering agent will yield the desired pH or pH range. As a source of nitrogen a variety of substances may be used including organic and inorganic nitrogen containing materials such as asparagine, alanine; phenylalanine,

peptone, tryptone and sodium nitrate. We prefer, however, for best results, to use tryptone as the nitrogen yielding substance. Representative compositions of preferred formulae are, for instance, the following:

1.5% Agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5 to 2% starch, 0.1 to 0.2% Asparagine;

1.5% Agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5% Starch, 1% Peptone;

1.5% Agar, 0.2% K_2HPO_4, 0.2% NaCl, 0.5% Starch, 1.0% Tryptone;

0.25% Agar, 0.2% K_2HPO_4, 0.2% NaCl, 1.5% Starch, 0.2% $NaNO_3$.

Cultures are preferably incubated at 25 to 35°C., substantially maintaining the pH at the indicated point or range. Growth proceeds rapidly and is accompanied by the formation of a soluble dark-brown pigment on organic media. It becomes rapidly covered with a white mycelium having a faint yellowish-green tinge. After 6 to 10 days' incubation, growth and production of actinomycin are usually complete.

We have found that the actinomycin in accordance with our invention possesses a selective bacteriostatic action with respect to different varieties of bacteria and that the markedly greater sensitivity of some bacteria to actinomycin than of others may be utilized for the purpose of isolating specific organisms from a mixed population. Actinomycin has a bacteriostatic effect in relatively small concentrations on gram-positive bacteria, while it requires much higher concentrations to be effective bacteriostatically with respect to gram-negative bacteria. Thus, for instance, when adding 0.1 mg. actinomycin to 10 cc. agar and incubating thereon a substrate such as milk or sewage containing both gram-positive and gram-negative bacteria, the growth of the gram-positive bacteria will be inhibited by the actinomycin. Generally, concentrations of

0.1 to 1.0 mg. per 10 cc. agar permit the selective growth of various bacteria by reason of the differentiation in sensitivity of varying amounts of the active actinomycin with respect to specific bacterial growth.

Both the actinomycin A and actinomycin B possess a relatively high molecular weight. Actinomycin A has the general empirical composition of 58.7 to 59.3% C, 6.5 to 7.5% H, 13.05 to 13.65% N, and a molecular weight in excess of 700.

Modifications may be made in carrying out the present invention without departing from the spirit and scope thereof, and we are to be limited only by the appended claims.

Having regard to the foregoing disclosure, the patent of which this specification forms part confers, subject to the conditions prescribed in the Patent Act, 1935, the exclusive right, privilege and/liberty of making, constructing, using and vending to others to be used, the invention as defined in claims submitted by the patentees as follows:

1. The process that comprises treating a culture of Actinomyces antibioticus with an organic solvent to thereby extract from said culture a concentrate comprising at least one antimicrobial substance of the class consisting of actinomycin A and actinomycin B.

2. The process that comprises treating a culture of Actinomyces antibioticus with a solvent comprising at least one member of the class consisting of ethyl ether, ethyl alcohol, ethyl acetate, carbon disulfide, acetone and chloroform to thereby extract actinomycin from said culture.

B

3. In the method of produding an antimicrobial substance the steps comprising treating a culture of Actinomyces antibioticus with ethyl ether to thereby extract actinomycin and recovering actinomycin from said ether extract.

4. In the method of producing an antimicrobial substance the steps comprising treating a culture of Actinomyces antibioticus with a solvent comprising at least one member selected from the group consisting of ethyl ether, ethyl alcohol, carbon bisulphide, acetone, and separating by selective solvent extraction actinomycin A and actinomycin B.

5. In the method of producing an antimicrobial substance the steps comprising treating a culture of Actinomyces antibioticus with petroleum ether to thereby extract actinomycin B.

6. An antimicrobial substance predominantly comprising a substantially ether-soluble concentrate, extracted from an Actinomyces antibioticus culture, and composed of a high molecular, substantially water-soluble, petroleum ether insoluble component and a substantially high molecular, water-insoluble, petroleum ether-soluble component, when prepared by a process according to claims 1, 2 or 3.

7. Crude actinomycin A, an antimicrobial substance predominantly comprising a high molecular, substantially ether- and water-soluble, petroleum ether-insoluble concentrate, extracted from an Actinomyces antibioticus culture, and recovered as a dark reddish solid exhibiting strong absorption at 450 mu and between 230 and 250 mu, when prepared by a process according to claims 1 or 4.

8. As a new product, pure actinomycin A which is identical with the actinomycin A extracted from cultures of Actinomyces antibioticus, which is a brick-red colored, crystalline, thermostable solid melting at approximately 250° C., and exhibiting strong absorption at 450 mμ and between 230 and 250 mμ, and which has the approximate composition of 59.01% C, 6.81% H, 13.38% N, and a molecular weight in excess of 700, when prepared by a process according to claims 1 or 4.

9. Crude actinomycin B, an antimicrobial substance predominantly comprising a high molecular, substantially water-insoluble, ether- and petroleum ether-soluble concentrate, extracted from an Actinomyces antibioticus culture, and recovered as a pale yellow oil, when prepared by a process according to claims 1, 4 or 5.

950756

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente

Patented June 15, 1948 2,443,485

UNITED STATES PATENT OFFICE

2,443,485

ANTIBACTERIAL SUBSTANCE AND METHOD OF PRODUCING IT

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No Drawing. Application January 19, 1943, Serial No. 472,846

4 Claims. (Cl. 260—236.5)

1

This invention relates to new and useful improvements in bio-chemical substances and, more particularly, to new and useful improvements in antibiotic substances.

In accordance with our invention, a new, powerful antibiotic substance, which we have called streptothricin, is prepared from cultures of certain Actinomycetes, notably certain strains of a type described as *Actinomyces lavendulae* (Waksman, Horning, Welsch, & Woodruff, Soil Science, vol. 54, pp. 281–296, 1942).

Our new substance, streptothricin, is an organic material, having the characteristics of a base, and of low nitrogen content. It is not affected by proteolytic enzymes. It is soluble in water, acid-alcohol, and in dilute mineral acid but not soluble in ether, petroleum ether, and chloroform. It is inactivated by concentrated acids and alkalis. Streptothricin is thermostable, being substantially resistant to heat at 100° C. for 15 minutes.

Streptothricin is active both bacteriostatically and bactericidally but does not exert a lytic effect, and higher concentrations are required for streptothricin to be effective bactericidally than are required for its bacteriostatic effectiveness. It is substantially non-toxic to animals when injected into the bloodstream or when taken orally.

The bacteriostatic action of streptothricin is unique, as compared with that of other antibiotic substances of microbial origin, such as actinomycin A or B, actinomycetin, lysozyme of Actinomyces, tyrothricin, tyrocidine, penicillin, pyocyanase, pyocyanin, etc., in that it is highly effective, in small concentrations, against certain gram-negative bacteria such as *E. coli*, and the Salmonella, Shigella, and Brucella groups, but only slightly against *Ps. fluorescens*. Also, streptothricin is highly active against certain gram-positive bacteria, such as *Bac. subtilis*, but not against other very closely related gram-positive bacteria, such as *Bac. mycoides*. In the selectivity of its action, streptothricin is entirely different from other antibiotic substances derived from Actinomyces.

On electrodialysis, streptothricin moves to the cathode at pH 7.0.

Streptothricin may be produced by inoculating a suitable medium with spores of *Actinomyces lavendulae*, and permitting growth to proceed for from about 6 to 12 days, at about 20–28° C.

After completion of growth, and filtration of the medium, streptothricin may be recovered from the filtrate.

According to one preferred embodiment of our invention, the recovery of the streptothricin may

2

be effected by treating the above-mentioned filtrate, at neutral or alkaline pH, with an adsorbent such as activated carbon or permutite, the streptothricin being completely adsorbed thereon. The adsorbate is then eluted with low-normality alcoholic mineral acid, such as alcoholic hydrochloric acid, for example, after which the adsorbent material may be filtered off. Upon treating the filtrate thus obtained with ether, an aqueous layer forms, which is removed and evaporated to dryness.

According to another embodiment of our invention, the acid-alcoholic eluate containing streptothricin may be neutralized, and evaporated to dryness.

According to still another embodiment of our invention, the eluate obtained by washing the acid-alcoholic adsorbate is neutralized and concentrated just to dryness. The residue thus obtained is then extracted with absolute alcohol, filtered, and the alcohol evaporated.

The products obtained according to the above procedures may be further purified, as, for instance, by the method outlined in the example given hereinafter.

A suitable medium for the growth of *Actinomyces lavendulae*, for the production of streptothricin, may comprise an aqueous medium containing tryptone, casein, peptone, dibasic potassium phosphate, sodium chloride, and a carbohydrate, such as glucose, starch, etc. Traces of iron salts, such as ferrous sulfate, for example, may also be present, and appear to exert a beneficial effect upon the growth of *Actinomyces lavendulae*. The presence of a small amount of agar, talc, or other similar material, to aid in maintaining a surface pellicle of growth, is helpful in the production of streptothricin.

We have discovered, furthermore, that streptothricin is formed when *Actinomyces lavendulae* is grown on certain simple nitrogenous compounds, such as glycocoll, alanine, aspartic acid, asparagine, and glutamic acid, in the presence of a small amount of a carbohydrate. In the case of some of the amino acids, such as asparagine and glycocoll, for example, streptothricin is formed even in the complete absence of the carbohydrate. However, when streptothricin is produced upon an amino acid alone, in the absence of carbohydrate, there is a gradual increase in alkalinity, which results in the destruction of streptothricin, and for that reason, the pH of the medium should be periodically adjusted to near the neutral point.

The following example illustrates a method of

carrying out the present invention, but it is to be understood that this example is given by way of illustration, and not of limitation.

Example

A medium consisting of

1.0% dextrose,
0.5% tryptone,
0.2% dibasic potassium phosphate,
0.2% sodium chloride, and

distilled water, is distributed in appropriate vessels to a depth of 1–2 inches. The medium is sterilized at 15 lbs. steam pressure for 15–20 minutes, and then cooled.

A water suspension of spores of a strain of *Actinomyces lavendulae* is prepared by scraping from agar slants. The medium is inoculated with a heavy suspension of the Actinomyces spores. Incubation is at a temperature of 20–28° C. Streptothricin elaboration is usually complete in 6–12 days. Flakes of growth are filtered off wit muslin, and the broth is treated as follows:

To a batch of approximately 100 liters of filtered streptothricin broth are added 1500 gms. of active charcoal. The mixture is stored for about 8–12 hours at 0–10°, and stirred up at about two-hour intervals. It is then filtered. A colorless filtrate is obtained, and discarded. The charcoal residue is then suspended in 10 liters of 95% ethanol made 0.15 normal with hydrochloric acid. It is placed in an ice bath, stirred for two hours, and then permitted to stand for 8–12 hours in the cold. The suspension is filtered, the charcoal residue discarded, and the brown clear filtrate obtained is poured into 100 liters of ether, with stirring. An aqueous layer separates, and is drawn off. It is a black thick liquid. One liter of water is then added to the alcohol ether solution with stirring. The aqueous layer, which is a dark brown solution, is then drawn off. The aqueous solutions are neutralized to pH 6–7.

The material thus obtained may be further purified by treatment thereof with a substance to adsorb anions (for example, "Amberlite IR–4") and then treating it with a substance to adsorb cations (for example, "Amberlite IR–I"). The material is then filtered, and the filtrate is treated with acid-washed permutite. The adsorbate is eluted with dilute mineral acid. A colorless solution containing streptothricin is thus obtained.

Modifications may be made in carrying out the present invention, without departing from the spirit and scope thereof, and we are to be limited only by the appended claims.

We claim:

1. A process for the production of streptothricin comprising cultivating a culture medium inoculated with spores of a streptothricin-producing strain of *Actinomyces lavendulae* for about 6 to 12 days, at 20–28° C., filtering, adsorbing streptothricin from the filtrate, and recovering the adsorbed streptothricin by eluting with low normality alcoholic mineral acid.

2. A process for the production of streptothricin comprising cultivating a culture medium inoculated with spores of a streptothricin-producing strain of *Actinomyces lavendulae* for about 6 to 12 days, at about 20–28° C., filtering, treating the filtrate thus obtained with an adsorbent material, eluting the adsorbate with low-normality alcoholic mineral acid, filtering, treating the filtrate with ether, and recovering streptothricin from the aqueous layer thus formed.

3. In a process of producing streptothricin by cultivation in a culture medium of spores of *Actinomyces lavendulae* the steps which comprise treating the filtered culture medium contain streptothricin with an adsorbent material to adsorb streptothricin, and recovering the adsorbed streptothricin by eluting with low normality alcoholic mineral acid.

4. As a new composition of matter, an organic, antibiotic substance which is thermostable; which has the characteristics of a base; which is soluble in water, acid-alcohol, and dilute mineral acids and insoluble in ether, petroleum ether, and chloroform; which is strongly active bacteriostatically against the gram-negative bacteria *E. coli* and the bacteria of the *Salmonella, Shigella,* and *Brucella* groups, and against the gram-positive bacteria *B. subtilis,* said activities being strong in comparison with the relative inactivity, in bacteriostatic respects, which characterizes the aforesaid antibiotic substance toward *Ps. fluorescens* and *B. mycoides;* and which is identical with antibiotic material, having the aforesaid bacteriostatic characteristics, that is produced by cultivation of organisms of the species *Actinomyces lavendulae* only in artificial media not naturally occurring in soil and under conditions favorable to such production by such organisms.

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HAROLD BOYD WOODRUFF.

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An Agency of Industry Canada / Un organisme d'industrie Canada

(11) CA 481107 (13) A
(40) 12.02.1952

(12)

(21) Application number: 481107D
(22) Date of filing: ..

(51) Int. Cl:

(71) Applicant: RUTGERS RES AND ENDOWMENT FOUN.

(72) Inventor: WAKSMAN SELMAN A ().
WOODRUFF H BOYD ().

(54) ANTI-BACTERIAL SUBSTANCES
(54) SUBSTANCES ANTI-BACTERIENNES

(57) **Abstract:**

This invention relates to new and useful improvements in bio-chemical substances and, more particularly, to new and useful improvements in antibiotic substances.

In accordance with our invention, a new, powerful antibiotic substance, which we have called streptothricin, is prepared from cultures of certain Actinomycetes, notably certain strains of a type described as Actinomyces lavendulae (Waksman, Horning, Welsch, & Woodruff, Soil Science, Vol. 54, pp. 281-296, 1942).

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centrated acids and alkalis. Streptothricin is thermostable, being substantially resistant to heat at 100°C for 15 minutes.

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The bacteriostatic action of streptothricin is unique, as compared with that of other antibiotic substances of microbial origin, such as actinomycin A or B, actinomycetin, lysozyme of Actinomyces, tyrothricin, tyrocidine, penicillin, pyocyanase, pyocyanin, etc., in that it is highly effective, in small concentrations, against certain gram-negative bacteria such as E. Coli, and the Salmonella, Shigella, and Brucella groups, but only slightly against Ps. Fluorescens. Also, streptothricin is highly active against certain gram-positive bacteria, such as Bac. subtilis, but not against other very closely related gram-positive bacteria, such as Bac. Mycoides. In the selectivity of its action, streptothricin is entirely different from other antibiotic substances derived from Actinomyces.

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After completion of growth, and filtration of the medium, streptothricin may be recovered from the filtrate.

According to one preferred embodiment of our invention, the recovery of the streptothricin may be effected by treating

the above-mentioned filtrate, at neutral or alkaline pH, with an adsorbent such as activated carbon or permutite, the streptothricin being completely adsorbed thereon. The adsorbate is then eluted with low-normality alcoholic mineral acid, such as alcoholic hydrochloric acid, for example, after which the adsorbent material may be filtered off. Upon treating the filtrate thus obtained with ether, an aqueous layer forms, which is removed and evaporated to dryness.

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According to still another embodiment of our invention, the eluate obtained by washing the acid-alcoholic adsorbate is neutralized and concentrated just to dryness. The residue thus obtained is then extracted with absolute alcohol, filtered, and the alcohol evaporated.

The products obtained according to the above procedures may be further purified, as, for instance, by the method outlined in the example given hereinafter.

A suitable medium for the growth of Actinomyces lavendulae, for the production of streptothricin, may comprise an aqueous medium containing tryptone, casein, peptone, dibasic potassium phosphate, sodium chloride, and a carbohydrate, such as glucose, starch, etc. Traces of iron salts, such as ferrous sulfate, for example, may also be present, and appear to exert a beneficial effect upon the growth of Actinomyces lavendulae. The presence of a small amount of agar, talc, or other similar material, to aid in maintaining a surface pellicle of growth, is helpful in the production of streptothricin.

We have discovered, furthermore, that streptothricin is formed when Actinomyces lavendulae is grown on certain simple nitrogenous compounds, such as glycocoll, alanine, aspartic acid, asparagine, and glutamic acid, in the presence of a small amount of a carbohydrate. In the case of some of the amino acids, such as asparagine and glycocoll, for example, streptothricin is formed even in the complete absence of the carbohydrate. However, when streptothricin is produced upon an amino acid alone, in the absence of carbohydrate, there is a gradual increase in alkalinity, which results in the destruction of streptothricin, and for that reason, the pH of the medium should be periodically adjusted to near the neutral point.

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Example

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0.5 % tryptone,
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To a batch of approximately 100 liters of filtered streptothricin broth are added 1500 gms. of active charcoal. The mixture is stored for about 8 - 12 hours at 0 - 10°, and stirred up at about two-hour intervals. It is then filtered. A colorless filtrate is obtained, and discarded. The charcoal residue is then suspended in 10 liters of 95% ethanol made 0.15 normal with hydrochloric acid. It is placed in an ice bath, stirred for two hours, and then permitted to stand for 8 - 12 hours in the cold. The suspension is filtered, the charcoal residue discarded, and the brown clear filtrate obtained is poured into 100 liters of ether, with stirring. An aqueous layer separates, and is drawn off. It is a black thick liquid. One liter of water is then added to the alcohol ether solution with stirring. The aqueous layer, which is a dark brown solution, is then drawn off. The aqueous solutions are neutralized to pH 6-7.

The material thus obtained may be further purified by treatment thereof with a substance to adsorb anions (for example, "Amberlite IR-4") and then treating it with a substance to adsorb cations (for example, "Amberlite IR-I"). The material is then filtered, and the filtrate is treated with acid-washed permutite. The adsorbate is eluted with dilute mineral acid. A colorless solution containing streptothricin is thus obtained.

Modifications may be made in carrying out the present invention, without departing from the spirit and scope thereof, and we are to be limited only by the appended claims.

While the term "Actinomyces" includes the organism used in producing streptothricin as herein described, the word Streptomyces is now known to more accurately define the genus organism with which the invention is concerned. Accordingly the latter term is used in the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. In the production an antibiotic substance which is soluble in water and insoluble in organic solvents by growing a culture of a Streptomyces to produce the antibiotic substance the method which comprises separating the Streptomyces growth from the culture broth, subjecting the culture broth in non-acid condition to an adsorbent on which the antibiotic substance is adsorbed, separating the adsorbent containing the antibiotic substance from the mixture and eluting the antibiotic substance from the adsorbent with an aqueous acid-alcohol reagent.

2. The process defined in claim 1 wherein the adsorbent is activated carbon.

3. In the production of the antibiotic substance streptothricin by growing a culture of a Streptomyces to produce streptothricin, the method which comprises separating the Streptomyces growth from the culture broth subjecting the culture broth in non-acidic condition to an adsorbent on which streptothricin is adsorbed, separating the adsorbent containing the streptothricin from the mixture and removing the streptothricin from the adsorbent by elution with acid-alcohol in which streptothricin is soluble.

4. Streptothricin.

95-1733

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

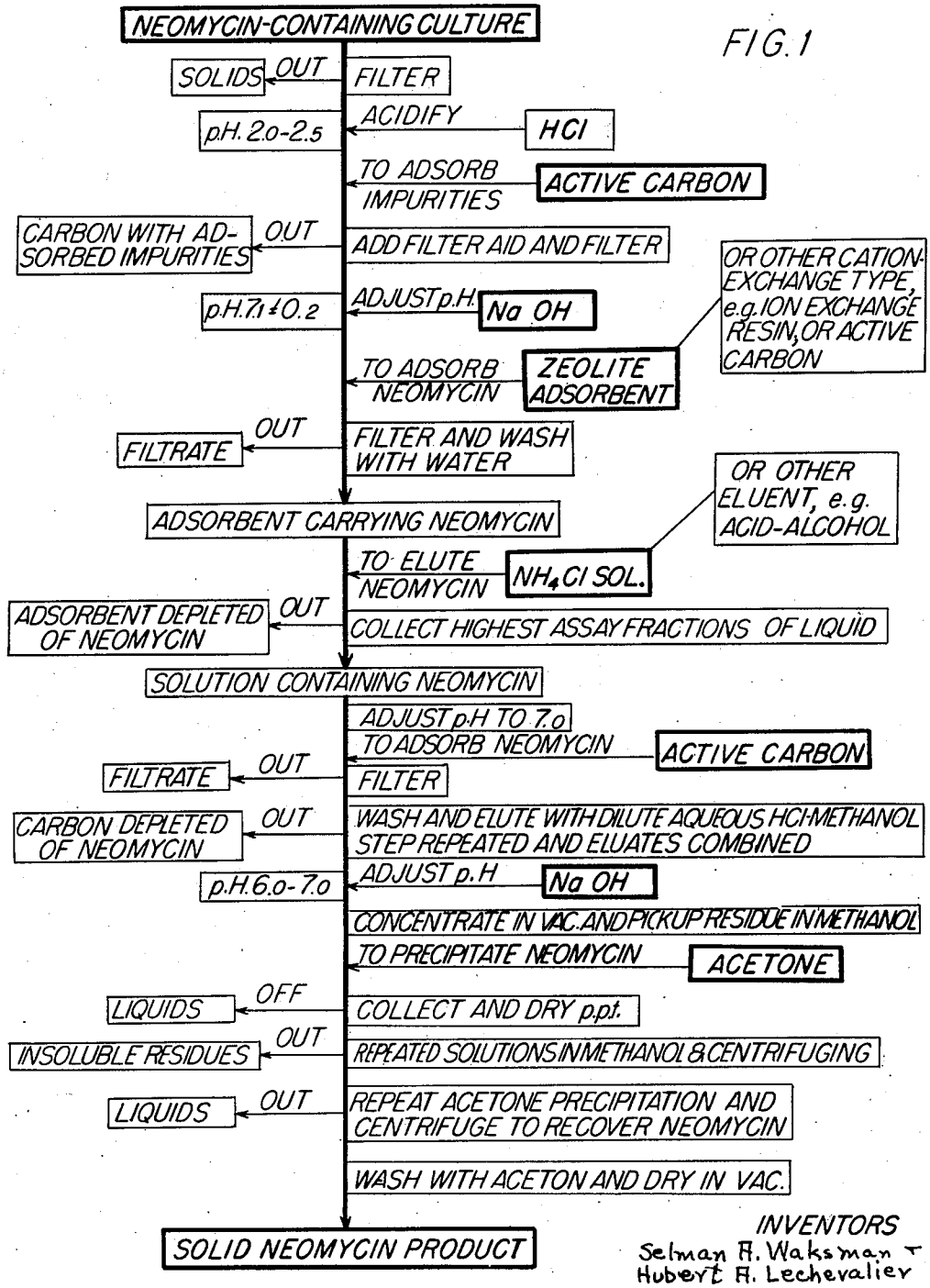
Cette Section est Absente

July 16, 1957 S. A. WAKSMAN ET AL 2,799,620

NEOMYCIN AND PROCESS OF PREPARATION

Filed June 29, 1956 4 Sheets-Sheet 1

FIG. 1



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NEOMYCIN AND PROCESS OF PREPARATION

Filed June 29, 1956 4 Sheets-Sheet 2

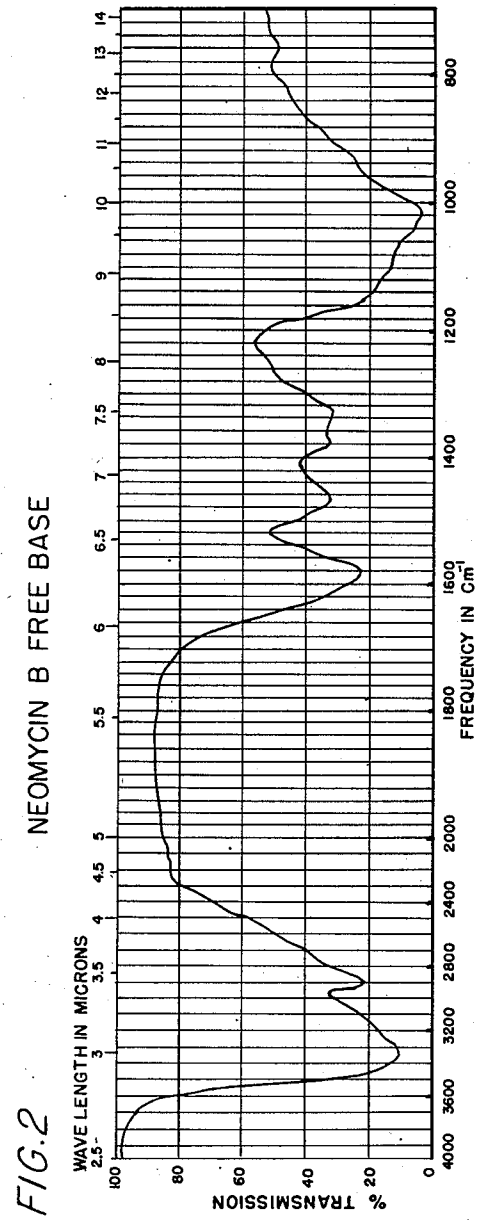

FIG. 2

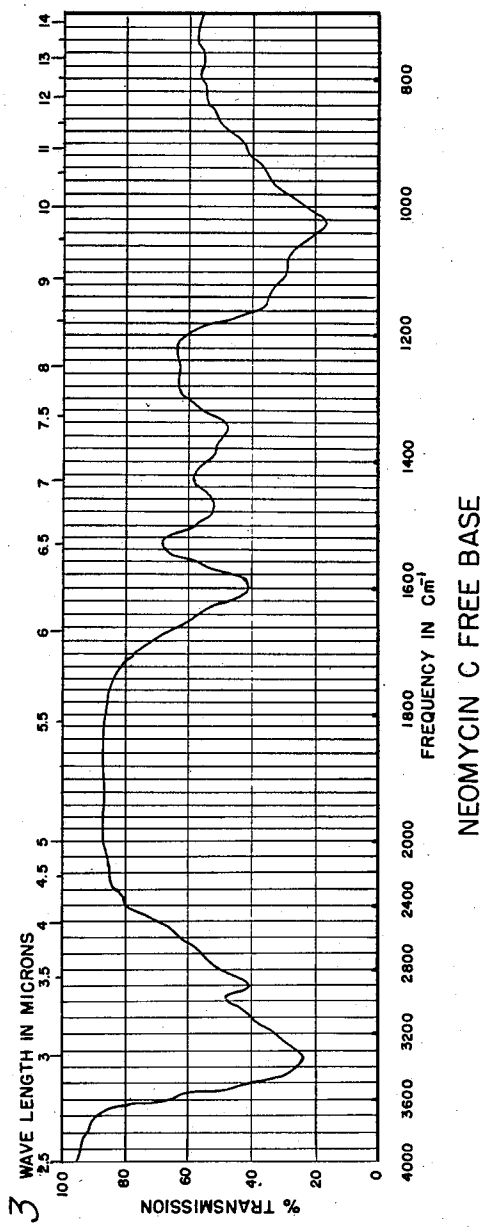

FIG. 3

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NEOMYCIN AND PROCESS OF PREPARATION

Filed June 29, 1956 4 Sheets-Sheet 3

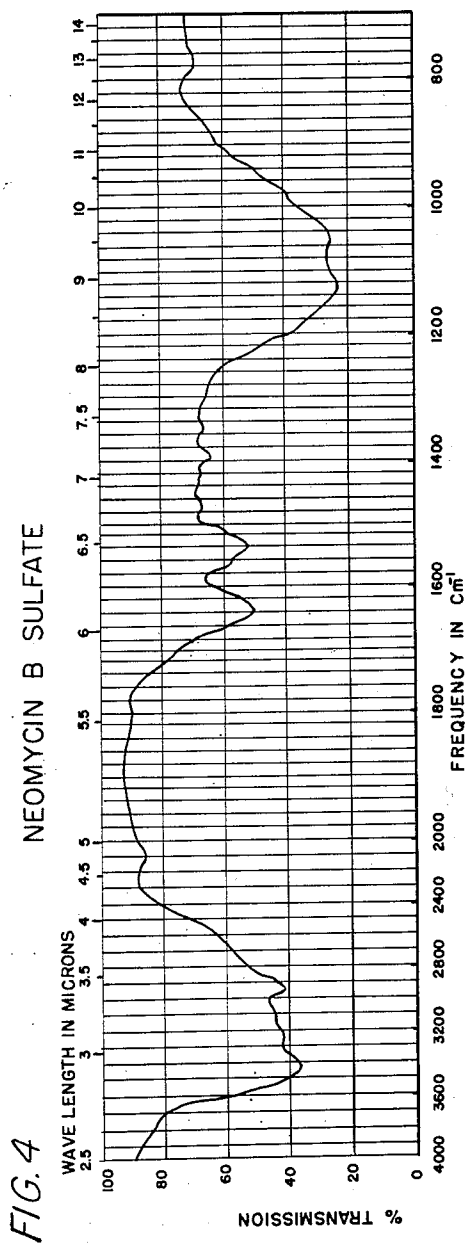

FIG. 4

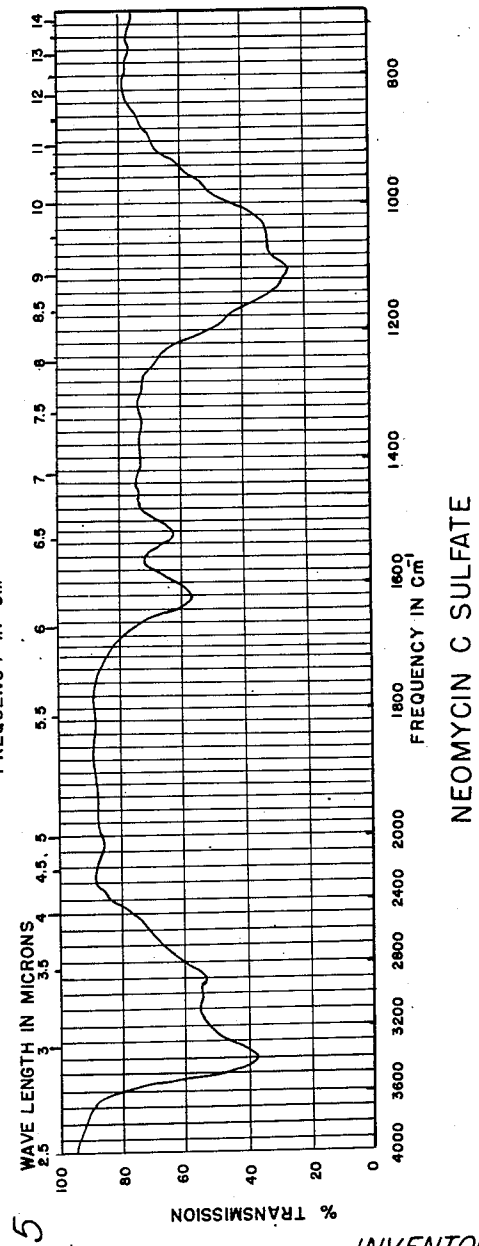

FIG. 5

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NEOMYCIN AND PROCESS OF PREPARATION

Filed June 29, 1956 4 Sheets-Sheet 4

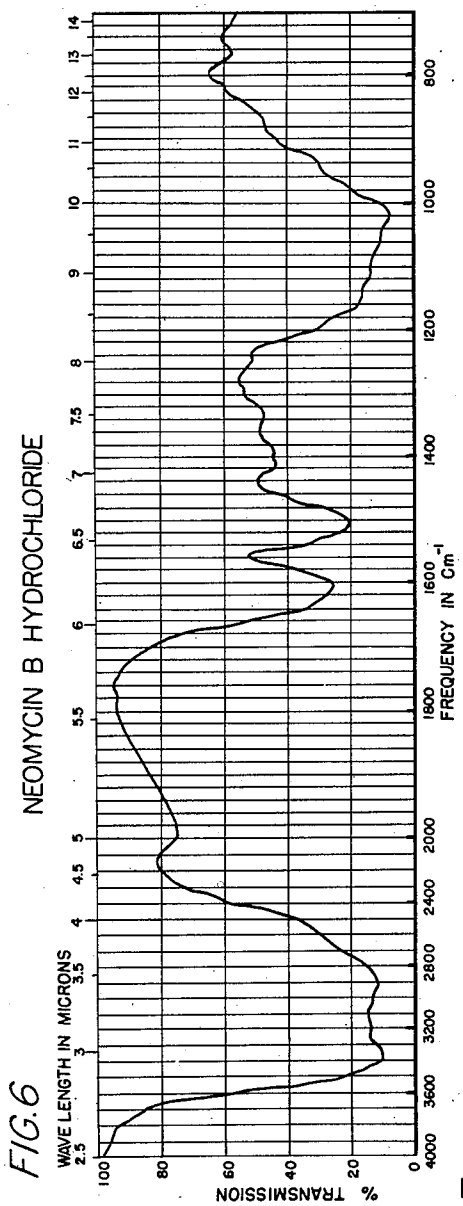

FIG. 6

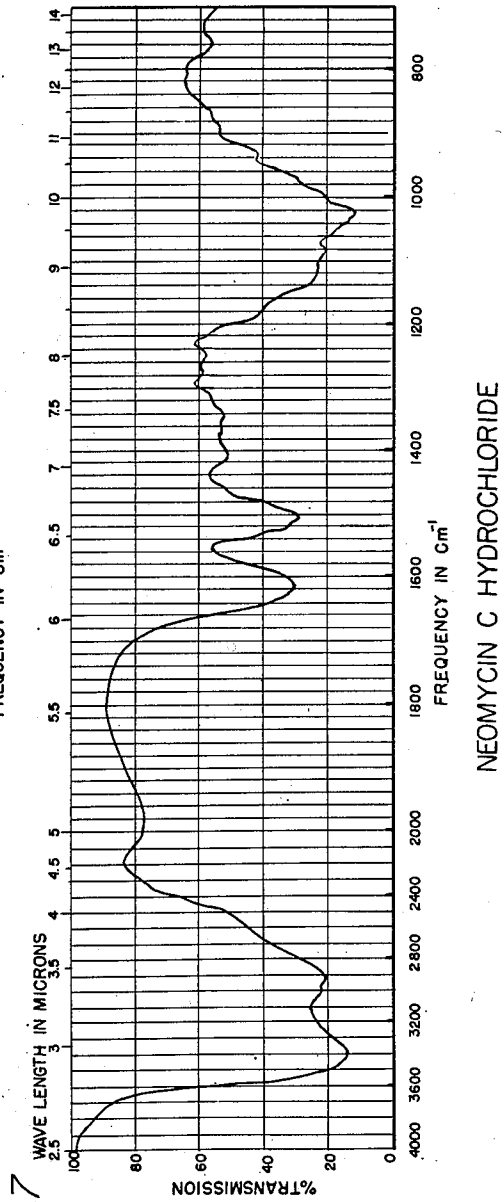

FIG. 7

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United States Patent Office

2,799,620

Patented July 16, 1957

2,799,620

NEOMYCIN AND PROCESS OF PREPARATION

Selman A. Waksman, New Brunswick, and Hubert A. Lechevalier, Martinsville, N. J., assignors to Rutgers Research and Educational Foundation, New Brunswick, N. J., a nonprofit corporation of New Jersey

Application June 29, 1956, Serial No. 594,987

23 Claims. (Cl. 167—65)

This invention relates to antibiotic substances and procedure for making them, and more particularly to a new and effective antibiotic substance that has now been produced by cultivation under artificial conditions of a micro-organism, further identified below, which is a species of the genus Streptomyces, i. e. one of the organisms classed as actinomycetes. The organism may also be aptly described as a strain of Streptomyces of the type of *Streptomyces fradiae*, the latter having been isolated from the soil in 1915 by Waksman (one of the present applicants) and Curtis, then designated as *Actinomyces fradii* but more recently listed in Bergey's Manual as *Streptomyces fradiae*. The present strain of organism of this nature, which at least closely resembles the one named, and which is employed for production of the new antibiotic as below described, has been deposited in the Rutgers University, Microbiology Department, Type Culture Collection, under the official number 3535 for the deposited culture, and has been deposited in the American Type Culture Collection as *Streptomyces fradiae* 3535; it may thus be conveniently and accurately identified as Streptomyces No. 3535.

Among the antibiotics now widely used, streptomycin has been particularly successful as a chemotherapeutic agent for a variety of purposes by reason of its activity against gram-negative as well as gram-positive bacteria, including the mycobacteria, and has been used effectively in the treatment and control of certain forms of human tuberculosis. There are, however, strains of some bacteria which are more or less resistant of streptomycin, the most significant manifestation of such resistance being its development, in certain cases, upon prolonged administration of the drug. That is to say, one or more resistant strains may develop or grow among the infectious organisms in such a case, which is usually a situation where the initial series of treatments has been only partially effective. It will therefore be appreciated that an especially useful antibiotic would be one having the following essential properties: (*a*) high activity against gram-negative bacteria and mycobacteria; (*b*) activity upon streptomycin-resistant strains of bacteria, especially of acid-fast bacteria; and (*c*) low toxicity to animals.

It has now been discovered, i. e. in accordance with the present invention, that the above-identified organisms (Streptomyces No. 3535) may be employed to produce a distinctively new and preeminently useful anti-bacterial substance which is of organic composition and which is

different, in both chemical and antibiotic respects, from other substances of its general class, such as streptomycin, streptothricin, grisein, and actinomycin.

The novel antibiotic composition, which has been named "neomycin" and may be conveniently so identified herein, is distinctively characterized by its possession, in substantial measure, of the desirable properties that are enumerated above and that are indicative of extraordinary utility for the substance. More complete reference to the anti-bacterial properties will be made below, but it may be noted at the outset that this substance has not only shown activity against a number of streptomycin-resistant strains of bacteria (including mycobacteria) but has also been less favorable to the development of resistant growth under circumstances where the latter, in the presence of another antibiotic, such as streptomycin, might be exepected. That is to say, among a number of organism types which include strains resistant respectively to other antibiotics, no significant difference in sensitivity to neomycin has been noted.

Referring to the drawings:

Fig. 1 is a flow sheet of a suitable process for extracting neomycin from a culture in which it is produced;

Fig. 2 is a curve of the infra-red spectrum of neomycin in the free base form, and specifically in the form of neomycin B, free base;

Fig. 3 is a curve of the infra-red spectrum of neomycin in the free base form, and specifically in the form of neomycin C, free base;

Fig. 4 is a curve of the infra-red spectrum of the sulfate of neomycin, specifically in the form of neomycin B sulfate;

Fig. 5 is a curve of the infra-red spectrum of the sulfate of neomycin, specifically in the form of neomycin C sulfate;

Fig. 6 is a curve of the infra-red spectrum of the hydrochloride of neomycin, specifically in the form of neomycin B hydrochloride; and

Fig. 7 is a curve of the infra-red spectrum of the hydrochloride of neomycin, specifically in the form of neomycin C hydrochloride.

This new antibiotic, neomycin, has been produced by growing the organism, Streptomyces No. 3535, in a suitable nutrient medium under appropriate stationary or submerged aerobic (viz. shaken) conditions, and then isolating and purifying the substance, e. g. by procedure of the sort hereinbelow described, including various steps of adsorption, recovery by elution, separation from impurities, and precipitation. So prepared, neomycin is found to be a basic compound (i. e. forms salts with acids and forms a reineckate), most active at an alkaline reaction. That is, its anti-microbial activity tends to be limited in acid conditions, but appears to rise as neutrality is reached, and is definitely favored by an alkaline condition of the medium, characteristics that are advantageous in view of the generally alkaline reaction of the blood. The isolated substance, i. e. neomycin, is readily soluble in water, in dilute acid solutions (e. g. dilute HCl), in aqueous methanol and in acid- alcohol of low acid normality, such as the ethanol-hydrochloric acid or methanol-formic acid solutions heretofore used for elution of streptomycin from adsorbents. It is rela-

tively insoluble in various organic solvents, such as ether, chloroform and acetone.

Neomycin has been found to be unusually thermostable, especially at neutral or slight acid or alkaline reaction, but also otherwise, and may thus be heated to 100° C. for substantial periods without destroying its activity. In one set of tests, separate batches of neomycin at pH 2, 7 and 10 respectively, were kept at a temperature of 100° C. for four hours, without significant loss of potency. Indeed this extraordinary heat stability of neomycin is a property of special utility and of definitive significance, differentiating the substance from chlortetracycline, penicillin and even streptomycin, and indicating the chemical distinctiveness of neomycin. It is also remarkably stable in solution, when stored for long periods of time such as a month or more. Solutions of it have been kept both at room temperature and under refrigeration, for at least three months without impairment of activity. Such further type of stability also affords a sharp distinction from substances such as penicillin and chlortetracycline, which deteriorate in solution in a matter of days, the latter of these being reported to be seriously impaired (even when refrigerated) in a few days. In the dry, i. e. crystalline or other finely divided state, neomycin can be safely kept for very long periods, practically indefinitely. It is not inactivated in the blood or in the presence of serum or other organic material, but it is inactivated by nucleic acid.

While the precise chemical structure of neomycin has not yet been determined, its novel composition seems fully demonstrated by its unique set of properties, including those mentioned above and elsewhere herein and especially including its antibiotic spectrum. Such properties are believed to be amply significant, even though the substance was not at first produced in a completely pure state, and has not been fully analyzed as to molecular structure. It appears, however, that neomycin does not give the maltol reaction as streptomycin does; nor does it give the Sakaguchi reaction for mono-substituted guanidine, its thus indicated lack of a guanidine group being a feature of distinction from various forms of streptomycin.

As stated, the antibiotic spectrum of neomycin is a specific and inherently unique characteristic, i. e. for purposes of identification relative to other antibiotic substances. Indeed it is generally recognized that such a spectrum, e. g. of bacteriostatic or like activity or inactivity toward a number of different organisms, constitutes a peculiarly effective means of characterizing and identifying materials of this class. That is to say, each antibiotic substance has a clearly determinable and defined spectrum which uniquely distinguishes it from other antibiotic materials without any necessity of numerical comparison among such substances as to their absolute activity toward any specific one or another of the many organisms against which they might be employed. Thus the spectrum of a given antibiotic consists, in effect, of points of relatively strong and weak activity (or inactivity), exhibited respectively toward various specific organisms and measured only in relation to each other; such a spectrum may be plotted or tabulated, and the configuration or shape of the resulting combination of peaks and valleys, so to speak, provides a positive identification of the substance and thus of its chemical as well as its antibiotic nature.

A typical spectrum for neomycin, to the extent of presently made tests, is set forth in detail hereinbelow, but certain features of it may be noted here. Generally speaking and relying on the determinations that have thus been effected up to the present, neomycin has a spectrum which involves activity toward the same important bacteria (including mycobacteria) to which streptomycin is effective, and inactivity toward fungi, again in the same way that streptomycin is relatively ineffective against such organisms. In consequence, neomycin is distinguished by its antibiotic spectrum from other materials (such as streptothricin, actinomycin, grisein, penicillin, clavacin) by most of the same general characteristics that distinguish streptomycin from them. The spectrum of neomycin also includes points of activity, so to speak, relative to a number of organisms that are insensitive to streptomycin; these regions of the spectrum which correspondingly afford distinction from the latter material are represented by streptomycin-resistant strains of *E. coli* and *M. tuberculosis*, and by the so-called Bodenheimer's organism.

The organism that has been employed to produce neomycin was isolated from the soil; as stated, it is one of the actinomycetes, has been identified as *Streptomyces fradiae*, and has been designated Streptomyces No. 3535. It gives a yellowish to brownish growth on synthetic and organic media, but no soluble pigment; it belongs, therefore, to the non-chromogenic group. It readily produces an aerial mycelium, especially on a synthetic medium, the mycelium being pigmented white colored to rose, sea shell pink or pink. The color of the mycelium may apparently be described as light russet-vinaceous, according to Ridgeway's nomenclature for color identification. Observations have also indicated that in the case of strain No. 3535 the sporulating hyphae are usually straight; some other neomycin-producing strains of *S. fradiae*, e. g. No. 3554 of the Rutgers collection, may produce spirals.

As indicated above, the new antibiotic substance has been produced by cultivation of the stated organism under highly artificial conditions, for example upon inoculating a suitable medium with spores of the organism or with submerged growth. In the light of experimental work with the process, it appears that considerable variation is permissible in the composition of the medium, preferably so long as suitable organic and inorganic constituents are present both to favor the growth of the organism and to stimulate production of the antibiotic. Thus it appears that best results are obtained when the culture is grown in a medium containing a suitable source of nitrogen, a carbohydrate, and inorganic or mineral elements such as provided by sodium chloride and other material as described below. A more specific enumeration of the types of components which can all be advantageously incorporated in the culture medium is as follows:

I. A peptone or protein digest; ordinary peptones, or protein digests such as casein digests seem to be satisfactory, and results have been obtained with materials such as soy bean peptone, sometimes called soya peptone. Particularly good results have been obtained with casein digests such as the material known as NZ amine. Another useful example is bacto peptone. A component of these types may advantageously be included, for example, in the proportion of 10 to 20 grams (or more, e. g. 25 grams) per liter of the medium. This component appears to be of special significance for the production of the antiobiotic, and may be generally described as peptone material.

II. Meat extract, or a similar type of material such as yeast extract or distiller's soluble, included in an amount, say, of 3 to 5 grams per liter or more, e. g. to 20 grams per liter. Although it is believed that this ingredient is of relatively less importance and may indeed be dispensed with in some cases, it appears to contribute materially to the desired results. This component may be described as extractive-type nutrient material, or alternatively as vitamin-containing material.

III. Carbohydrate material, preferably represented by glucose or a similar sugar such as maltose. Glucose or the like may be used in an amount, for instance, of 5 to 10 grams per liter and appears to be primarily helpful for good growth of the organism, although not, in a direct or strict sense, critically essential for production of neomycin. Indeed glucose can be omitted entirely in many media for neomycin production.

IV. Certain mineral elements, especially when such are found lacking in the water used to make up the medium or broth. An element that seems particularly important is zinc in the form of a zinc salt, such as zinc sulfate, included in relatively small amount, e. g. 10 parts per million or thereabouts. It also appears that sodium chloride is an extremely desirable component, say in the proportion of about 5 grams per liter.

V. Water, usually in the form of ordinary tap water. While distilled water may be used, e. g. by supplementing it with a zinc salt as indicated above, tap water which naturally contains a trace of combined zinc has been found entirely suitable and avoids any need for addition of mineral components other than a salt such as sodium chloride.

Using a medium of the sort just described and containing all five (5) of the listed ingredients, the pH is preferably adjusted with NaOH to about 7 or 7.2. While it is possible to begin with an acid medium, e. g. having a pH as low as 6, experience indicates that the production of the antibiotic is then materially delayed, and indeed an increase of acidity may ensue. In fact it appears that glucose or the like tends to produce or favor an increase of acidity and a consequent delay in production of neomycin. This effect of glucose has been overcome by employing peptone or casein hydrolysate in relatively substantial amount, i. e. toward the upper end of the range of proportions indicated above. Diminution of the unfavorable effects of glucose in the production of the antibiotic may also be achieved, at least in part, by decreasing the amount of the glucose (preferably while increasing the proportion of peptone as just explained) or by specifically adding a neutralizing agent such as calcium carbonate. For the latter purpose calcium carbonate has the further advantage of being self-neutralizing; while other substances such as sodium carbonate might be employed, it then becomes necessary to make daily or other regular additions of such agent, in order to maintain conditions favoring the production of the antibiotic. Alternatively, glucose can be omitted entirely, provided that the protein hydrolysate is increased, as above.

Using media of the foregoing or equivalent character, neomycin can be produced with either stationary or shaken cultures. In the case of stationary cultures, present indication is that a satisfactory yield of the antibiotic requires incubation at room temperature, e. g. within a range of 22° to 28° C. (preferably 25° to 28° C.), for a period of 7 to 12 days. For stationary cultures, the medium should be very shallow; even then, the spores of this organism tend to drop, i. e. to become submerged, and thus to fail in production of the antibiotic. It appears that this organism is characterized by a heaviness or greater specific gravity than organisms such as *Streptomyces griseus,* with which less difficulty is experienced in the incubation of stationary cultures. Preferred results are obtainable by somewhat thickening the medium, as by incorporating ¼ to ½% of agar to afford a semi-solid or semi-liquid constituency. With such modification of the medium, the organism appears to have a suitable support so that the culture grows on the surface as a pellicle, receiving the necessary quantity of air for elaboration of the antibiotic substance.

Considerably more rapid incubation, for satisfactory yield of neomycin, has been achieved with shaken cultures, i. e. relatively deep bodies of the culture medium to which suitable agitation is imparted for effective aeration. Thus 3 to 5 days at a temperature of about 25° C. have been found sufficient for useful production, the preferred temperature range being 25° to 28° C. It appears that for optimum results the extent of agitation, or more strictly the extent of aeration, should be rather carefully controlled. It is, of course, extremely important that considerable air be caused to permeate the medium, e. g. during the shaking operation; while it is difficult to define quantitatively the amount of air or aeration necessary, the control of the latter to the desired point in actual practice is a very easy matter, involving at the most a few simple tests. While the times of incubation stated above are those heretofore found optimum in the experimental practice of the invention, it is understood that other times may be used or required in special cases. For instance, experience in the art of antibiotic production has generally indicated that the incubation period may be materially reduced by using a relatively large inoculum.

While various procedures may be adopted for separating the antibiotic material from the culture broth and for subsequently purifying it, preliminary account may be given of certain operations found especially convenient. Generally stated, the procedure involves steps of adsorption and elution, including supplemental purification steps, and extending, if desired, to chromatographic separation. By way of general example of the process, the culture medium, after the desired period of incubation, may be filtered and the filtrate treated with a suitable adsorbent such as activated charcoal or other active carbon, which adsorbs the neomycin. Upon separation of the adsorbate, i. e. the carbon, it can be treated with ethanol having a hydrochloric acid content of 2% (for example, 72% ethanol and the balance water, with HCl amounting to 2% of the total) to elute the antibiotic, or by other acid alcohol such as methanol-formic acid, for like purpose. Subsequent purification steps may include further adsorption and elution of the antibiotic, followed ultimately by precipitation with an organic liquid such as acetone and final washing and drying of the precipitate at appropriately moderate temperature.

Alternatively, the first adsorption of the neomycin may be effected with an adsorbent of the cation-exchange type. In such case (as likewise when carbon is used to adsorb neomycin) there may be a preliminary treatment of the culture medium after filtration, by strongly acidifying it and introducing activated carbon to adsorb impurities, it being understood that neomycin is not adsorbed under strongly acid conditions. For use of the cation-exchange type of adsorbent (or similarly when the carbon type of adsorbent is employed after the preliminary cleaning) the pH of the filtrate resulting from separation of the impurity-adsorbent should first be brought to an approximately neutral value. The antibiotic is then adsorbed by treatment with the stated adsorbent, and the latter, i. e. a neomycin-containing adsorbate is filtered and subjected to elution for separation of the antibiotic. Examples of cation-exchange adsorbents are zeolites, such as Decalso (a sodium aluminum silicate) and ion exchange resins, e. g. of sulfonic acid type and especially those of the carboxylic acid type, an especially useful instance of the latter being the product known as Amberlite IRC–50, which is stated to be a copolymer as disclosed in Patent No. 2,340,111, issued January 25, 1944, it being further understood that any of the specific examples of copolymers therein described would be suitable. In the case of a cation-exchange type of adsorbent, such elution may be effected with ammonium chloride solution or with dilute HCl. The resulting liquid in which the antibiotic is dissolved may thereafter be subjected to successive adsorption and elution operations, terminating with appropriate procedure for precipitation and physical separation and drying of the desired, active material, i. e. neomycin.

A more generic description of adsorbent materials useful for separation of neomycin is material selected from the class consisting of active carbon and cation-exchange adsorbents; as indicated above, however, the cation-exchange materials have exhibited unusual effectiveness.

Neomycin, e. g. produced by methods of this kind,

has been found to possess a remarkably wide antibiotic spectrum, showing extremely useful activity against both gram-positive and gram-negative bacteria, and including significant activity against mycobacteria, both of the streptomycin-sensitive and the streptomycin-resistant types. Furthermore, in tests of a sort which have shown contrary results with streptomycin, there has been lesser development of resistance to neomycin among the tested organisms, such results tending to indicate the limited number of strains of such organisms which might be specifically resistant to neomycin as distinguished from streptomycin, for example.

The following table represents an antibiotic spectrum of neomycin, compared to those of streptomycin and streptothricin, the determinations for neomycin being made by the agar-streak method (i. e. an agar plate dilution method) with the isolated antibiotic material such as prepared in accordance with the procedure of the specific example set forth below. Specific values of activity in this table should of course be read with the understanding that the determinations may be improved or otherwise affected in degree upon further purification of the antibiotic, and especially with the understanding that different strains of the various test organisms may result in some differences in strength of activity. However, it is believed that the over-all nature or configuration of the spectrum is not specifically affected by such variations. For general purposes of identification of the antibiotic by the shape of its spectrum, it will likewise be understood that no particular meaning need be attached to a comparison of the absolute activities of neomycin on the one hand, and streptomycin or streptothricin on the other hand, i. e. for any given organism against which neomycin and one or both of the others are substantially active. The chief significance of the spectrum is to exhibit the over-all relationship, for each substance, of the various activities and inactivities. It may be noted, however, that according to present experience, neomycin is a highly active or potent substance, permitting economy in the amount required, and facilitating actual administration, when it may be used for control of various infections.

TABLE I

Antibiotic spectrum of crude neomycin, as compared to that of crude streptomycin and streptothricin

[Expressed in dilution units per gram of crude preparation required to inhibit growth of test organisms]

Organism	Neomycin ×1,000	Streptomycin ×1,000	Streptothricin ×1,000
1. *Bacillus subtilis*	150–750	125	125
2. *Bacillus mycoides*	20–150	20	<0.8
3. *Bacillus cereus*	20–60	30	<0.8
4. *Staphylococcus aureus*	100–250	15	50
5. *Sarcina lutea*	10	100	37.5
6. *Escherichia coli* SS	25	25	25
7. *Escherichia coli* RS	20	0	Active
8. *Pseudomonas aeruginosa*	2.5	1	<0.8
9. *Proteus vulgaris*	25	10	12.5
10. Bodenheimer's organism	15	0	Active
11. *Serratia marcescens*	20	25	1.2
12. *Mycobacterium tuberculosis hominis* 607 (SS)	80–250	Active	Active
13. *Mycobacterium tuberculosis hominis* 607 (RS)	50–150	Inactive	Active
14. *Mycobacterium avium*	50–150	Active	Active
15. *Mycobacterium ranae*	150	Active	Active
16. *Mycobacterium phlei*	300	100	12.5
17. *Trichophyton metagrophytes*	<0.3	<0.3	Active
18. *Cryptococcus albicans*	<0.3	<0.3	Active
19. *Penicillium notatum*	<0.3	<0.3	Active

In the above table and elsewhere herein, the symbol SS means a streptomycin-sensitive strain and RS, a streptomycin-resistant strain. It will be understood that the reference to dilution units (which are the figures listed in the table, multiplied by 1000 in each instance) expresses the number of times that the given preparation of the given antibiotic may be diluted and still inhibit growth of the organism under test. Thus in the table high values represent strong activity, while the very lowest values represent relative inactivity. For convenience and brevity, the several organisms in the table may be identified by the numbers arbitrarily assigned to them in the left-hand column. A standard unit of activity is that amount of material which will inhibit the growth of a selected standard organism under selected standard conditions, in one milliliter of medium.

As shown, neomycin is fully active against all of organisms numbered 1 to 16 inclusive in the table, including activity against Nos. 1 to 6 inclusive, 8, 9, 11, 12 and 14 to 16 inclusive, against which streptomycin is similarly effective, and in contrast with streptothricin which exhibits relatively small or no activity against organisms 2, 3, 8 and 11. On the other hand, while streptothricin is active against various fungi, e. g. organisms 17, 18 and 19 of the table, neomycin is relatively inactive. It may be noted in passing that whereas the numerical values in the table represent determinations made on a basis warranting numerical comparison, some of the activities, e. g. of streptomycin and streptothricin, were determined by tests of different grades of the substance or under other circumstances, and for the results of such other tests, the characteristics are simply reported as "active" or "inactive." It is believed that the characteristics of activity and inactivity thus reported are amply significant for purposes of comparison and spectral designation in the table.

An especially significant feature of the spectrum of neomycin, distinguishing it from streptomycin, is its activity relative to organisms 7, 10 and 13, i. e. of the same character of activity as toward many other organisms in the table, and in comparison with the relative inactivity of neomycin toward organisms 17, 18 and 19. Bodenheimer's organism, Number 10 in the table, has been employed in assaying antibiotic substances, specifically to determine streptothricin in the presence of streptomycin, the latter exhibiting relatively no activity toward this organism. Sensitivity of organisms 7 and 13 toward neomycin is of special utility, i. e. representing the capability of neomycin to control these streptomycin-resistant strains of bacteria. As will be appreciated, organisms 12 and 13 are among those responsible for tuberculosis in human beings, it being thus apparent that neomycin has a field of effectiveness, e. g. against resistant strains, to which streptomycin does not extend.

The antagonism of neomycin toward strains of organisms against which streptomycin is ineffective has been demonstrated in other tests beyond those summarized in Table I. Thus for example, when plates containing different concentrations of neomycin were streaked with streptomycin-sensitive, streptomycin-resistant and streptomycin-dependent strains of *E. coli,* the first two were found to be sensitive alike to neomycin while the last exhibited no growth at all. Upon conducting the same test with streptomycin, only the first strain of organism showed sensitivity to the antibiotic effect, the second organism being essentially unaffected and the third yielding the growth for which it requires (according to previous discovery) the presence of streptomycin. These tests are clearly significant of difference in the biological and chemical nature of neomycin, from streptomycin.

When broth or agar cultures of various bacteria containing sufficient neomycin to inhibit growth were incubated for relatively long periods of time, no further development of the bacteria occurred; thus the stability of the antibiotic was further demonstrated, in contrast to other substances such as chlortetracycline.

For additional evidence of the action of neomycin on mycobacteria, determinations were made of the sensitivity of various streptomycin-sensitive and streptomycin-resistant strains of these organisms by the use of turbidimetric procedures in the Dubos Tween medium. The following table summarizes the results of such tests with a number of organisms or strains of organisms of this type,

including the human pathogenic culture of *M. tuberculosis* H37Rv.

TABLE II

Effect of neomycin upon the growth of different strains of M. tuberculosis *in Dubos medium*

[Incubation at 37° C. for 14 days]

Test Organism	Growth inhibition units/ml.
M. avium (SS)	4.0
M. avium R (RS)	4.0
M. tuberculosis H37Rv (SS)	0.2
M. tuberculosis H37RvR (RS)	0.2
M. tuberculosis H37Rv (SS)	0.5–1.0
M. tuberculosis H37RvR (RS)	1.0
M. tuberculosis 607 (SS)	0.1
M. tuberculosis 607R (RS)	0.25

It was found that neomycin was active against all of the listed types of organisms, and as shown in the table, in each instance the streptomycin-resistant and streptomycin-sensitive strains were in effect equally sensitive to neomycin.

The fact that neomycin does not appear to favor the ready development of resistant cultures, i. e. strains of organism which are peculiarly resistant to neomycin, has been indicated by reliable tests. In one such set of tests a twenty-hour-old agar culture of *E. coli* was suspended in water and plated out in nutrient agar containing various amounts of neomycin. After 9 days incubation at 28° C., only one colony of a yeast, but not of the bacteria, developed out of 22 billion cells on a plate containing 25 u./ml. (units of activity per milliliter) of neomycin and only two colonies, likewise of yeasts and not bacteria, on a plate carrying 5 u./ml. of neomycin, no colonies at all being noted on a third plate carrying 10 u./ml. Thus it was evident that no resistant culture had been developed in any case. It was found later, however that a few colonies will develop in the presence of two to four units of neomycin, but much fewer (namely one-tenth to $\frac{1}{100}$) than in the presence of similar concentrations of streptomycin. When pieces of agar were removed from these plates and inoculated into sterile agar plates, only some of the pieces from the plate that had carried 5 u./ml. neomycin exhibited growth; the pieces from the plates that had contained 10 u./ml. and 25 u./ml. of neomycin yielded no growth at all. These, as well as other tests, reveal that neomycin is highly bactericidal as well as bacteriostatic.

Another and extremely important property of neomycin is that it appears to be relatively non-toxic, or to have extremely low toxicity. Tests on animals and similar tests (e. g. on egg embryos) have not only afforded confirmation, in such tests, of the relatively strong activity of neomycin against various bacteria, but have also indicated that the toxicity, if any, of the antibiotic to animals is at most very limited. In these tests the activity against bacteria has been shown with respect to both gram-positive and gram-negative organisms and against both streptomycin-sensitive and streptomycin-resistant organisms, examples being streptomycin-sensitive strains of *Staphylococcus aureus, Klebsiella pneumoniae,* and *Shigella pullorum* and streptomycin-resistant strains of *Salmonella schottmülleri* and *Staphylococcus aureus.* The tests revealed an effective capacity of neomycin to control each of these organisms. With respect to toxicity, the tests on animals, e. g. mice, indicated that no appreciably toxic or other adverse effects occured even though the treatment was increased to from 20 to 80 times the effective dose.

From what has been said hereinabove, it will be appreciated that the culture medium employed for production of the antibiotic may vary considerably, dependent on conditions, nutrient substances available and the like. Simply by way of example, the following are instances of media which have been employed for the cultivation of *Streptomyces fradiae* (No. 3535) and production of neomycin thereby:

CULTURE MEDIUM—EXAMPLE 1

Soy bean peptone	g—	20
Meat extract	g—	5
Glucose	g—	5–10
Sodium chloride	g—	5

One liter of tap water (as described above).

CULTURE MEDIUM—EXAMPLE 2

Soy bean peptone	g—	10
Meat extract	g—	5
Glucose	g—	10
Calcium carbonate (fine powder)	g—	10
Sodium chloride	g—	5

One liter of tap water (as described above).

CULTURE MEDIUM—EXAMPLE 3

Casein hydrolysate (NZ-amine)	g—	25
Beef extract	g—	10

One liter of tap water (as described above).

CULTURE MEDIUM—EXAMPLE 4

Casein hydrolysate (NZ-amine)	g—	25
Distiller's soluble	g—	20

One liter of tap water (as described above).

As explained above, the medium of Example 2, which has a relatively low peptone content and a relatively high glucose content, also includes a quantity of calcium carbonate to prevent possible undesirable effects of increased acidity during incubation. While each of the above media has been usefully employed, Examples 3 and 4 have been found to yield very superior results. In each case, shaken cultures were used. In each instance, the pH is adjusted to approximately 7 before inoculation with spores or submerged growth of the organism S. No. 3535.

By way of further and more specific example of a set of operations for separating and partially purifying neomycin, the following procedure is set forth, as shown in Fig. 1. After preliminary filtration to remove suspended solids, the culture medium or broth was acidified with concentrated hydrochloric acid, reducing its pH to a value of 2.0 to 2.5. Activated charcoal, specifically the kind identified as Darco G–60, was added in an amount of 5 grams per liter and the mixture stirred for about 30 minutes. Thereafter a suitable filter-aid, for example a material of the nature of diatomaceous earth, specifically a filtration-assisting material known as Hyflo Super-Cel, was stirred into the mixture, in the amount of 5 grams per liter, and the mixture was then filtered through a pad of the same material, e. g. Hyflo Super-Cel, in like amount. Since neomycin is not found to be adsorbed at strongly acid reaction, the result of this treatment with charcoal and subsequent filtration provided a preliminary removal of certain impurities, it being understood that an expedited process may in some cases omit this preliminary treatment.

The filtrate from the treatment just described thus constitutes a preliminary purified filtrate of culture broth and contains the desired neomycin. The pH of this solution was then adjusted to 7.1 ± 0.2 with 40% sodium hydroxide solution. An adsorbent material, specifically of the zeolite type (sodium-aluminum silicate) was then introduced, a suitable substance being the product known as Decalso. The amount of this adsorbent may vary, effective results having been had where it was introduced in a proportion of 1 gram for each 7000 activity units of neomycin in the solution. The mixture was then stirred for about 30 minutes and the adsorbate, i. e. the adsorbent-containing neomycin, was filtered off, washed with water and permitted to dry in air overnight.

The dried Decalso adsorbate was then placed in a column having an inside diameter of 30 mm. and fitted with a glass wool plug. A 10% aqueous ammonium chloride solution was percolated through the adsorbate (viz. the zeolite material containing the adsorbed neomycin) and successive fractions were collected for assay. Thus the neomycin was effectively eluted from the adsorbent. The assay of the several fractions can be effected either by the streak method using *E. coli,* or by the agar cup method, then conveniently using *B. subtilis.* The fractions having the highest potencies were combined to make a single body of solution and the pH of the mixture was adjusted to 7.0 A further quantity of activated charcoal, e. g. Darco G–60 in amount of 10 grams per 100 ml. was added and the mixture was stirred for 30 minutes. The adsorbate, viz. the charcoal-containing neomycin, was filtered off, washed with water and dried with air. The antibiotic was eluted from this adsorbate by stirring the latter for 30 minutes with 0.05 N hydrochloric acid in 50% methanol, using 5 ml. of the dilute acid alcohol per gram of the charcoal. The eluted charcoal was filtered off and subjected to a second elution in the same manner. The eluates were combined, the pH of the combination being then adjusted to 6.0–7.0 with aqueous sodium hydroxide. Such solution, containing the neomycin, was thereupon concentrated in vacuo to a volume of about 3 ml.

The described small residue of liquid, containing the antibiotic, was picked up in methanol; the resulting mixture was treated with 8 to 10 volumes of acetone to precipitate the neomycin. The precipitate was collected and dried. It was then treated with about 10 ml. of methanol and centrifuged to remove insoluble material. This material was again treated with 10–15 ml. of methanol. Again the insoluble residue was centrifuged off. The neomycin-containing solutions, viz. the supernatant liquids at the end of each centrifuging operation, were then treated with 8 to 10 volumes of acetone to precipitate the neomycin. The resulting suspensions were permitted to stand a few hours at 5° C., whereupon they were again centrifuged. The separated solid materials this time constituting the neomycin, were finally washed with acetone and dried in vacuo, to yield dry, rather finely divided products, constituting isolated and separated, though still somewhat crude bodies of the new antibiotic substance, neomycin. As indicated above, further purification steps may be followed, leading to a product of high purity corresponding to the quality of certain other antibiotics now commercially available. However, the product resulting from the procedure specifically described has been found to possess the new and desirable properties herein set forth, both antibiotically and otherwise.

For reference purposes herein, a unit of neomycin is the minimum amount of such substance that will completely inhibit the growth of a selected standard strain of *Escherichia coli,* namely *E. coli* A. T. C. C. 9637, in one ml. of nutrient agar.

By way of further example of the spectrum of neomycin, the following Table III presents a somewhat expanded or more elaborate comparison of the antibiotic spectra of neomycin and streptomycin, it being understood that the explanatory or qualifying remarks preceding Table I hereinabove apply equally here. In the case of Table III the values for neomycin were found with the somewhat crude complex, obtained as described above. In this table the quantities set forth are the amounts of the antibiotic required to inhibit growth of the respective organisms, in 1 ml. of culture, the neomycin values being given in units per ml. and the streptomycin values in micrograms (of pure streptomycin) per ml. Thus unlike Table I (wherein high numbers represented strong activity), Table III is directly expressed in quantities of antibiotic substance, so that low numerical values indicate strong activity of the substance, while very high values mean relative inactivity.

TABLE III

Comparative antibiotic spectra of neomycin and streptomycin

[Expressed in amounts required to inhibit growth of organism.]

Organism	Neomycin (units/ml.)	Streptomycin (micrograms/ml.)
Aerobacter aerogenes	0.625	0.5–2.5
Bacillus anthracis	0.156	0.375
B. mycoides	0.1–0.5	0.1–3.8
B. subtilis	0.02–0.1	0.12–1.0
Brucella abortus	1.25–5.0	0.5–3.75
B. melitensis	0.625–2.5	0.5
B. suis	0.312–2.5	0.5
Clostridium perfringens	>10.0	>104
Corynebacterium diphtheriae	0.156	0.375–3.75
Escherichia coli	1.25–2.5	0.3–3.75
E. coli RS	1.5–5.0	>1,000
Hemophilus influenzae	1.25–2.5	1.56–5.0
H. pertussis	2.5	1.25–3.0
Klebsiella pneumoniae	0.312–0.625	0.625–8.0
K. pneumoniae RS	0.312	>1,000
Malleomyces mallei	>10.0	10–>10.0
Mycobacterium avium	0.1–0.3	10
M. phlei	0.05–0.078	0.12
M. tuberculosis	<0.5	1.0–5.0
M. tuberculosis RS	<0.5	>100
Neisseria intracellularis	1.25–2.5	5.0
Pasteurella pestis	0.625	0.75–1.5
P. tularensis	0.156	0.15–0.3
Phytomonas pruni	0.1	0.25
Proteus vulgaris	1.25–2.5	0.4–3.0
Pseudomonas aeruginosa	12.5–25.0	2.5–25.0
Salmonella typhosa	0.1–0.625	1.0–37.5
S. schottmülleri	0.4–0.7	2.0
Sarcina lutea	2.5	0.25
Serratia marcescens	1.25	1.0
Shigella paradysenteriae	0.25–0.5	0.25–3.75
Staphylococcus aereus	0.156–0.625	0.5–>16.0
Streptococcus faecalis	5.0	50.0
Vibrio comma	2.5	6.0–37.5
Various fungi	>10.0	>10.0

A further inherent feature of the antimicrobial properties of neomycin is that it is inactive against Rickettsiae, such as Rocky Mountain spotted fever (*Rickettsia rickettsii*). Additional properties of neomycin, likewise inherently characteristic of the substance, are that it gives a negative response to test by the biuret reaction, that it is not inactivated by hydroxylamine or cysteine, and that its potency is not affected by sodium chloride, for example even when sodium chloride is present to the amount of 20% in solutions of neomycin used for antibacterial test. Neomycin gives positive Molisch and carbazole tests for carbohydrate residues and a negative Elson-Morgan test for glucosamine. It gives a positive ninhydrin test, and shows no acidic or carbonyl groups. It does not reduce Fehling's or Tollens' reagents. It contains no methoxyl groups.

Counter-current distributions of neomycin in a number of solvent systems and at various values of pH, have been made, using a hydrochloride of neomycin (prepared as described hereinabove) and employing the so-called Craig 24-plate counter-current distribution machine (see Craig, in J. Biol. Chem., vol. 155, page 519, for 1944). The distributions were effected between a buffered aqueous phase and pentasol, using stearic acid as carrier. Three systems that were employed respectively involved borate buffer at pH 7.3, 7.6 and 7.8, the procedure being as described by E. A. Swart (The use of counter-current distribution for the characterization of Streptomyces antibiotics, in Journal of the American Chemical Society, vol. 71, page 2942). Like distribution in the system of Plaut and McCormack (Journal Amer. Chem. Soc., vol. 71, page 2264, 1944) was also effected, i. e. between sodium bicarbonate buffer and pentasol, again using stearic acid as carrier. To provide reference points, streptomycin and streptothricin were distributed in the four systems, it being noted that the results with respect to principal, significant peaks in the distribution curves were the same for the three substances (neomycin, streptomycin and streptothricin) whethr distributed in a mixture or separately. As will be understood, the distribution in each case involved 24 transfers.

The distribution curves were plotted from suitable bio-assays of the contents of the 24 tubes in the machine; in every case it was found that each substance was represented by a single, major peak. The following summarizes the results obtained, it being noted that the stated bicarbonate-buffer system is inherently buffered at pH of approximately 7.6, which therefore need not be specified:

TABLE IV

Buffer	Location (Tube Number) of Peak		
	Neomycin	Streptomycin	Streptothricin
Borate at pH 7.3	14	3	2
Borate at pH 7.6	21	9	8
Borate at pH 7.8	22	16	14
Bicarbonate	15	21	8

The noted pattern of peaks for neomycin is distinctive and reproducible, and is understood to represent an inherent characteristic of such substance. A careful repetition of these distribution tests has also been made, with precisely the same solvent systems and the same distribution procedure. The peak concentrations of the several antibiotics (each being distributed separately in each of the solvent systems) were determined both by bio-assays and chemical assays. A standard cup-assay method was used for the biological assays. For the chemical analysis of neomycin and streptothricin distributions the ninhydrin reaction was employed, while the Sakaguchi method was used for the streptomycin distributions. The biological and chemical analyses were found to agree with each other perfectly. The distributions at pH 7.3 were made at temperatures (C.) of about 28° (neomycin), 35° (streptomycin) and 31° (streptothricin); those for pH 7.6 at about 29°; and the remainder at about 24° to 26°, except that the temperature of the streptothricin distribution at pH 7.8 was about 20°. The results of these twelve distributions of the three substances (each as hydrochloride) are shown by the following tabulation in the manner of Table IV:

TABLE V

Buffer	Location (Tube Number) of Peak		
	Neomycin	Streptomycin	Streptothricin
Borate at pH 7.3	13	6	3
Borate at pH 7.6	21	10	11
Borate at pH 7.8	22	18	14
Bicarbonate	14	21	9

As will be noted, Table V is in good agreement with Table IV, wherefore the approximate pattern of either table, especially as to the peak locations of neomycin and their general relation to the peak locations for the other antibiotics in the same systems, may be taken as a significant characteristic of neomycin. Minor variations of peak locations occurred, as are to be expected in scientific experiments.

As will be apaprent from description hereinabove, neomycin can be produced in the free base form, and also as acid salts, of which specific examples already noted or indicated are the hydrochloride, formate and picrate. Another such readily produced salt is the sulfate.

Neomycin has been found to contain, e. g. in its free base form, the following approximate proportions of the following elements, carbon 44.95%, hydrogen 7.55%, nitrogen 14.46%, oxygen 33.04%. This analysis indicates the following empirical formula:

$C_{29}H_{58}N_8O_{16}$

It will be understood that the acid salts, such as mentioned above, are formed in a conventional manner.

Studies have indicated that neomycin as produced by the specific procedures defined above usually consists very predominantly of a single specific compound which has been designated neomycin B. It has also been indicated that neomycin appears in what is understood to be an isomeric form, which has been called neomycin C. Each of the isomers is understood to have all of the chemical, physical and biological properties described hereinabove, including the stated empirical formula, the formation of acid salts as explained, and the significant antibiotic characteristics that have been pointed out. Methanolysis of neomycin hydrochloride has been found to yield two fragments. The first fragment is an amorphous hydrochloride which is devoid of reducing power, which yields all its nitrogen as amino nitrogen and which is understood to have the empirical formula:

$C_{12}H_{24}N_4O_6.4HCl$

This substance is called neamine and has also been called neomycin A, although it is not true neomycin but is a degradation product which exhibits relatively little antibiotic activity, by dilution tests, against gram-negative organisms (examples of such organisms being *S. marcescens*, Bodenheimer's organism, *E. coli* and *A. aerogenes*), i. e. in comparison with the activity of true neomycin toward gram-negative bacteria and also in comparison with the activity of neamine against gram-positive organisms.

The other fragment of methanolysis of neomycin is a compound called methyl neobiosaminide which upon acid hydrolysis yields a reducing diamine having (as at present understood) the empirical formula:

$C_6H_{14}N_2O_3.2HCl$

The remainder of the methyl neobiosaminide is a pentose as shown by the production of furfural on acid hydrolysis. The methyl neobiosaminide produced by methanolysis of neomycin C is an isomer of the same compound produced from neomycin B and the resulting diamines are similarly isomers of each other. As indicated, neomycin produced by the specific procedures outlined above is usually found to consist very largely of neomycin B, accompanied in most cases by a small proportion of neomycin C. Neamine is distinguished from true neomycin in a number of ways, e. g. as indicated above, and also in that upon counter-current distribution in the borate-buffer-pentasol-stearic acid system at pH 7.6, it exhibits a peak at about tube 15 (24 transfers) whereas true neomycin appears at about tube 21.

The optical rotation, $[\alpha]^{D}_{25}$, of neomycin B hydrochloride is +54°, neomycin B sulfate +58°, and neomycin C hydrochloride +80° (all rotation measurements taken in water, c.=0.5%).

Infra-red absorption spectra have been obtained with respect to neomycin, in various forms, and are illustrated in the drawings, Figs. 2 and 3 showing the spectrum of the free base respectively for the forms of neomycin B and neomycin C, Figs. 4 and 5 showing the spectra for neomycin B and neomycin C sulfates, and Figs. 6 and 7 showing the spectra for neomycin B and neomycin hydrochlorides. It will be noted at the outset that the spectra for neomycin B and neomycin C are very closely alike, indicating their near-identity and presenting what are believed to be a number of distinguishing characteristics (the same for both compounds) in relation to other substances.

These spectra were made with the respective compounds embodied in pressed potassium bromide disks, i. e. by taking mixtures of the particular neomycin compound in each case, with finely divided potassium bromide, the intimate, uniform admixture being compressed to a disk or tablet of appropriately transparent or translucent character for infra-red radiation. The spectra were taken in conventional fashion, with various points on the curves representing percent transmission and the

curves being extended through a wide range of frequencies or wave lengths. The frequencies are conveniently stated in reciprocal centimeters ($cm.^{-1}$). Translation of the values to wave length in microns can be readily effected, if desired, in conventional manner.

It is noted that each of the spectra, i. e. for the free base, sulfate and hydrochloride of both neomycin B and neomycin C, exhibits broad intense absorption maximum bands at 3600 to 2400 $cm.^{-1}$ and 1200 to 900 $cm.^{-1}$. These bands appear to be distinctively characteristic of neomycin.

By way of preliminary chemical interpretation of the spectrum, it may be noted that in the case of the free base spectra, the strong broad absorption extending over the spectral range 3800 $cm.^{-1}$ to 2200 $cm.^{-1}$ indicates hydrogen bonded OH and NH groupings. Likewise in the free base spectra, absorption near 1590 $cm.^{-1}$ demonstrates at least a part of the nitrogen in the compound to be in primary amino grouping or groupings. A strong absorption in the vicinity of 1000 $cm.^{-1}$ confirms the presence of OH groupings in the structure.

In addition to the broad bands mentioned above, further details of significant features of the spectra are as follows, again expressed as approximate locations of maxima and minima at frequencies designated in reciprocal centimeters:

With respect to the spectra for the neomycin free base (Figs. 2 and 3) both neomycin B and neomycin C show intense absorption maxima at 2900, 1460 and 1015 $cm.^{-1}$, and absorption minima at 2890, 1525, 1425, 1210 and 800 $cm.^{-1}$. The neomycin B free base shows intense absorption maxima at 3380, 1583 and 1325 $cm.^{-1}$, and a distinct absorption maximum at 1378 $cm.^{-1}$. Neomycin C free base shows intense absorption maxima at 3340, 1590 and 1335 $cm.^{-1}$. In addition, it may be noted that the absorptions around 3150 $cm.^{-1}$ and around 1040 $cm.^{-1}$ are stronger for neomycin B than for neomycin C.

With respect to the spectra for neomycin sulfate (Figs. 4 and 5) both neomycin B and neomycin C show intense absorption maxima at 3400, 2910, 1625 and 1525 $cm.^{-1}$, and absorption minima at 2250, 1570 and 800 $cm.^{-1}$. Neomycin B sulfate shows intense absorption with a single maximum at 1110 $cm.^{-1}$, whereas neomycin C sulfate shows two absorption maxima respectively at 1120 and 1100 $cm.^{-1}$. Neomycin B sulfate also shows weak absorption maxima at 1463, 1408, 1381 and 1337 $cm.^{-1}$. It is also observed that neomycin B sulfate shows relatively stronger absorption near 3240 and 3105 $cm.^{-1}$, than neomycin C sulfate.

With respect to the spectra for neomycin hydrochloride (Figs. 6 and 7) both neomycin B and neomycin C show intense absorption maxima at 3400, 2900 and 1500 $cm.^{-1}$, and absorption minima at 1750, 1150, 1430 and 800 $cm.^{-1}$. Neomycin B hydrochloride shows intense absorption maxima at 1595 and 1010 $cm.^{-1}$ while neomycin C hydrochloride shows such maxima at 1603 and 1025 $cm.^{-1}$. In addition, neomycin B hydrochloride exhibits weak absorption maxima at 1381, 1325 and 812 $cm.^{-1}$, while neomycin C hydrochloride has a weak absorption maximum at 1343 $cm.^{-1}$. It also appears that neomycin B hydrochloride has relatively stronger absorption near 3220 $cm.^{-1}$ and in the vicinity of 2550 $cm.^{-1}$ than neomycin C hydrochloride.

As indicated above, purification of neomycin preparations may include chromatography, which may be utilized to effect a separation of neomycin C in purified form, relative to neomycin B. For example, upon subjecting neomycin which contains a major amount of B and a minor amount of C (e. g. produced as explained above), in appropriate acid salt form, to carbon chromatography, the major eluate fraction contains neomycin B, while a small fraction eluted prior to the main fraction removes neomycin C, which may thus be obtained in purified form and in quantity, from the collected preliminary fractions of a considerable number of carbon chromatograms. By way of example, a suitable column can be prepared, say to a bed depth of 42 inches, with an acidified aqueous slurry of 2 kilograms of activated carbon (Darco G–60) and one kilogram of diatomaceous earth. 400 grams of neomycin sulfate (containing both B and C) are prepared in an aqueous solution, 760 ml., at pH 2.5 (sulfuric acid), with the addition of some of the same materials as used in the column (total 300 g.) to form a slurry. The slurry is introduced into the top of the column and the column is developed with water, with the active fractions collected in 50 ml. cuts. After withdrawal of optically inactive liquid representing liquid holdup, the active material (in the further eluate fractions) is identified by optical rotation and represents first a small eluate containing neomycin C, and a main eluate from which neomycin B (sulfate) is recovered by suitable drying. The small neomycin C sulfate fractions recovered from a considerable number of carbon chromatograms as described above are combined and subjected to appropriate further purification, including carbon chromatography of exactly the same kind, yielding, from the optically significant and uniform fractions, a purified product of neomycin C sulfate (optical rotation, $+82°$).

It will be understood that where neomycin is produced in the form of a particular acid salt, the free base form may be obtained by suitable or conventional procedure, and other acid salts readily obtained from the free base. For instance, by treating the sulfate with a strongly basic anion exchange resin, the neomycin base is obtained. From the latter, other salts may be produced, for example, the hydrochloride being obtained by dissolving the base in dilute hydrochloric acid. These operations are equally applicable to neomycin in its B, C or mixed forms.

It will be understood that other procedures may be employed for the production of neomycin, and for its isolation and purification, for instance in accordance with variations of the sort indicated hereinabove, as well as in other ways consonant with the nature of the substance and its characteristics of solubility and of response to reagents appropriate for use in procedures of this general character. It is therefore to be understood that the invention is not limited to the specific steps and compositions herein described but may be embodied in other ways without departure from its spirit.

This application is a continuation-in-part of our copending application Serial No. 526,753, filed August 5, 1955, which was in turn a continuation-in-part of an application that was copending therewith, viz. Serial No. 131,686, filed December 7, 1949, now abandoned. Said last-mentioned application, Serial No. 131,686, was in turn a continuation-in-part of an application that was copending therewith, viz. Serial No. 76,184, filed February 12, 1949, now abandoned.

We claim:

1. In a process for producing the antibiotic substance neomycin, the steps of growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous medium favorable to such production and containing peptone material and a nutrient extract, to form said substance, said medium including a minor mineral content favorable to the production of neomycin, and separating the substance from the culture medium.

2. In a process for producing the antibiotic substance neomycin, the steps of growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous medium favorable to such production and containing peptone material, a nutrient extract, and a small amount of combined zinc, to form said substance, and separating the substance from the culture medium.

3. In a process for producing the antibiotic substance neomycin, the steps of forming said substance by growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous nutrient medium favorable to such production and containing a small amount of

combined zinc, and separating the substance from the culture medium.

4. In a process for producing the antibiotic substance neomycin, the steps of forming said substance by growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous nutrient medium favorable to such production and containing glucose, said medium also including peptone material in sufficient amount to prevent the retardation of neomycin production which might otherwise be the effect of the glucose, said medium including a minor mineral content favorable to the production of neomycin, and separating the substance from the culture medium.

5. In a process for producing the antibiotic substance neomycin, the steps of forming the substance by growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous nutrient medium favorable to such production and containing glucose in amount greater than 5 grams per liter, said medium also including calcium carbonate to prevent the retardation of neomycin production which might otherwise be the effect of the glucose, the aqueous content of said medium being tap water, and separating the substance from the culture medium.

6. In a process for producing the antibiotic substance neomycin, the steps of forming the substance by growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous medium favorable to such production and containing peptone material, material of the class consisting of meat extract and yeast extract, and a small amount of combined zinc, and separating the substance from the culture medium.

7. In a process for producing the antibiotic substance neomycin, the steps of growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous nutrient medium favorable to such production and under submerged conditions with aeration, to form said substance, while controlling the extent of aeration to a limited value to provide an essentially maximum production for the aforesaid culture and conditions, said medium including a minor mineral content favorable to the production of neomycin, and separating the substance from the culture medium.

8. Procedure for recovering neomycin from a culture medium in which it has been produced, comprising reducing the pH of the neomycin-containing medium to a strongly acid value, treating the medium with an adsorbent to adsorb impurities therefrom, separating the treated medium from said adsorbent, adjusting the pH of said separated medium to a value at least as high as about 7, treating said adjusted medium with an adsorbent to adsorb neomycin therefrom, and eluting neomycin from said last-mentioned adsorbent.

9. In a process for producing the antibiotic substance neomycin by growing a culture of a neomycin-producing organism under conditions favorable to the formation of neomycin, the steps in separating the substance from the culture medium containing it, which comprise preliminarily purifying the medium by acidulating it to a pH of about 2, treating it with an adsorbent to adsorb impurities therefrom, and separating the treated medium from said adsorbent, and thereafter establishing the pH of the medium at a value at least as high as about 7, treating the medium with an adsorbent to adsorb the substance, and eluting the substance from the adsorbent with an aqueous solvent.

10. Procedure for recovering neomycin from a culture medium in which it has been produced, comprising acidulating the medium to a pH of about 2, treating it with active carbon to adsorb impurities therefrom, filtering the impurity-containing carbon from the medium, thereafter adjusting the pH of the medium to a value of about 7, treating the neomycin-containing medium with a cation-exchange adsorbent material to adsorb neomycin therefrom, and eluting neomycin from the adsorbent material.

11. In a process for producing the antibiotic substance neomycin, the steps of growing a culture of a neomycin-producing strain of *Streptomyces fradiae,* under submerged aerobic conditions, in an aqueous medium favorable to such production and containing nutrient material, to form said substance, said medium including a minor mineral content favorable to the production of neomycin, and separating the substance from the culture medium.

12. In a process for producing the antibiotic substance neomycin, the steps of growing a culture of a neomycin-producing strain of *Streptomyces fradiae* in an aqueous, nutrient-containing medium favorable to such production, having a pH greater than 6, and containing a source of nitrogen, to form said substance, said medium including a minor mineral content favorable to the production of neomycin, and separating the substance from the culture medium.

13. Procedure for recovering neomycin from a culture medium in which it has been produced, comprising acidulating the medium to a pH of about 2, treating it with active carbon to adsorb impurities therefrom, filtering the impurity-containing carbon from the medium, thereafter adjusting the pH of the medium to a value of about 7, treating the neomycin-containing medium with active carbon to adsorb neomycin therefrom, and eluting neomycin from the adsorbent material.

14. The method of producing neomycin comprising culturing a neomycin-producing strain of *Streptomyces fradiae,* adjusting the pH of the culture medium to about 2.0, filtering the acidified culture medium, neutralizing the filtrate, adding activated carbon to the neutralized filtrate to adsorb the antibiotic, and eluting the antibiotic from the charcoal with an acid solution.

15. The method of producing neomycin comprising culturing a neomycin-producing strain of *Streptomyces fardiae,* adjusting the pH of the culture medium to about 2.0, then treating the acidified culture medium with active carbon to adsorb impurities therefrom, filtering the acidified culture medium, neutralizing the filtrate, adding activated carbon to the neutralized filtrate to adsorb the antibiotic, and eluting the antibiotic from the second-mentioned carbon with an acid solution.

16. The method of producing neomycin comprising culturing a neomycin-producing strain of *Streptomyces fradiae,* adjusting the pH of the culture medium to about 2.0, filtering the acidified culture medium, neutralizing the filtrate, adding an adsorbent to the neutralized filtrate to adsorb neomycin therefrom, and eluting neomycin from said adsorbent.

17. The method of producing neomycin comprising culturing a neomycin-producing strain of *Streptomyces fradiae,* adjusting the pH of the culture medium to about 2.0, then treating the acidified culture medium with an adsorbent to adsorb impurities therefrom, filtering the acidified culture medium, neutralizing the filtrate, adding an adsorbent to the neutralized filtrate to adsorb neomycin therefrom, and eluting neomycin from said last-mentioned adsorbent.

18. An organic antibiotic composition selected from the group consisting of neomycin B, neomycin C and the acid salts of said neomycins, each of said neomycins being a substance: which is thermostable, soluble in water, dilute hydrochloric acid and acid-alcohol of low acid normality, insoluble in ether, chloroform and acetone, essentially non-toxic to animals in amounts effective to control infection, stable in aqueous solution for at least one month, and incapable of inactivation by hydroxylamine; and which is characterized by: negative reaction to test for guanidine; failure to give the maltol reaction; negative responses to the biuret reaction test and to test for glucosamine; positive responses to the ninhydrin test and to tests for carbohydrate residues; absence of acidic, carbonyl and methoxyl groups; production, on methanolysis,

of neamine and neobiosaminide; containing the following elements in the following approximate proportions, C. 44.95%, H 7.55%, N 14.46%, O 33.04%; substantial antibiotic activity against

B. subtilis
B. mycoides
B. cereus
S. marcescens
Bodenheimer's organism

and against both streptomycin-sensitive and streptomycin-resistant strains of

E. coli
S. aureus
M. tuberculosis
M. avium

and relative inactivity against fungi and *Rickettsia rickettsii*; further properties of said neomycins being that: neomycin B hydrochloride and neomycin C hydrochloride exhibit optical rotation, in water, of +54° and +80° respectively; and said neomycins have characteristic infra-red absorption, observed with the substances in pressed potassium bromide disks, and measured in frequencies of the infra-red region expressed in reciprocal centimeters, as follows: both of said neomycins in free base form and their hydrochlorides exhibit broad intense absorption maximum bands at 3600 to 2400 $cm.^{-1}$ and 1200 to 900 $cm.^{-1}$; each of said neomycins in free base form shows

intense absorption maxima at 2900, 1460 and 1015 $cm.^{-1}$, and absorption minima at 2980, 1525, 1425, 1210 and 800 $cm.^{-1}$; neomycin B hydrochloride and neomycin C hydrochloride each have intense absorption maxima at 3400, 2900 and 1500 $cm.^{-1}$, and absorption minima at 1750, 1550, 1430 and 800 $cm.^{-1}$; neomycin B in free base form has intense absorption maxima at 3380, 1583 and 1325 $cm.^{-1}$ and a distinct absorption maximum at 1378 $cm.^{-1}$; neomycin C in free base form has intense absorption maxima at 3340, 1590 and 1335 $cm.^{-1}$; neomycin B hydrochloride has intense absorption maxima at 1595 and 1010 $cm.^{-1}$ and weak absorption maxima at 1381, 1325 and 812 $cm.^{-1}$; and neomycin C hydrochloride has intense absorption maxima at 1603 and 1025 $cm.^{-1}$ and a weak absorption maximum at 1343 $cm.^{-1}$.

19. Neomycin B free base, as defined in claim 18.

20. An acid salt of neomycin B, as defined in claim 18.

21. Neomycin B hydrochloride, as defined in claim 18.

22. Neomycin B formate, as defined in claim 18.

23. A method of producing neomycin in high yields without the use of added buffering agent, which comprises culturing *Streptomyces fradiae* in contact with an aqueous nutrient medium essentially comprising an assimilable source of nitrogenous and growth-promoting substances and an assimilable carbohydrate component, a substantial proportion of said carbohydrate component being maltose.

No references cited.

U. S. DEPARTMENT OF COMMERCE

PATENT OFFICE

CERTIFICATE OF CORRECTION

Patent No. 2,799,620 July 16, 1957

Selma A. Waksman et al.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction and that the said Letters Patent should read as corrected below.

Column 1, line 44, for "resistant of" read -- resistant to --; column 14, line 60, after "neomycin", second occurrence, insert -- C --; column 15, line 30, for "2890" read -- 2980 --; line 53, for "1150" read -- 1550 --.

Signed and sealed this 8th day of April 1958.

(SEAL)

Attest:

KARL H. AXLINE

Attesting Officer

ROBERT C. WATSON

Commissioner of Patents

Patented Feb. 5, 1946 2,394,031

UNITED STATES PATENT OFFICE

2,394,031

PROCESS FOR THE PRODUCTION OF CITRIC ACID

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No Drawing. Application March 14, 1942, Serial No. 434,786

13 Claims. (Cl. 195—36)

This invention relates to fermentation and to processes of fermentation for the production of citric acid.

The production of citric acid by fungi, particularly by certain species of Penicillium and by strains of *Aspergillus niger*, has been long known, but the obtainment of commercial quantities of citric acid by fermentation has hitherto required the practice of surface or shallow processes, which necessitated the growth of the fungi in a stationary position for the formation of a surface pellicle. Surface processes of fermentation are disadvantageous in that they require an extensive plant, and are therefore generally less efficient industrially than deep or submerged processes.

Prior to the present invention, a satisfactory economic industrial process for the production of citric acid, substantially free from other acids, by the submerged growth of fungi, has not been achieved. Wells and Ward, of the Bureau of Chemistry and Soils, United States Department of Agriculture, in "Industrial and Engineering Chemistry," vol. 31, February, 1939, page 175, considered the prevailing evidence of the impossibility of developing a submerged fermentation process for citric acid, stating:

"Considerable research effort has been expended on attempts to develop a submerged fermentation process for citric acid, and undoubtedly a large amount of unpublished work has been done on the problem. All reliable evidence indicates the impossibility of such technique. The reason for this failure is not definitely known, but it appears likely that some vital derangement in the enzyme system is responsible."

An object of this invention is to provide processes for the successful production of citric acid on an industrial scale by submerged fungal growth.

Another object of the invention is the utilization, in a submerged condition, of a species of Aspergillus, namely, *A. wentii*, which has not been hitherto employed in citric acid fermentation processes.

A further object is the utilization of this fungus in a form of growth peculiarly adapted to intense fermentative activity while in a submerged state.

Another object is to provide a comparatively simple and easily operable process for the fermentation of carbohydrates by means of this fungus, in a submerged condition, in the presence of suitable nutritional materials, and under such other environmental requirements of agitation, aeration, pressure and temperature, as are most conducive to a high yield of citric acid to the substantial exclusion of other acids.

Other objects will be apparent and a fuller understanding of this invention will be had by reference to the following description and claims.

In the present invention the applicants have developed a number of important procedures for the specific production of citric acid by the submerged process. Although each of these procedures may be modified from the exact specifications given herein, they are effective as a group and preferably as a sequence for the success of this process.

I. *Choice of organism.*—The applicants have found that, among the numerous organisms capable of producing citric acid and belonging to the Aspergillus and Penicillium groups, none is so effective as a strain of *Aspergillus wentii*.

The organism *Aspergillus wentii*, which one of these applicants isolated from the soil in 1915, was first described by Wehmer in 1896. Thom and Church more recently described it in "The Aspergilli" (Williams and Wilkins Co., Baltimore, Md., 1926).

An agar medium which is especially favorable to the development of *A. wentii* in the isolation and the identification of this organism and in the production of abundant spore material is one containing 5 grams of peptone, 10 grams of glucose, 1 gram of potassium dihydrophosphate, ½ gram of crystalline magnesium sulfate, and 15 to 25 grams of agar, per liter. The medium is sterilized at 10 pounds pressure for 30 minutes, and inoculated with a tube culture of *A. wentii*. The cultures are incubated, at 25° to 35° C., for about 7 to 20 days.

Cane sugar, or purified molasses containing an equivalent quantity of fermentable carbohydrates, may be used instead of glucose in an agar medium for the production of cell material and spores.

This organism, *A. wentii*, has three distinct advantages over the *Aspergillus niger* group, namely: (1) it grows rapidly under submerged conditions, as well as under surface conditions; (2) it produces citric acid abundantly; (3) it has only a slight tendency to produce oxalic and gluconic acids, and this tendency is substantially suppressed under the conditions of the processes herein described. A further advantage which *A. wentii* has is that it can withstand high acidity, whereas the members of Penicillium usually cannot. These facts, in relation to *A. wentii* and *A. niger*, are brought out in the following two illustrations:

(a) In the appended table is indicated the influence of the duration of the incubation upon citric acid production by *A. wentii* in comparison with the acid production by a vigorous strain of *A. niger*, measured in terms of the alkali required for the neutralization of the acid produced:

Days of growth	Ml. of 0.25N NaOH needed to neutralize 5 ml. of the culture solution	
	A. wentii	*A. niger*
3	1.2	0.7
5	2.9	1.0
9	4.3	1.3

(b) The following tabulation presents the relative data as to the acid-production capacity of *A. wentii* and various citric-acid-producing strains of *A. niger*, under comparable conditions:

Organism	Total acid produced, ml. of 0.25N NaOH needed to neutralize 5 ml. of culture solution	Per cent conversion of sugar to acid
A. wentii	9.5	25.8
A. niger 5	2.0	5.5
A. niger 6	1.3	3.2
A. niger 7	1.7	4.8
A. niger 9	0.6	1.4
A. niger 29	5.4	13.0
A. niger 32	1.7	4.8
A. niger 35	2.1	6.2
A. niger 37	0.1	
A. niger 39	2.6	8.2
A. niger 40	0.7	1.8

The numbers given to *A. niger* in this table are laboratory numbers of different cultures.

The differences found in the capacity to produce citric acid per se were actually greater than those shown in the foregoing illustrations of total acid production, because many of the *A. niger* strains produced oxalic acid as well as citric acid, whereas *A. wentii* produced practically only citric acid.

II. *Composition of medium.*—From the results of a large number of experiments it was found that a very favorable medium for the growth of *A. wentii* and the production of citric acid by this organism, under submerged conditions, is one having the following composition:

Cane sugar	grams	150
Urea	do	1.0
Magnesium sulfate ($MgSO_4.7H_2O$)	do	0.5
Potassium dihydrophosphate (KH_2PO_4)	do	0.08
Potassium chloride	do	0.15
Manganese sulfate ($MnSO_4.7H_2O$)	do	0.02
Zinc sulfate ($ZnSO_4.7H_2O$)	do	0.01
Water to make	cc	1000

It has been found experimentally that urea can be used as a very satisfactory source of nitrogen. Glucose can be used in place of cane sugar.

III. *Acidification of medium.*—The pH of the medium is adjusted to 2.0 with hydrochloric acid, or other acid, just before the inoculation with the spores of *A. wentii*, in view of the fact that it has been found that a pH of about 2.0 to 3.0 is the optimum factor for the growth of the fungus in the most desirable vegetative type, for the production of citric acid by these submerged processes, and that this pH range is also a factor which is conducive to a substantially complete suppression of oxalic acid formation.

IV. *Oxygenation and agitation of medium.*—The results obtained by the applicants in studies of the effect of aeration of the medium described under "Composition of medium" by means of oxygen and of air, and of the effect of the absence of aeration, upon the production of citric acid by *A. wentii*, under submerged conditions and appropriate gentle agitation, at a temperature of 28° C., are illustrated by the following tabulation:

Days of fermentation	Acid produced by treatment with (titratable acidity, Ml)—		
	Oxygen	Air	No aeration
5	3.0	1.2	0.9
7	4.1	1.4	1.1
12	7.4	2.0	1.4

Agitation of the culture medium is requisite throughout the course of the processes herein described, and, depending on the type of the chamber in which the fermentation is conducted, may be brought about in any suitable way, as by gentle stirring, rotating, rocking, or shaking, or by a combination of such procedures.

V. *Conditions of incubation.*—In the preparation of cultures of *A. wentii*, an optimum temperature for the incubation of the spores has been found to be 25° to 35° C., although temperatures of about 18° to 37° C. may be employed. The period of incubation varies from 7 to 15 days.

VI. *Form of fungus growth.*—In the attainment of the best results in every phase of the process, and in the development of the fungus growth which is most suitable in the practice of the process, it is important to obtain a granular, preferably a pearl-like or bead-like, type of growth. Flocculent and filamentous growth greatly reduces the effectiveness of the fungus. The best growth is bead-like in size and form, and is gray to yellowish or almost greenish in color. The formation and maintenance of this type of growth, which is most active in producing citric acid under submerged conditions, is governed primarily by the four factors outlined under I–IV. The optimum stage of development of the fungal growth can be determined by examination of the bead-like growth and the size of the beads, and by observation of their color.

VII. *Replacement of medium.*—When the optimum growth stage of the fungus has been substantially attained, the residue of the original medium is replaced. From the results of a large number of experiments, it has been found that a favorable medium for replacement is one having the following composition, which is adjusted to a pH of 2.0:

Cane sugar	grams	150
Urea	do	0.5
Potassium dihydrophosphate	do	0.05
Potassium chloride	do	0.15
Manganese sulfate ($MnSO_4.4H_2O$)	do	0.02
Zinc sulfate ($ZnSO_4.7H_2O$)	do	0.01
Water to make	cc	1000

This replacement medium may also contain magnesium sulfate, and glucose can be used instead of cane sugar.

Such a replacement of the medium effects considerable savings in the time and the sugar required to produce the fungal growth. The production of citric acid sets in immediately, and much higher yields are obtained, as the follow-

ing example of the production of citric acid by *A. wentii*, submerged during the growth stage and the replacement stage, illustrates:

Days of fermentation	Growth stage (medium as described under "Composition of medium")		Replacement stage (medium as described under: "Replacement of medium")	
	Acid produced, ml. of 0.25N NaOH needed to neutralize 5 ml. of culture solution	Sugar converted, per cent	Acid produced, ml. of 0.25N NaOH needed to neutralize 5 ml. of culture solution	Sugar converted, per cent
4	0.3		5.2	32.9
6	5.4	51.5	9.3	41.2
8	9.9	53.2	12.2	47.1
10	11.6	52.9	15.2	52.2

VIII. *Partial neutralization of acid.*—It has been found that either calcium carbonate or calcium hydroxide is decidedly helpful as a neutralizing agent in the practice of this process, since oxalic acid is produced when caustic soda or caustic potash is used as a neutralizing agent. Partial neutralization up to about one-third of the titratable acidity of the medium can be carried out with such calcium compound as a means of increasing the production of the citric acid. The pH should not exceed 3.0.

IX. *Effect of combining the steps related.*—It has been further found that a high yield of citric acid can be obtained by combining the several treatments already indicated. This is brought out in the following figures of the yields of citric acid obtained by the action of a submerged growth of *A. wentii* in a partially neutralized sugar medium:

Days of fermentation	Citric acid produced	Sugar consumed (total sugar in medium, 15 gm.)	Conversion of sugar to acid
	Grams	*Grams*	*Percent*
3	3.46	8.06	42.9
5	5.01	11.44	43.9
7	7.13	13.58	52.5

X. *Production of pure citric acid.*—In the industrial practice of a submerged process for producing citric acid, it is highly essential that only this acid is formed. In this connection, it has been found that the degree or the extent of the neutralization of the citric acid formed during the fermentation, and the kind of neutralizing agent used, control the result in that they prevent the formation of other acids. When caustic soda or caustic potash is used for the neutralization of the acid, there is a pronounced inducement of oxalic acid formation.

XI. *Use of molasses in place of sugar.*—Molasses can be used in place of sugar for the production of citric acid by the submerged process of fermentation herein described. The use of a purified, or "high-test," molasses is preferred, however, as a means of producing large and economical yields of citric acid, inasmuch as certain types of grades of molasses have been found to contain various substances which may tend to impair the fullest activity of the process. The "purification" of the molasses is mainly directed to the removal of most of the ash or mineral as well as some of the nitrogen compounds found in varying amounts in some molasses.

The following composition, adjusted to pH 2.0, provides a satisfactory medium utilizing a purified molasses:

Purified molasses, equivalent to total sugars of	grams	150
Urea	do	1.0
Magnesium sulfate ($MgSO_4.7H_2O$)	do	0.5
Potassium dihydrophosphate	do	0.08
Potassium chloride	do	0.15
Manganese sulfate ($MnSO_4.4H_2O$)	do	0.02
Zinc sulfate ($ZnSO_4.7H_2O$)	do	0.01
Water to make	cc	1000

XII. *Purification of molasses.*—In view of the fact that certain grades of molasses afford comparatively low yields of citric acid in the submerged process, it has been found that such molasses can be advantageously purified by various agents, such as by bauxite, bone char, "Suchar CSP" (activated carbon), and by certain resins which are made available under the name of "Amberlites." Molasses, purified by any one or by two or more of these purifying agents, affords an increased yield of citric acid, during both the growth phase and the replacement phase of the fermentation process.

The "Amberlites" are synthetic ion-exchange resins. "Amberlite" 1R1 is a cation-exchange resin of the phenol-formaldehyde type, containing free phenolic hydroxyl groups. "Amberlite" 1R4 is an anion-exchange resin of the amine-formaldehyde type, containing amino groups capable of forming amine salts.

The following tables illustrate the effect of the purification of molasses upon the yield of citric acid, as compared with the yield of citric acid from cane sugar, in processes of submerged fermentation by *A. wentii:*

Table I

	Acid produced after 8 days' incubation. Ml. of 0.25N NaOH needed to neutralize 5 ml. of culture solution	Sugar converted to citric acid
		Per cent
Untreated molasses	3.3	7.2
Molasses filtered twice through bone char	7.5	20.5
Molasses filtered through resins	8.8	28.0
Cane sugar	13.2	37.6

Table II

	Titratable acidity, cc. 0.25N NaOH	Sugar consumed	Sugar converted to citric acid
		Grams	*Per cent*
Untreated molasses	9.0	15.00	19.4
Molasses previously treated with bone char, "Suchar," and resins	21.7	11.33	61.9
Cane sugar	23.35	11.25	67.1

Fermentation 12 days.

The process of producing citric acid by *A. wentii* under submerged conditions can be conducted by the following steps preferably in their sequence and combination:

Example I

Aspergillus wentii is first grown on a suitable agar medium, such as that which has been indicated herein, for a period of 7 to 20 days, for the production of an abundance of spore mate-

rial. One of the described media for the production of citric acid is now sterilized, adjusted to pH 2.0, and inoculated with a heavy suspension of spores in sterile water. The inoculated medium is then placed in a suitable chamber, or the medium may be sterilized in the chamber and then inoculated. Any chamber having an inner surface of enamel, or stainless steel, or other metal resistant to the medium and to citric acid, may be used. Next, the growth phase is initiated. The contents of the chamber are now continuously agitated, while either air under pressure, or oxygen at atmospheric pressure, or a mixture of oxygen and air under pressure, is simultaneously admitted into the chamber. The contents of the chamber are maintained at a temperature of about 25° to 35° C. As soon as a substantial quantity of citric acid has been formed, a sterile suspension of calcium carbonate or calcium hydroxide is added to neutralize such a quantity of the formed acid that the pH does not exceed 3.0. Agitation and aeration are continued for a period of 7 to 15 days. When the fungal growth has attained an optimum stage of development, as previously indicated, and when most of the sugar has been converted to citric acid, the acid solution is withdrawn from the chamber, and the replacement phase is begun. The withdrawn solution is replaced with a supply of the replacement medium. The fermentation is resumed for another 5 to 10 days, under similar conditions. Further such replacements are made as long as the growth of the fungus continues to be adequately operative.

Example II

Cuban invert molasses is diluted with distilled water to afford a solution containing about 15 per cent of total carbohydrates. The solution is treated with bone char, "Suchar CSP," and "Amberlites" 1R1 and 1R4. The pale yellow liquor is decanted or filtered from the resulting mixture. To this liquor are now added urea and inorganic salts in the concentrations indicated in the foregoing formula under the "Use of molasses in place of sugar." The medium thus prepared is sterilized by heat, adjusted to a pH of 2.0, and inoculated with the cell material of *Aspergillus wentii* previously developed on a cane sugar or other carbohydrate medium. Submerged fermentation in a deep culture tank is then conducted, under continuous agitation and aeration under pressure or oxygenation at atmospheric pressure, at a temperature of about 25° to 35° C. and at a pH value not exceeding 3.00, as described in the foregoing example. Practically all the carbohydrate is converted into citric acid in about 12 days.

The references herein to "sugar," "cane sugar," "glucose," and "carbohydrate" are intended to imply the operativeness in these processes of all monosaccharides and disaccharides, or mixtures thereof. Mixtures of monosaccharides, disaccharides and starch or starches can also be used. Starch or starches, without sugar, can be utilized, due regard being had to the viscosity of a starchy dispersion. In this regard, the practical limit of starch concentration has been found to be at about 5 per cent, preferably about 3 per cent. Inulin may also be employed in these processes.

The examples herein are given by way of illustration and not of limitation, as it is obvious that certain modifications may be made in the compositions of the media and in the steps of the fermentation process, and in the kinds and proportions of the materials employed, without departing from the spirit and the scope of the invention and the purview of the claims.

We claim:

1. Process of producing citric acid comprising forming a medium of carbohydrate selected from the group consisting of monosaccharides, disaccharides, starches, inulin and materials containing these carbohydrates, and nutrient inorganic and nitrogen-containing substances, supplying thereto citric-acid-producing strains of *Aspergillus wentii*, and maintaining agitation and aeration such as to disperse oxygen through the medium and to induce and maintain the growth of the said strains in a submerged condition throughout the medium.

2. Process for the production of citric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, by means of citric-acid-producing strains of *Aspergillus wentii*, which comprises the successive steps of sterilizing a medium containing nutrient materials and such carbohydrates, adjusting the pH value of the said medium to 2.0, inoculating the said medium with the said strains of *Aspergillus wentii*, continuously agitating and simultaneously aerating the resulting inoculated medium under pressure, meanwhile developing fungal growth in a submerged condition in the said inoculated medium at a temperature range of approximately 18° to 37° C., adding to the resulting nutrient culture solution a member of the group consisting of calcium carbonate and calcium hydroxide to raise the pH value to not more than 3.0 after a substantial quantity of citric acid has been produced therein, meanwhile subjecting the said nutrient culture solution to similar conditions of agitation, aeration, pressure and temperature, removing substantially the liquid part of the residue of the said nutrient culture solution, adding a fresh supply of medium of carbohydrate and nutrient materials to the residual masses of fungal mycelium, subjecting the renewed medium and the fungal mycelium submerged therein to similar conditions of agitation, aeration, pressure and temperature, meanwhile maintaining the pH value of the medium at not more than 3.0, and conducting the fermentation to substantial completion.

3. Process for the production of citric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, by means of citric-acid-producing strains of *Aspergillus wentii*, which comprises the successive steps of sterilizing a medium containing nutrient materials and such carbohydrates, adjusting the pH value of the said medium to 2.0, inoculating the said medium with the said strains of *Aspergillus wentii*, continuously agitating and simultaneously supplying oxygen at atmospheric pressure to the resulting inoculated medium, meanwhile developing fungal growth in a submerged condition in the said inoculated medium at a temperature range of approximately 18° to 37° C., adding to the resulting nutrient culture solution a member of the group consisting of calcium carbonate and calcium hydroxide to raise the pH value to not more than 3.0 after a substantial quantity of citric acid has been produced therein, meanwhile subjecting the said nutrient culture solution to similar

conditions of agitation, temperature, and oxygenation at atmospheric pressure, removing substantially the liquid part of the residue of the said nutrient culture solution, adding a fresh supply of medium of carbohydrate and nutrient materials to the residual masses of fungal mycelium, subjecting the renewed medium and the fungal mycelium submerged therein to similar conditions of agitation, temperature, and oxygenation at atmospheric pressure, meanwhile maintaining the pH value of the medium at not more than 3.0, and conducting the fermentation to substantial completion.

4. Process for the production of citric acid by fungal fermentation comprising the successive steps of preparing an aqueous solution of purified molasses to afford a total carbohydrate concentration of about 5 to 20 per cent, preferably 15 per cent, supplementing such solution with nutrient materials, sterilizing the resulting nutrient medium by heat, adjusting the pH value of this nutrient medium to 2.0, inoculating the sterile nutrient medium with a pure culture of citric-acid-producing strains of *Aspergillus wentii*, incubating the resulting inoculated nutrient medium from about 4 to 12 days, at about 25° to 35° C., until the maximum mycelial growth has been attained, meanwhile supplying air under gentle agitation under pressure, adding a sterile suspension of a member of the group consisting of calcium carbonate and calcium hydroxide to neutralize a part of the formed acid and to raise the pH of the nutrient medium to not more than 3.0, removing the residual medium from the fermenting chamber when the fungal growth of mycelium becomes characterized by small bead-like forms, adding to the residual fungal growth a replacement supply of liquid medium containing nutrient materials and purified molasses to afford a total carbohydrate concentration of about 5 to 20 per cent, preferably 15 per cent, allowing the fungal growth to act upon the said renewed medium for a period varying from 4 to 12 days, under similar conditions of agitation, aeration, pressure and temperature, removing the residual medium and replacing it by successive fresh supplies of media containing nutrient materials and purified molasses in similar concentration until the citric-acid-producing capacity of the fungal growth is substantially exhausted, meanwhile maintaining the pH value of the added media at not more than 3.0.

5. In a process for the production of citric acid from carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, by means of citric-acid-producing strains of *Aspergillus wentii*, involving the operations of preparing a carbohydrate solution containing any of the said carbohydrates and nutrient inorganic and nitrogen-containing materials, and inoculating the said solution with the said strains, the steps of inducing the growth and maintenance of the fungal mass of the said strains in small bead-like forms, and of inhibiting flocculent and filamentous formations of the said strains, by continual agitation and aeration of the inoculated carbohydrate nutrient solution during the growth stage and throughout the entire fermentation periods, at a temperature range of about 18° to 37° C.

6. In a process for the production of citric acid, the steps of growing bead-like forms of citric-acid-producing strains of *Aspergillus wentii* in a submerged condition, which comprise preparing a culture solution of carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, in concentrations up to about 3 to 20 per cent of total carbohydrates, the said solution also containing urea, magnesium sulfate, potassium dihydrophosphate, potassium chloride, manganese sulfate and zinc sulfate, sterilizing the said culture solution by heat, adjusting the reaction to a pH value of 2.0, inoculating the sterilized culture solution with a pure culture of citric-acid-producing strains of *Aspergillus wentii*, incubating the resulting inoculated culture solution for about 4 to 12 days, at about 25° to 35° C. until the maximum mycelial growth in the form of beads has been attained, and meanwhile agitating the inoculated culture solution and supplying air thereto under pressure.

7. In a process for the production of citric acid from carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, by means of citric-acid-producing strains of *Aspergillus wentii*, involving the operations of preparing a carbohydrate solution containing nutrient materials and inoculating such nutrient solution with such strains, the step of adjusting the pH value of the said solution to 2.0 just before the inoculation of the said solution with the spores of *Aspergillus wentii*, whereby the optimum growth of such strains in vegetative bead-like forms is obtained and the formation of oxalic acid is substantially suppressed.

8. In a process for the production of citric acid from carbohydrates selected from the group consisting of monosaccharides, disaccharides, starches, inulin, and materials containing these carbohydrates, by means of citric-acid-producing strains of *Aspergillus wentii*, involving the operations of preparing a carbohydrate solution containing nutrient materials and inoculating said solution with such strains, the step which consists in adding to the culture medium a neutralizing agent of the group consisting of calcium carbonate and calcium hydroxide to raise the pH value of the said medium to not more than 3.0, after the formation of citric acid has definitely progressed in the said medium, whereby the production of citric acid is increased and the formation of oxalic acid is substantially suppressed.

9. In a process for the production of citric acid from molasses, involving the operations of preparing a medium containing purified molasses and nutrient materials and inoculating the said medium with citric-acid-producing strains of *Aspergillus wentii*, the steps which consist in first treating unpurified molasses with bone char, Suchar, and resins, then filtering the resulting product, dissolving the said nutrient materials in the filtrate, and adjusting the pH value of the resulting solution by acidification to 2.0.

10. Process of producing citric acid comprising forming a medium of cane sugar and inorganic and nitrogen-containing substances, supplying thereto cirtric-acid-producing strains of *Aspergillus wentii*, and maintaining agitation and aeration such as to disperse oxygen through the medium and to induce and maintain the growth of the said strains in a submerged condition throughout the medium.

11. Process of producing citric acid comprising forming a medium of glucose and inorganic

and nitrogen-containing substances, supplying thereto citric-acid-producing strains of *Aspergillus wentii*, and maintaining agitation and aeration such as to disperse oxygen through the medium and to induce and maintain the growth of the said strains in a submerged condition throughout the medium.

12. Process of producing citric acid comprising forming a medium of molasses and inorganic and nitrogen-containing substances, supplying thereto citric-acid-producing strains of *Aspergillus wentii*, and maintaining agitation and aeration such as to disperse oxygen through the medium and to induce and maintain the growth of the said strains in a submerged condition throughout the medium.

13. Process for the production of citric acid which comprises inoculating with a citric-acid-producing strain of *Aspergillus wentii* an aqueous medium comprising urea, magnesium sulfate, potassium dihydrophosphate, potassium chloride, manganese sulfate, zinc sulfate, and carbohydrate selected from the group consisting of monosaccharides, disaccharides, starches, inulin and materials containing these carbohydrates, and then agitating and aerating the inoculated medium so as to permit submerged, aerobic mold growth and fermentation.

SELMAN A. WAKSMAN.
EDWARD OTTO KAROW.

Certificate of Correction

Patent No. 2,394,031. February 5, 1946.

SELMAN A. WAKSMAN ET AL.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction as follows: Page 2, first column, line 58, for "($MnSO_4.7H_2O$)" read ($MnSO_4.4H_2O$); and that the said Letters Patent should be read with this correction therein that the same may conform to the record of the case in the Patent Office.

Signed and sealed this 28th day of May, A. D. 1946.

[SEAL]

LESLIE FRAZER,
First Assistant Commissioner of Patents.

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[SEAL]

LESLIE FRAZER,
First Assistant Commissioner of Patents.

Patented Aug. 17, 1943 2,326,986

UNITED STATES PATENT OFFICE

2,326,986

PROCESS FOR THE PRODUCTION OF FUMARIC ACID

Selman A. Waksman, Highland Park, N. J., assignor, by direct and mesne assignments, of one-half to Merck & Co. Inc., Rahway, N. J., a corporation of New Jersey, and one-half to Chas. Pfizer & Co., Inc., Brooklyn, N. Y., a corporation of New Jersey

No Drawing. Application July 1, 1939, Serial No. 282,423

18 Claims. (Cl. 195—36)

This invention relates to processes for the production of fumaric acid from various carbohydrates and carbohydrate-containing materials, by means of certain strains of fungi.

The production of fumaric acid by certain fungi, particularly by *Rhizopus nigricans*, is known. However, the reported yields were either variable or insufficient for the utilization of the processes for industrial purposes. These variations were due not only to a lack of a realization of the existence of a specific fungus capable of producing fumaric acid to the substantial exclusion of other acids but also to an insufficient recognition of the fact that an economic production of fumaric acid depends strictly on a knowledge of the physiology of the specific organism and on certain conditions of culture.

Though the importance of the specific strains of fungi, from a standpoint of merely a tendency to form fumaric acid, has hitherto been recognized, it has not been previously perceived that the ability to form fumaric acid is not a property characteristic of all strains of Rhizopus; that there is not only a quantitative difference but also a qualitative difference in acid-forming capacity between strains of Rhizopus; and that the various morphologically identical strains of *Rhizopus nigricans*, as well as the male and female races of even the same strain of this species, may differ strikingly in their acid-forming capacities.

I have now invented processes for the production of fumaric acid which give consistently high yields of fumaric acid under standardized specified conditions of fungal growth, treatment, and control.

My processes are carried out in two distinct ways—the one whereby the solution in which the fungal growth takes place is removed and is replaced by one or more solutions in which the fermentation for the production of fumaric acid predominates; the other whereby the growth of the fungus and the entire production of the fumaric acid take place in the same solution. Aseptic technique is practised throughout.

In my processes I preferably use certain selected strains of species of fungi belonging to the genus Rhizopus. These are strains of *Rhizopus nigricans*. These strains are selected as possessing in particular an abundance of the enzyme mechanism by which they are able, when suitably grown and treated according to my processes, to produce fumaric acid in industrially important quantities. However, though the abundant production of fumaric acid by fungi is limited largely to species of the genus Rhizopus and is even still further narrowed by economic considerations to certain strains of the species *Rhizopus nigricans* possessing a distinctive physiological specificity, I have found that other organisms belonging to the order Mucorales are also capable of forming fumaric acid in industrially important quantities according to my processes herein described.

The isolation and identification of the fumaric acid producing strain of *Rhizopus nigricans* can be accomplished as follows:

Various natural materials such as soil, decaying organic material, spoiled bread and other spoiled foodstuffs can be used as a source of the organism. This material is plated out on certain bacteriological media, especially those favorable to the development of fungi. The fungus medium of Waksman can be used for this purpose. This medium contains, per liter, 5 gms. peptone, 10 gms. glucose, 1 gm. KH_2PO_4, ½ gm. $MgSO_4.7H_2O$ and 25 gms. agar. The reaction of this medium is adjusted to pH 4.0. The medium is sterilized at 10 pounds pressure for 30 minutes. The natural material is plated out in this medium in various dilutions and allowed to incubate at 25° C. for a period of 2 to 3 days. The colonies of Rhizopus, which can easily be recognized by the trained observer, are then picked and transferred, either to the same medium in test tubes or to any other medium suitable for the growth of this fungus. Pure cultures are thus obtained. These cultures are now grown on liquid media having the composition and under the conditions outlined in this specification. The medium is tested for fumaric acid by known chemical methods after various intervals of incubation at 25° C. to 37° C. Those strains, which, under conditions comparable to those of my processes, give rapid and abundant growth and show a strong fumaric acid producing capacity, to the practical exclusion of undesirable acids, are selected for the purposes of my processes.

Suitable strains of other members of the Mucorales, such as certain species of Mucor, Cunninghamella, and Circinella, can be similarly selected. These strains and those of *Rhizopus nigricans* will be generally referred to as fumaric acid forming strains.

I have found that the carbohydrate:nitrogen ratio present in the nutrient culture medium is of great importance in the fumaric acid producing capacity of the described strains of *Rhizopus nigricans* as well as other Mucorales which were

found to be capable of producing fumaric acid. In general the lower the carbohydrate:nitrogen ratio the more unfavorable are the conditions of fumaric acid accumulation, and conversely, up to a certain maximum, a higher ratio is more favorable for the conditions of fumaric acid accumulation. These carbohydrate (as carbon): nitrogen ratios have been ascertained to range between about 25:1 and 300:1, respectively. However, it has been found further that these conditions become considerably modified by the presence (or addition) of zinc ion to the medium so that in general this element greatly reduces the yields of fumaric acid especially during the period of active growth. This effect is much reduced at higher carbohydrate:nitrogen ratios, as described more fully later.

The most favorable sources of nitrogen have been found to be ammonium sulfate and other salts of ammonia, as well as urea and other compounds of nitrogen, though the influence of urea on fumaric acid formation is less favorable than that of ammonium sulfate.

As it is highly important to balance the nitrogen concentration in the culture medium, since an excessive amount of nitrogen will considerably delay the production of fumaric acid and will reduce or even prevent the yield of fumaric acid, I have determined that, for example, the presence of 200 mgs. to 500 mgs. of nitrogen per liter of medium containing 50 to 150 gms. of carbohydrate gives favorable results. Maximum mycelial growth is attained, accompanied and followed by the production of fumaric acid.

The other constituents of the basal medium, besides carbohydrate, as a source of carbon, comprise, in certain cases, to be described, nutrient mineral salts, and a neutralizing agent such as calcium carbonate.

These nutrient mineral salts are used by me in various combinations and concentrations, substantially the following representing a typical composition: $(NH_4)_2SO_4$, 0.2%; $MgSO_4.7H_2O$, 0.05%; K_2HPO_4, 0.5%. In the case of crude carbohydrate materials these required salt concentrations may be less.

I have further found that the presence of certain catalytic or trace elements in the culture medium markedly modifies the physiology of Rhizopus. These elements have a decided effect both upon the growth of the fungus and upon the production of fumaric acid.

Specifically, zinc has a decided stimulating effect upon mycelial growth of Rhizopus at the expense of the accumulation of fumaric acid, whereas iron has a decided stimulating effect upon the production of fumaric acid by Rhizopus with a repressive effect upon its mycelial growth.

It has also been found that it is necessary to balance the nutrient culture medium in such a way as to produce the optimum mycelial growth by accelerating such growth through the presence of zinc, so that this growth will be accompanied and immediately followed by the maximum formation of fumaric acid.

In attaining this balance, I add a salt of zinc, such as zinc sulfate, $ZnSO_4.7H_2O$, to the culture medium in concentrations of about 1 to 10 mgs. per liter of medium containing 50 to 150 gms. of carbohydrate, and balance the effect of the zinc with a salt of iron, such as ferric sulfate, $Fe_2(SO_4)_3$, in concentrations of about 1 to 20 mgs. per liter of medium containing 50 to 150 gms. of carbohydrate. Optimum conditions—insofar as they are inducible by these trace elements—are thus obtained for both the mycelial growth and the fumaric acid production steps, which will be described in the examples to follow.

It is to be emphasized that the stated amounts of zinc and iron salts may not all have to be added to the medium and that somewhat higher concentrations of zinc and iron salts are not eliminated by the figures here given. The minute amounts of these elements which are required render it quite likely that they may be contained in larger or smaller quantities as impurities in some or all of the other major constituents of the medium. The fact remains, however, that zinc and iron are important elements in my processes.

The stated amounts of these elements, zinc and iron, are intended to apply when chemically pure nutrient salts are used together with a crystalline carbohydrate of a high degree of purity.

Analyses of the materials for contents of zinc, iron, and other heavy metals, and supplementary additions thereof, if the contents of them are deficient, provide accurate control of the critical ratio of zinc to carbohydrate in the growth stage, as well as control of the factors involving the requirements of iron or other heavy metals.

I have found that up to 20 mgs. of $Fe_2(SO_4)_3$ per liter of culture solution, depending on the carbohydrate:nitrogen ratio, provides a satisfactory concentration of iron for the acid-forming capacity of my selected strains of Rhizopus.

It has further been found that, on the one hand, the associative nature, or, on the other hand, the antagonistic nature of these two trace elements, zinc and iron, depends on the ratio in which they are present in the culture medium, their associative nature being manifested when the zinc and the iron exist in a ratio of substantially 1 to 2 of their stated salts.

In addition to iron, trace amounts of maganese and copper may be introduced in order to accentuate the described specific effect of zinc. Such traces do not require such rigid control as do those of zinc and iron.

It is to be especially noted that the addition of zinc as well as maganese and copper and minute quantities of iron may be highly desirable in order to obtain as rapidly as possible an abundant mass of cell substance, especially when a pure carbohydrate is used. This cell substance would be formed without the simultaneous high yields of fumaric acid, since the presence of these heavy metals promotes the conversion of the carbon of the consumed carbohydrate into cell substance rather than allowing the carbon to accumulate in the form of fumaric acid. Such desirability depends upon the economic interest. Such addition may be particularly desirable when the mature growth substance is supplied with replacement sugar solutions. The cell substance will convert the sugar under such conditions into fumaric acid at a high rate. This procedure is described below.

Moreover, the carbohydrate concentration of the medium influences the intensity of the effects of zinc and iron, and I have determined that the net effect of zinc and iron on fumaric acid production is associative when the initial carbohydrate concentration of the medium is high (i. e. 20 to 30 percent), but that the net effect of zinc and iron on fumaric acid production is antagonistic when the initial carbohydrate concentration of the medium is low (i. e. below 20 per cent).

I have found that small amounts of certain inorganic salts, namely, phosphates, and sulfates, accelerate both the process of fumaric acid production and the yield of fumaric acid if these salts are added to the culture liquid during the second and later stages of the fumaric acid production step, and that they serve as efficiently as iron in this respect and may be substituted therefor. For purposes of this acid-forming acceleration I may therefore use instead of iron, for example, dipotassium phosphate, K_2HPO_4, and magnesium sulfate, $MgSO_4.7H_2O$, in concentrations of about 0.3 to 1 gm. each per liter of solution containing about 200 gms. of carbohydrate. Very small amounts of nitrogen may also be added.

I have further found that the mycelial mass of Rhizopus can produce free fumaric acid in large amounts even in the complete absence of a neutralizing agent. The free acid accumulates as such in the culture medium and eventually attains concentrations sufficient to crystallize out in the culture as free acid. This accumulation of fumaric acid has been found to lower the pH of the medium to about 2.4, at which point the crystallization of the acid occurs. The capacity of Rhizopus to produce fumaric acid at this high hydrogen ion concentration is noteworthy, as this pH range is much below that of the activity of most living systems.

However, the removal of the free fumaric acid, by its neutralization with calcium carbonate or other such agent, is decidedly conducive to an abundant mycelial growth especially in the presence of zinc. I, therefore, use a suitable neutralizing agent for this purpose as an essential step toward the attainment of optimum mycelial growth.

In view of the fact that an alkaline environment has a depressive effect on the germination of Rhizopus spores, calcium carbonate is added to the culture after mycelial growth has fairly well advanced, i. e., shortly after spore germination.

The accumulation of free acid soon tends to retard both the total amount and the rate of fumaric acid formation. Therefore, it is desirable to neutralize this acidity. I have found that calcium carbonate or magnesium carbonate serves very satisfactorily to neutralize this acid and results in a more rapid conversion of sugar to fumaric acid and at a higher rate. This is true during both the original growth phase and during the subsequent replacement phase. The nature of these phases will become evident from the examples to follow.

I have found further that the soluble calcium fumarate salt reaches a concentration which exceeds its solubility and it crystallizes out abundantly throughout the medium and throughout the cell mass until the whole culture presents a solid mass of crystalline material. Under these conditions the conversion of sugar to acid ceases. It is, therefore, desirable to neutralize the acid with alkalies whose fumarate salts are more soluble and hence would not crystallize so readily. I have found that KOH or NaOH can be used quite effectively to obviate this difficulty. Care should be exercised when using the soluble alkalies not to add enough to render the pH above 7.0. It is preferable to adjust the pH periodically at frequent intervals to approximately 5.0 to 6.5.

I have further found that I can readily substitute for pure sugar or other pure carbohydrate a cheap crude material, such as molasses or starch materials, even in the first or growth-phase of my processes. In this way, fumaric acid yields are sacrificed in order to obtain a rapid and abundant cell substance for use in the replacement phase.

Extensive experiments in respect to the use of submerged growths of Rhizopus have been made, and I have determined that the utilization of the fermentation processes by means of this fungus for fumaric acid production is industrially practicable.

Selected strains of *Rhizopus nigricans* can be grown rapidly and abundantly, in a submerged condition, in large containers corresponding to tank conditions, with the aid of rapid aeration or under air pressure and adequate agitation, with or without calcium carbonate or other neutralizing agents. The rate of growth under these conditions is greatly accelerated (twenty-four to forty-eight hours as compared to five to seven days for stationary surface growth) and the yields of fumaric acid obtained have practical importance. The advantages of a deep-tank fungus fermentation process over shallow-pan processes are obvious. The original nutrient culture solution may be drained off through the false bottom used for aeration or otherwise filtered off, leaving the mass of fungal mycelium in the container, which is then recharged with carbohydrate solution and, if desired, with calcium carbonate, or neutralized periodically with soluble alkalies. The fermentation of this replaced sugar by the preformed mycelium begins immediately at a high rate, resulting in considerable economy of time. The mass of fungal mycelium still possesses a high fermentation capacity through several sugar replacements although with gradually diminishing vigor.

If during submerged fermentation some interference with the process should tend to arise from the crystallization and the solidification of the culture solution as a result of the limited solubility of calcium fumarate, such interference can be readily overcome by the use of lower concentrations of carbohydrate in the replacement solutions, or by the use of soluble alkalies, such as KOH or NaOH.

The use of pure sugar solutions in submerged fermentation results in the occurrence of a product of a high degree of purity, and the relative insolubility of fumaric acid permits the liberation of the free fumaric acid directly from the culture solution merely by concentration and by addition of a mineral acid, such as HNO_3 or HCl.

The temperature optimum for mycelial growth, with complete disregard for acid production, is about 35° C. However, the optimum for fumaric acid production during the replacement phase is about 28° C. It is consequently advisable to conduct growth at about 28°–35° C.; fermentation is best conducted at about 28° C.

The maximum growth of the fungus can be empirically observed to have occurred by the extent of the proliferation; or it can be determined from the nitrogen consumed; or it can be roughly ascertained by the removal from the process container and the weighing of a given area of the pellicle in the case of stationary cultures, or by the removal of aliquot portions of the contents of the container and the weighing of the fungus material contained therein in the case of submerged growths.

The several successive process steps which I have found to be, by virtue of their specific combination and sequence, valuable for the success-

ful and economic production of fumeric acid will now be described.

Example I

A culture solution, containing glucose or another carbohydrate or a mixture of carbohydrates, in concentrations of about 5 to 15 per cent total carbohydrate, is supplemented with the described nutrient salts and salts of catalytic or trace elements, and is sterilized by heat.

This culture solution is inoculated with a pure culture of selected strains of the fungus *Rhizopus nigricans*. Inoculation is effected by means of either the fungal spores or a predeveloped suspension of germinated spores, the latter resulting in considerable economy of time. The culture is incubated at about 28°–35° C.

In respect to the first stage, or growth phase, of this process, the culture is now allowed to grow either in a stationary manner, wherein a surface mass of growth or pellicle develops, or in a submerged condition, wherein the culture is gently agitated with air or with a mechanical stirrer, being held at atmospheric pressure or under increased air pressure, for 24 to 60 hours.

A lattice-like or other suitable support for the growing fungus is essential for the rapid development of an abundant mass of mycelial growth into a heavy pellicle in the stationary vessels, especially where a large surface area of liquid is to be covered by the growth. The lattice should preferably be approximately parallel to the liquid surface.

Calcium carbonate or other alkali is added to the culture liquid, stoichiometrically and aseptically, to neutralize the fumaric acid which the fungus has produced from the carbohydrate.

In stationary gowth tanks the liquid is agitated under the culture and care is taken not to injure the thin pellicle of growth which has formed on the surface.

The submerged culture is subjected to aeration either in rotating or in stationary tanks with air passing through the culture or under increased pressure.

When the growth of the fungus has reached its maximum at about 28°–35° C., which is usually within 2 to 7 days, depending on the relative concentration of the nutrient materials, trace elements and conditions of growth, such as temperature, the nutrient culture liquid in the containers in which growth has occurred is removed as completely as possible by drainage, by siphoning or by centrifuging.

This removed culture liquid contains the equivalent of about 10 to 45 per cent of fumaric acid on the basis of the carbohydrate consumed by the fungus, and this liquid is saved for the recovery of the fumaric acid therein.

The cell substance remaining in the containers (such substance being called "pellicles" in the case of the growths in stationary cultures) is then washed with sterile water to remove the last traces of nutrient salts, and this wash solution is drained off. This step is not essential for successful production of fumaric acid.

In respect to the second stage, or the replacement phase, of the process of fumaric acid production, the growth culture liquid is now substituted by the addition to the preformed cell substance of a solution of carbohydrate in concentrations containing up to about 20 per cent of carbohydrate, without supplementary nitrogen or other nutrient mineral salts. Simultaneously, calcium carbonate, substantially stoichiometrical to the anticipated fumaric acid yield, which may be up to about 50 per cent of the carbohydrate consumed by the fungus, is added to the fresh culture liquid, or neutralization is effected periodically by KOH or NaOH.

Accelerators of fumaric acid formation are also added at this step, for example: $Fe_2(SO_4)_3$ and $MgSO_4.7H_2O$, each in concentrations of 0.1 gm. to 1.0 gm. per liter of solution containing up to 20 per cent of carbohydrate.

The mass of fungal growth, which abundantly provides a strong enzyme mechanism for producing fumaric acid, is now allowed to act upon this carbohydrate solution for a period varying from 1 to 7 days, being aided by agitation and aeration or air pressure similar to that described.

The rates of growth and fumaric acid production are greatly accelerated by the presence of a surface support in the stationary cultures and by aeration, by air pressure and by agitation of the submerged type of growth.

Upon the initiation of the second stage the temperature is kept at about 28° C., which appears to be about optimum for fumaric acid production.

The culture solution is removed, as before, and the replacement of the carbohydrate by new solutions is repeated several times until the enzyme system of the Rhizopus mycelium is substantially exhausted.

The mature cell substance retains for some time its function as an enzyme system under these conditions, converting the carbohydrate and so continuing to produce fumaric acid with gradually reduced vigor, however, to the practically complete exclusion of other acids, from as many as three or four successive replacements with new carbohydrate solutions.

No vegetative growth, or very little, occurs in the replacement phase since nitrogen and other essential growth elements are lacking.

The addition of calcium carbonate or other neutralizing agent in the replacement phase is not absolutely essential in this process, because a substantially pure fumaric acid is formed and it crystallizes from the culture liquid owing to its low solubility. However, in order to bring about a high rate and an increased yield of fumaric acid formation, calcium carbonate or other neutralizing agents may be stoichiometrically added in this second stage.

Small amounts of other acids may be produced during both stages.

The final removal of the culture liquid containing calcium fumarate or other salts of fumaric acid and of the precipitated crystalline mass produced in the vessel completes the process. The fumaric acid is recovered as described.

Example II

The several steps of the first stage or growth phase, of the process described in Example I are followed, but the replacement of the culture medium solution by successive carbohydrate solutions, as described in the second stage of the process of Example I, is avoided by utilizing an excess of carbohydrate (such as 20 to 30 per cent) over nitrogen in the medium in its initial preparation. This excess of carbohydrate over nitrogen functions in the manner already stated. Aeration is effected as previously described.

Thus, this process is continuous up to the point of substantial enzyme-system exhaustion of the fungal mycelium.

As in Example I, the presence of calcium carbonate as a neutralizing agent is not essential, though it or other neutralizing agents may be used in substantially stoichiometrical proportions for the purpose of increasing the yield of fumaric acid.

As also in Example I, removal of the liquid from the substantially exhausted fungal mycelium and removal of the crystalline mass formed in the vessel complete the process.

Example III

The several successive steps of the first stage, or growth phase, of the process described in Example I are followed, except that a cheap crude carbohydrate-rich material, such as molasses or starch materials, is used in place of pure sugar and nutrient salts.

This crude material is diluted with water to afford a concentration from 5 to about 30 per cent of carbohydrate content, depending on the nature of the material, and, for reasons stated, it is supplemented with a nitrogen salt and a zinc salt, especially if these are lacking, as shown by analysis of the crude carbohydrate material. Excellent mycelial growth is produced on this medium.

As to the second stage of this process, this growth is now treated for the production of fumaric acid by the addition of a solution of sugar or other carbohydrate, in a concentration up to about 20 per cent of carbohydrate, as well as calcium carbonate or other neutralizing agent previously stated. An iron salt, and potassium phosphates and magnesium sulfate in proportions previously indicated may also be introduced. Aeration is effected as previously described.

By the introduction of pure sugar solutions in the second stage of this process, a fumaric acid of high purity can be obtained in each of several successive treatments of the mass of fungal mycelium, until the latter's acid-producing activity is substantially exhausted.

As in the process of Example I, the presence of calcium carbonate during the second stage is not essential, as enough slightly soluble fumaric acid goes into solution and attains concentration to crystallize from the culture liquid. A neutralizing agent may be used, however, to cause a considerably increased yield of fumaric acid.

As also in Example I, final removal of the liquid from the fungal mycelium and removal of the crystalline mass formed in the vessel complete the process.

References made herein to "various carbohydrates and carbohydrate-containing materials," "other pure carbohydrate," "solution of carbohydrate," "carbohydrate-rich material," and all expressions concerning the use of carbohydrate or carbohydrates in these processes, relate to glucose, or sucrose, or starch, or a mixture of these carbohydrates, or materials containing them, in solution or suspension.

These examples are given by way of illustration and not of limitation, as it is obvious that certain modifications may be made in the steps of these processes, and in the kinds and proportions of the materials employed, without departing from the spirit and scope of the invention and the purview of the claims.

I claim:

1. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of a fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with the said strains, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5–6.5, and conducting the fermentation at about 28° C. to substantial completion.

2. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of Rhizopus, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric acid producing strains of Rhizopus, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5–6.5, and conducting the fermentation at about 28° C. to substantial completion.

3. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of *Rhizopus nigricans*, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric acid producing strains of *Rhizopus nigricans*, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5–6.5, and conducting the fermentation at about 28° C. to substantial completion.

4. Process for the production of fumaric acid by fungal fermentation of carbohydrate material, comprising successive steps of preparing a culture solution of fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates in concentrations up to about 5 to 15 per cent total carbohydrate, supplementing such culture solution with nutrient salts and salts of members of the group consisting of manganese, copper and zinc, sterilizing said culture solution by heat, inoculating the sterile culture solution with a pure culture of fumaric-acid-producing strains of species of a genus of the order Mucorales, incubating the resulting inoculated culture solution from about 24 to 60 hours at about 28° to 35° C. until maximum mycelial growth has been attained, meanwhile supplying air under gentle agitation at not less than atmospheric pressure, adding an alkaline agent to neutralize the formed acid and to favor fungal growth, removing the culture solution from the container when fungal growth has reached the optimum growth of mycelium, adding to the fungal growth a solution of carbohydrate in concentrations containing up to about 20 per cent of carbohydrate

and a neutralizing agent, all in the presence of phosphates and sulfates, allowing the mass of fungal growth to act upon the said renewed carbohydrate solution for a period varying from 1 to 7 days at about 28 C., removing the culture solution and replacing it by successive fresh solutions of carbohydrate material until the fumaric acid forming capacity of the fungal growth is substantially exhausted.

5. Process described in claim 4, with the following modifications: utilizing an excess of fermentable carbohydrate selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates, over nitrogen in the medium in its initial preparation, whereby the replacement of the culture medium solution by successive carbohydrate solutions is avoided and whereby the process of fumaric acid formation is continuous up to the point of substantial enzyme system exhaustion of the mycelial growth.

6. Process described in claim 4, with the following modifications: substituting crude for pure fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates and nutrient salts, diluting such crude material with water, supplementing such solution with a nitrogen salt and a zinc salt, developing substantially maximum fungal growth on this medium, treating this growth for the production of fumaric acid by the addition of a solution of fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates in a concentration up to about 20 per cent of carbohydrate.

7. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of a fumaric-acid-producing strains of a species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient materials and inoculating such solution with such fungi, according to claim 1, the added step which consists in growing the fungi in a submerged condition in deep culture tanks.

8. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements and inoculating such solution with such strains, the step which consists in balancing the nitrogen concentrations in the culture medium so that it will contain substantially 200 mgs. to 500 mgs. of nitrogen per litre of medium containing 50 to 150 gms. of carbohydrate.

9. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements and inoculating such solution with such strains, the step which consists in adding to the culture medium a zinc salt in substantially the concentrations represented by 1 to 10 mgs. of $ZnSO_4.7H_2O$ per litre of medium containing 50 to 150 gms. of carbohydrate, whereby mycelial growth is stimulated.

10. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucaroles, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc salt and inoculating such solution with such strains, the step which consists in adding to the culture medium an iron salt in substantially the concentrations represented by 1 to 20 mgs. of $Fe_2(SO_4)_3$ per litre of medium containing 50 to 150 gms. of carbohydrate, whereby an associative balanced concentration of iron and zinc salts is attained, and whereby fumaric acid formation is stimulated.

11. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc and iron salts and inoculating such solution with such strains, the step which consists in balancing the initial carbohydrate concentration of the medium by providing a carbohydrate content of 20 to 30 per cent, whereby the net effect of the zinc and the iron is associative in the medium in concentrations substantially represented by the relation of 1 to 10 mgs. of $ZnSO_4.7H_2O$ to 1 to 20 mgs. of $Fe_2(SO_4)_3$ per litre of medium.

12. In a process for the production of fumaric acid from fermentable carbohydrates selected selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc salt and inoculating such solution with such strains, the step which consists in adding to the culture medium phosphates and sulfates in substantially the typical concentrations of about 0.3 to 1 gm. of dipotassium phosphate, K_2HPO_4, and about 0.3 to 1 gm. of magnesium sulfate, $MgSO_4.7H_2O$, per litre of solution containing about 200 gms. of carbohydrate, whereby the effect of zinc is neutralized and fumaric acid formation is stimulated.

13. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements and inoculating such solution with such strains, the step which consists in partially neutralizing the formed fumaric acid with alkalies, whose fumarate salts are more soluble than calcium fumarate, to a pH range of about 5.0 to 6.5, whereby the process of fermentation is not hindered by the occurrence of a solid mass of crystalline material within the container.

14. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates, by

means of fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the successive steps of inoculating such a carbohydrate solution containing nutrient materials with the said strains, developing fungal growth in a submerged condition in the culture solution at not less than atmospheric pressure in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., meanwhile subjecting the nutrient culture solution to aeration, removing that solution, supplying the residual fungal mycelium with a fresh solution of such carbohydrate in the presence of an iron salt, conducting the fermentation at about 28° C. to substantial completion, and meanwhile subjecting the renewed solution to similar aeration.

15. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of Cunninghamella, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric-acid-producing strains of Cunninghamella, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5–6.5, and conducting the fermentation at about 28° C. to substantial completion.

16. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of Circinella, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric-acid-producing strains of Circinella, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5–6.5, and conducting the fermentation at abou. 28° C. to substantial completion.

17. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with the said strains, developing fungal growth in the presence of a zinc salt, removing the nutrient culture solution, and supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt.

18. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates, by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the steps of inoculating such a carbohydrate solution containing nutrient materials with the said strains, developing fungal growth in a submerged condition in the culture solution in the presence of a zinc salt, meanwhile subjecting the culture solution to aeration, removing that solution, supplying the residual fungal mycelium with a fresh solution of such carbohydrate in the presence of an iron salt, and meanwhile subjecting the renewed solution to similar aeration.

SELMAN A. WAKSMAN.

CERTIFICATE OF CORRECTION.

Patent No. 2,326,986. August 17, 1943.

SELMAN A. WAKSMAN.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction as follows: Page 2, first column, line 44, for "0.5%" read --0.05%--; and second column, line 26, for "or" before "other" read --and--; lines 41 and 47, for "maganese" read --manganese--; page 4, first column, line 1, for "fumeric" read --fumaric--; line 37, for "gowth" read --growth--; page 5, second column, line 2, claim 1, strike out "a" before "fumaric-acid-producing"; line 45, claim 3, for "nycelium" read --mycelium--; page 6, first column, line 41, claim 7, strike out "a" before "fumaric-acid-producing"; and second column, line 9, claim 10, for "Mucaroles" read --Mucorales--; line 40, claim 12, strike out "selected"; and that the said Letters Patent should be read with this correction therein that the same may conform to the record of the case in the Patent Office.

Signed and sealed this 16th day of November, A. D. 1943.

Henry Van Arsdale,
Acting Commissioner of Patents.

(Seal)

Patented Oct. 17, 1922. 1,432,312

UNITED STATES PATENT OFFICE.

SELMAN A. WAKSMAN, OF NEW YORK, N. Y.

PROCESS OF TREATING SILK FIBERS.

No Drawing. Application filed February 3, 1921. Serial No. 442,277.

To all whom it may concern:

Be it known that I, SELMAN A. WAKSMAN, a citizen of the United States, residing in the city of New York, county of Bronx, and State of New York, have made new and useful Improvements in Processes of Treating Silk Fibers, of which the following is a specification.

This invention relates to a process of treating and de-gumming silk fibers through certain bacterial agencies whereby a more definite and expeditious result is obtained than has heretofore been possible.

In the treatment of silk fibers preparatory to de-gumming, it has been customary to soften the gum by immersing the raw silk fibers in warm water, or in a mixture of soap and oil, for a required period of time. After this preliminary softening treatment, the fiber is then immersed in a concentrated soap solution for the purpose of dissolving the gum. This process is not only expensive, but is, to some extent, injurious to the delicate silk fibers. Further, by this process, all the gum will be removed, and it is not possible to leave a certain amount of gum, as is often desirable for different grades of silk.

To, therefore, obviate these difficulties and disadvantages, it has heretofore been proposed to substitute various enzymes for the soap treatment. But all such proposed processes have met with certain difficulties, and the soap treatment is today still quite generally practiced.

I have discovered that by first softening the silk gum or sericin in any suitable manner, and under suitable conditions, it will then become available as a food for certain proteolytic bacteria which will, under favorable conditions, thrive and multiply rapidly, and in so doing, will cause disintegration of the softened gum.

My invention, therefore, includes the preparation and supplying of proper nutrient and stimulating materials in a prepared solution into which a pure culture of the selected and prepared strongly proteolytic bacterium is introduced. The supply of nutrient and stimulating material is aimed to be sufficient to cause a rapid growth of the bacteria which thereafter will continue their activity on the softened gum. My complete process, therefore, includes the softening of the gum or sericin, under proper conditions. This may be done by soaking the silk in a mixture of neat's-foot oil and olive oil soap, or in a mixture of oil and soap, which will result in softening the gum. In place of a mixture of oil and soap, hot water at about 90° to 95° C. may be used for a period of 20 to 30 minutes. The silk thus treated is then washed with warm water or steam and put into suitable vats. These vats are fitted with aerating apparatus for the purpose of supplying oxygen to the bacterial growth, and with steam pipes for regulating the temperature. The vats may be made of any suitable material not injurious to the bacterial growth, as wood, glass, metal, or the like. The raw silk fiber is then suspended in a suitable manner as on sticks, so as to hang down into the vats. Either before the silk fiber is introduced into the vat, or after the preparation is made, the vat is filled with water to which has been added the proper and necessary nutrients for stimulating bacterial growth. These nutrients may consist of peptones, potassium phosphate and magnesium sulfate. Any other salt which is useful for stimulating bacterial growth may obviously be substituted for those named above. The concentration of these nutrients varies somewhat, depending on the gum content of the silk, and on the water which is used, and the quantity in all cases should be sufficient to start a rapid growth of the bacteria which are then introduced. After the solution has been prepared and the silk softened as stated above, a pure culture of a strongly proteolytic bacterium is introduced. This culture has previously been prepared by the usual and well known bacteriological methods, and may preferably be put up in suitable containers and in suitable quantities for the silk to be treated.

The quantity of the bacterial culture to be added to the vat should be sufficient to thoroughly inoculate the solution. Any strong proteolytic bacterium may be used, such as Bacillus florescens liquefaciens, and Bacterium mycoides or other spore forming or non-spore forming types, and it is immaterial whether these are isolated from the soil, water, air or foodstuffs, the important feature being that the culture must be pure.

A suitable temperature is maintained. The entire liquid is kept preferably at 30° to 37° C. Soon after introducing the cul-

ture, the bacteria begin to multiply rapidly, first using the nutrients which have been introduced into the solution, and then attacking the sericin of the silk and decomposing it.

It is my purpose, in all cases, to introduce just sufficient nutrients to start bacterial growth, and the quantity of nutrients introduced should in no case be more than is required for this purpose. The bacteria will not attack the pure silk fiber (fibroin) or in any way injure the same. In a period of 24 to 48 hours, all the gum, or sericin, of the silk will have been dissolved off. The solution may then be removed, or, if preferred, the silk may be taken from the vats and washed with water or steam. At this point, it may in some cases be necessary to supplement the treatment with a weak soap solution, for the purpose of removing any small particles of gum and impurities that may still adhere to the fiber. In such cases, it is necessary to use but a very weak solution, and I find that one to three pounds per one hundred pounds of silk will ordinarily be sufficient. I also find that because of the practically entire disappearance of the gum, this bath can be used several times, thus greatly reducing the expense, even when a separate treatment of a soap solution is necessary. On the other hand, with the old process, it was necessary to use 20 to 30 pounds of soap for each hundred pounds of silk treated, and usually this solution could be used only once. I have found that by varying the time of incubation or silk treatment, any desired quantity of gum may be removed.

My process, therefore, results in a great saving in the cost of the soap, if soap is used at all, and in a definite result which is obtained in a very short period of time. I find that by using the bacterial process, the silk fiber will also be stronger than in the case where the soap treatment and strong alkalies are necessary.

For practical purposes, I prepare a pure culture of any one of the above named bacteria, or of any other bacteria, possesssing the desired properties, and place the same into suitable containers, in quantities sufficient to inoculate a given amount of solution properly prepared, as stated, and for degumming a definite quantity of silk fiber.

By so providing a definite culture medium for treating a definite quantity of silk under suitable conditions, as indicated above, I am enabled to standardize, not only the culture employed, but also the mode of operation, to thereby greatly facilitate the degumming process, so as to make the same certain and definite at a great saving of time and expense.

Having now set forth the object and nature of my invention, and the manner of carrying the same into operation, what I claim as new and useful and of my own invention, and desire to secure by Letters Patent, is:

1. The process of subjecting raw silk fiber to the action of strongly proteolytic bacteria to thereby degum said fibers.

2. The process of treating raw silk fibers, for the purpose of degumming the same, and improving their texture, which comprises subjecting the raw silk fibers to the action of strongly proteolytic bacteria under conditions to thereby cause a rapid development of the bacteria, and permitting said bacteria to act on the gum to free the said fibers from the gum.

3. The process of treating silk fibers for the purpose of dissolving the sericin and loosening said fibers, which consists in preparing said fibers, then subjecting the prepared fibers to the action of strongly proteolytic bacteria under proper conditions, and finally washing the silk fibers.

4. The process of treating silk material containing silk fibers and undesirable products for the purpose of freeing said fibers from the undesirable products and improving the texture, which comprises treating said material under favorable conditions, in a solution containing bacteria possessing strongly proteolytic properties and sufficient nutrients to stimulate rapid bacterial development.

5. The process of treating silk material containing silk fibers and sericin, for the purpose of freeing the fibers from the sericin, and for loosening their texture, which includes softening the silk material and treating the same in a solution containing a pure culture of bacteria possessing strongly proteolytic properties, and having been developed in said solution for the purpose of attacking the sericin and freeing the silk fibers therefom.

6. A bacterial culture preparation having strongly proteolytic properties and in concentrated form and of sufficient quantitiy to thoroughly and quickly inoculate a definite and prepared solution containing stimulating nutrients for degumming a given quantity of silk fiber placed in said prepared and inoculated solution.

In testimony whereof I have hereunto set my hand on this 15th day of December. A. D., 1920.

SELMAN A. WAKSMAN.

Patented May 14, 1946 2,400,143

UNITED STATES PATENT OFFICE

2,400,143

PROCESSES OF FERMENTATION

Selman A. Waksman, Highland Park, N. J., assignor to Merck & Co., Inc., Rahway, N. J., a corporation of New Jersey

No Drawing. Application May 8, 1943, Serial No. 486,248

11 Claims. (Cl. 195—36)

This invention relates to a process for the production of citric acid, and particularly to a surface process for such production by fermentation.

It has been known, since Wehmer's work in 1892 on the *Citromyces* group of fungi, and since Zahorski's work in 1913 and Currie's later work on the *Aspergillus niger* group, that some fungi or molds are capable of producing citric acid from sugar and sugar-containing materials. However, the citric acid which has been so produced has been commonly accompanied by the production of other acids, such as oxalic and gluconic.

The objects of this invention concern the use of a fungal organism, which has not been previously recognized as a producer of citric acid, under suitable conditions of nutrition and by procedures which result in the production of citric acid in high yield, to the substantial exclusion of other acids.

These and other objects will be apparent and a fuller understanding of this invention will be had by reference to the following description and claims.

The organism employed in this invention is *Aspergillus wentii*, which belongs to the group of yellow to brown molds. It is characterized by certain well defined morphological features, which distinguish it from other molds, especially those belonging to the *Aspergillus niger* or black group of molds, as is clear from the comparative descriptions which are given of these two groups in "The Aspergilli," by Thom and Church (Williams & Wilkins Co., Baltimore, Md., 1926).

The process herein which the applicant has developed for the production of citric acid by *Aspergillus wentii* advantageously comprises a growth phase and a fermentation phase.

For growth, a medium is provided which is favorable for the rapid and the extensive growth of the fungal organism, *A. wentii*. It has been found that this medium must have certain properties such as those afforded by the supply of a good source of energy (sucrose, dextrose, the sugars of molasses, as instances), a source of nitrogen (for examples, ammonium sulfate, sodium nitrate, ammonium nitrate, urea, or peptone), and certain minerals comprising those essential to the growth of the organism (especially, phosphate, sulfate, calcium, magnesium, potassium, iron and zinc).

In connection with the provision of the growth elements, the iron and zinc are presented in such a balanced concentration as to favor growth but to retard sporulation of the fungus. Such effects are realized by keeping down the iron concentration to a minimum necessary for growth and by maintaining the zinc concentration at a maximum tolerable by the fungus, as indicated in the culture medium formula below, which illustrates such a balance.

Thus, a typical culture medium which is favorable for the surface aerobic growth of *Aspergillus wentii* is one composed of:

Cane sugar	grams	150
Ammonium nitrate	grams	3
Peptone	gram	0.5
Magnesium sulfate ($MgSO_4.7H_2O$)	gram	1.0
Dipotassium hydrogen phosphate (K_2HPO_4)	gram	0.5
Potassium chloride	gram	0.5
Zinc sulfate ($ZnSO_4.7H_2O$)	gram	0.3
Ferric chloride ($FeCl_3.6H_2O$)	gram	0.03
Calcium chloride	gram	0.1
Distilled water to make	c. c	1000

It has been found that the best results are attained when the culture medium is adjusted to about pH 2.0.

The concentrations of the constituents of this medium are not absolute; considerable variation still affords favorable results. For instance, the cane sugar content may vary from about 10 per cent to about 20 per cent, or molasses may be utilized to provide about 10 per cent to about 20 per cent of total carbohydrates required for the growth of the fungus and the production of citric acid.

This culture medium is sterilized by heat and is then inoculated with an aqueous suspension of spores of *Aspergillus wentii* which has been grown upon an appropriate solid or liquid medium adapted for extensive spore production, such as a nutrient agar medium comprising, as a typical example:

	Grams
Peptone	5
Glucose	10
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5
Magnesium sulfate ($MgSO_4.7H_2O$)	0.5
Agar	15
Water to make	1000

As a saving in time, the spores of this fungus may be pregerminated in a water suspension or in a dilute solution of this nutrient agar medium, for about 24 hours, at about 25° to 32° C. This pregermination procedure can be carried out in small sterile flasks or other sterile containers.

The process may be illustrated by the following

example, though it is to be understood that this example is given in illustration and not in limitation:

Large shallow vessels containing the described culture medium, which has been inoculated with the suspension of spores or with the pregerminated spores of *A. wentii*, are now placed in an incubating chamber at about 25° to 32° C. This range of temperature is favorable for the growth of the fungus. Within about 3 days, a good surface pellicle of the fungus, white in color and almost free from brown spores, is obtained. The period of growth depends upon the temperature and the composition of the culture medium. Such period may be completed in 2 or 3 days, or it may need to be extended to 4 or 5 days.

The described growth phase thus results in the production of a firm, well-developed pellicle of *A. wentii*. An appreciable amount of citric acid is also produced during this period. However, the more active production of citric acid occurs during the subsequent fermentation.

After that pellicle has been obtained, the residual culture medium is drawn off and is then available for the recovery of citric acid therefrom by known methods.

The main fermentation is now begun by replacing the drawn-off residual culture medium by a solution containing from about 15 per cent to 20 per cent of total carbohydrate or carbohydrates, such as sucrose, maltose, lactose, glucose or dextrose, levulose, invert sugar, or the sugars contained in molasses, or mixtures of these carbohydrates.

During this main fermentation the temperature of the carbohydrate solution, which has been introduced below the formed fungus pellicle, is maintained at about the range of that which prevailed during the growth phase, i. e., about 25° to 32° C. The formation of citric acid begins to take place immediately.

In order to prevent an undue accumulation of free citric acid in the shallow vessels, the prevalence of which would tend to interfere with the activity of the mycelium, the fermenting liquid is neutralized from time to time by the addition of a sufficient quantity of powdered calcium carbonate. An excess of calcium carbonate in the vessels is undesirable; the amount of calcium carbonate added should be sufficient to neutralize excess acidity without raising the pH of the medium above 3.0.

It has been found by the applicant that even an alkali-metal hydroxide, such as caustic soda or caustic potash, can be used as a neutralizing agent in this process, provided that it is used under carefully controlled conditions. This is surprising, since the use of an alkali-metal hydroxide ordinarily induces the formation of oxalic acid. It is therefore necessary to add the alkali-metal hydroxide solution, for example, a normal solution of sodium hydroxide or potassium hydroxide, every few hours, or in a slow continuous stream, so that the pH value of the fermenting liquid is adjusted to not more than 3.0, preferably from 2.0 to 3.0, to avoid an excess of such alkali. If this addition of alkali-metal hydroxide solution is made by the stirring of the latter below the pellicle or fungus pad, the pH value is kept not in excess of 3.0, with the results that any tendency to the formation of oxalic acid is substantially suppressed and the injurious effect upon the mycelium of a large accumulation of free citric acid in the shallow vessels is avoided.

In view of the fact that the production of citric acid involves an aerobic process, it is essential either that the solution or the dispersion of carbohydrate should be very shallow, that is, about 1 to 3 inches in depth, or that, where deeper vessels are used, air either should be pumped under pressure into the fermenting liquid below the fungus pellicle or should be supplied above the growth of the fungus, until the completion of the process.

During this fermentation phase, active citric acid formation occurs, with a yield of about 30 to 60 per cent, substantially corresponding to the amount of carbohydrate or carbohydrates and the kind or kinds thereof used. The citric acid is obtained from its resulting salt by known methods.

The references herein to "dextrose," "glucose," "levulose," "invert sugar," "sugars of molasses," "sugars contained in molasses," "sucrose," "cane sugar," "maltose," "lactose," "carbohydrate," and "carbohydrates," are intended to include all monosaccharides, disaccharides, or mixtures thereof. Any mixtures of monosaccharides, disaccharides and/or starches can also be used. Starch or starches, as well as dextrins, without monosaccharide or without disaccharide, can be utilized. When starch is used due regard must be had to the viscosity of a starchy dispersion. In this latter respect, the practical limit of starch concentration has been found to be about 5 per cent, preferably about 3 per cent. Inulin may also be employed in these processes, in a manner similar to that of the use of starch.

When molasses is used in the described process, careful prior purification of the molasses is not essential, though some purification of some grades of molasses is advantageous by way of diminishing their content of mineral and some nitrogen compounds which, when excessive, may adversely affect the yield of citric acid.

The foregoing examples have been given by way of illustration, and not of limitation, as it is obvious that certain modifications may be made in the composition of the culture medium and in the kinds and proportions of the materials employed, and in the steps of the fermentation process, without departing from the spirit and the scope of the invention and the purview of the claims.

I claim:

1. Process for the production of a member selected from the group consisting of citric acid and its salts, comprising the fermentation of an aqueous carbohydrate substrate containing nutrient inorganic and nitrogen-containing materials by means of a surface aerobic growth of *Aspergillus wentii*, in the presence of iron and zinc salts.

2. Process for the production of a member selected from the group consisting of citric acid and its salts which comprises inoculating a medium containing a carbohydrate and nutrient inorganic and nitrogen-containing materials with *Aspergillus wentii*, in the presence of iron and zinc salts, then aerating the medium so as to permit surface aerobic growth of the said fungus and to promote fermentation, and adding a neutralizing agent during the course of the fermentation to control acidity.

3. Process for the production of a member selected from the group consisting of citric acid and its salts by means of a surface aerobic growth of *Aspergillus wentii*, which comprises the steps of sterilizing a culture medium containing nutrient inorganic and nitrogen-containing materials and carbohydrates, inoculating the said medium with *Aspergillus wentii*, developing mycelial

growth in the presence of iron and zinc at a temperature range of approximately 25° to 32° C., under free access of air, removing the residual nutrient culture medium, supplying the developed mycelium with a solution of carbohydrate, adding a neutralizing agent to control acidity, and conducting the fermentation at about 25° to 32° C. under free access of air to substantial completion.

4. In a process for producing citric acid by the fermentative action of a surface aerobic growth of *Aspergillus wentii*, the step of promoting the mycelial growth of the said fungus by growing the spores of the said fungus in a medium comprising nutrient inorganic and nitrogen-containing materials, salts of iron and zinc, and carbohydrate.

5. In a process for producing citric acid by means of a surface aerobic growth of *Aspergillus wentii*, involving the operations of preparing a culture solution containing nutrient inorganic and nitrogen-containing materials, and carbohydrate, and inoculating such solution with the said fungus, the step which consists in adding to the said culture solution soluble salts of iron and zinc in substantially the concentrations represented by 0.03 gram of crystalline ferric chloride ($FeCl_3.6H_2O$) and 0.3 gram of crystalline zinc sulfate ($ZnSO_4.7H_2O$) per litre of the said culture solution, whereby a balanced ratio of iron and zinc salts is attained, and whereby the growth of the fungus is favored and the sporulation of the fungus is retarded.

6. In a process for producing citric acid by means of a surface aerobic growth of *Aspergillus wentii*, involving the operations of preparing a culture solution containing nutrient inorganic and nitrogen-containing materials, iron, zinc, and carbohydrate, and inoculating such solution with the said fungus, the step which consists in adding to the fermenting liquid a solution of an alkali-metal hydroxide in sufficient quantity to maintain the pH of the fermenting liquid at a value of not more than 3.0, whereby the formation of oxalic acid is substantially avoided.

7. Process for the production of citric acid, comprising the fermentation by means of a surface aerobic growth of *Aspergillus wentii* of an aqueous substrate containing per litre:

	Grams, about—
Carbohydrate	30 to 200
Ammonium nitrate	3
Peptone	0.5
Magnesium sulfate ($MgSO_4.7H_2O$)	1.0
Dipotassium hydrogen phosphate (K_2HPO_4)	0.5
Potassium chloride	0.5
Zinc sulfate ($ZnSO_4.7H_2O$)	0.3
Ferric chloride ($FeCl_3.6H_2O$)	0.03
Calcium chloride	0.1

the said substrate being adjusted to a pH value of about 2.0.

8. Process for the production of citric acid, comprising the fermentation, by means of a surface aerobic growth of *Aspergillus wentii*, of an aqueous substrate containing a carbohydrate, ammonium nitrate, peptone, magnesium sulfate, dipotassium hydrogen phosphate, potassium chloride, zinc sulfate, ferric chloride, and calcium chloride.

9. Process for the production of a member selected from the group consisting of citric acid and its salts, comprising the fermentation of an aqueous carbohydrate substrate containing nutrient inorganic and nitrogen-containing materials by means of a surface aerobic growth of *Aspergillus wentii* in the presence of iron and zinc salts, removing the residual medium after the development of a pellicle, supplying a carbohydrate solution to the thus developed pellicle, and conducting further surface aerobic fermentation by means of the said pellicle.

10. Process for the production of a member selected from the group consisting of citric acid and its salts by means of a surface aerobic growth of *Aspergillus wentii*, which comprises the steps of inoculating a solution containing nutrient inorganic and nitrogen-containing materials and about 10 per cent to 20 per cent of total carbohydrate with a suspension of pregerminated spores of the said fungus, the said nutrient inorganic and nitrogen-containing materials comprising compounds of nitrogen, phosphorus, sulfur, calcium, potassium, magnesium, iron and zinc, conducting the growth of said fungus at a temperature range of about 25° to 32° C. for about 2 to 5 days, in the presence of an abundant air supply, for the production of a pellicle, then removing the residual culture solution and replacing it by a solution containing about 3 per cent to 20 per cent of total carbohydrate, adding to that solution of carbohydrate a neutralizing agent to maintain a pH value not in excess of 3.0 during the fermentation, and conducting the fermentation at a temperature range of about 25° to 32° C. to substantial completion, in the presence of an abundant air supply.

11. Process for the production of a member selected from the group consisting of citric acid and its salts by means of a surface aerobic growth of *Aspergillus wentii*, which comprises the steps of inoculating a solution containing nutrient inorganic and nitrogen-containing materials and about 10 per cent to 20 per cent of total carbohydrate with a suspension of pregerminated spores of the said fungus, the said nutrient inorganic and nitrogen-containing materials comprising compounds of nitrogen, phosphorus, sulfur, calcium, potassium, magnesium, iron and zinc, conducting the growth of the said fungus at a temperature range of about 25° to 32° C. for about 2 to 5 days, for the production of a pellicle, then removing the residual culture solution and replacing it by a solution containing about 3 per cent to 20 per cent of total carbohydrate, adding to that solution of carbohydrate a neutralizing agent to maintain a pH value not in excess of 3.0 during the fermentation, meanwhile supplying air under pressure into the substrate, and conducting the fermentation at a temperature range of about 25° to 32° C. to substantial completion.

SELMAN A. WAKSMAN.

Patented Dec. 21, 1926. 1,611,700

UNITED STATES PATENT OFFICE.

SELMAN A. WAKSMAN, OF NEW YORK, N. Y.

PROCESS OF MAKING PROTEOLYTIC ENZYMES.

No Drawing. Application filed January 19, 1920. Serial No. 352,553.

The object of this invention is to prepare a fungus culture capable of developing a high degree of proteolytic power.

My invention is based upon the utilization of the property possessed by certain fungi when properly cultivated on suitable media or substrata under proper conditions of treatment of developing proteolytic power.

The first step in the process employed in carrying out my invention is the development of a proper culture capable of being developed or propagated under proper conditions into a condition possessing strong proteolytic action. In the development of the desired culture possessing the capacity referred to certain microscopic fungi can be employed, particularly organisms belonging to *Aspergillus flavus*, *Aspergillus oryzæ* and *Aspergillus wentii* groups.

The organism is cultivated in accordance with the principle of my invention for a series of successive generations upon a medium or substratum which is rich in protein in order to still further increase or augment the proteolytic power acquired by such organism in the cultivation thereof.

Various media or substrata may be employed for the successive generations of the selected organism. In practice I have found that a medium or substratum composed of one part of rice and two parts of soya bean cake are satisfactory for this purpose. The rice and soya bean cake are mixed together and cooked with water so that the water content forms approximately forty per cent of the total mass. I do not desire, however, to be limited in respect to the specific percentage of the water content. After cooking the mass it is suitably sterilized, as, for example, with steam pressure, or any other suitable manner.

Other media or substrata may be used with good results. In practice a medium which is poor in carbohydrates and rich in proteins in the presence of the necessary salts such as potassium or magnesium salts and phosphates, and media containing peptone, pure protein, such as casein, and the like, well answers the purpose. The culture after propagation on a medium of the nature above referred to is resown on fresh masses of the medium for successive generations, allowing a sufficient time for each propagation for the development of the spores.

The second step in carrying out my invention is the development of a mass of growth of the culture, after its propagation, as above described, which furnishes a sufficient quantity of spores to inoculate large batches of medium or substratum for the production of enzymes.

The medium or substratum for the development of the culture in quantity may have the same composition as that above described for the initial successive generations of the culture. If desired, however, other media may answer the purpose. I have found that this medium can be prepared by mixing together two parts of wheat bran and two parts of soya bean cake and one part of alfalfa meal. The soya bean cake should be ground. To the mixture of the wheat bran, soya bean cake and alfalfa meal is added about forty per cent of water, and the mixture is then steamed for a sufficient length of time to sterilize it. I have found that steaming for a period of two hours is sufficient. If, however, the mixture is not sufficiently steamed to completely sterilize it, sterilization under pressure, fifteen pounds for a period of an hour and a half, may be resorted to. After sterilizing the mass it is permitted to cool and is then inoculated with the culture produced by the propagation and successive generation as above described. The culture is then allowed to develop and grow under suitable conditions of temperature and for a sufficiently long period of time to permit an abundance of spore production. I have found in practice that an abundance of spore production results when the inoculated mass is maintained at a temperature of about 30° C. for a period of about seven days. My invention, however, is not to be limited or restricted in respect to this temperature or length of time.

At the end of a proper period the spores of the fungus may be utilized for the production of a growth in order to secure a substance possessing the desired proteolytic power. The mass or medium upon which the spore production has been secured may itself be employed as an inoculum for a medium or substratum on which the final propagation is effected. Instead of using the mass containing the fungus growth as the inoculum the mass upon which the spores have been grown may be dried and sifted in order to separate out the spores for utilization in the subsequent operations of the invention. Instead of employing the me-

dium directly or sifting the medium in its dry condition, the mass may be agitated with water in order to detach the spores from the particles of the medium upon which they have been developed and grown, the detached spores becoming suspended in the water. This water containing the spores is then strained, or otherwise manipulated, to remove any of the coarse material of the stratum upon which the spores have been developed, and the strained water carrying in suspension therein the seed spores detached from the mass may be used to inoculate the medium employed for final propagation.

The third step in the operation in carrying out my invention is the production of a growth from the spores developed and grown as above described, which growth results in the production of a substance possessing the desired proteolytic power. Any suitable medium or substratum may be employed upon which this production growth is effected. In practice I prefer to employ a substance or a mixture of substances which are rich in proteins. I have found that a mixture of one part of wheat or other bran with one to two parts of bean cake, for example, soya bean cake, which has been freed from oil and ground, to which may or may not be added one-half part of ground alfalfa meal is suitable. To this mixture water of about forty per cent is added. Instead of soya bean cake other substances may serve the purpose, such for example, as linseed oil cake, cotton seed meal, dried blood, etc., which are rich in proteins. The ground alfalfa meal employed in this mixture serves partly as a filler or body and partly as a nutrient.

The mass produced as above described is steamed from one to two hours. After the steamed mass has been permitted to cool it is inoculated with the spore material prepared and in the form above described. This inoculated mass is maintained under suitable conditions of ventilation and temperature and for the desired period of time for the fungus to develop its maximum enzymatic power. I have found it convenient to place the inoculated mass about one inch in thickness in trays, which are placed in a convenient chamber having means to provide sufficient ventilation and maintained at a suitable temperature, for example, from 28° C. to 32° C., for a period of from twenty-four to forty-eight hours. At the expiration of this time the fungus has developed its maximum enzymatic power and then its development or growth is arrested.

The next step of the process embodying my invention relates to the extraction of the enzymes for utilization for the various purposes for which enzymes possessing a high proteolytic power are desired.

The enzymes developed in the final step of propagation can be utilized in various ways. For example, the mass containing the growth can be dried at a low temperature, say below 45° C., and ground, and in that form utilized for the enzymatic power it possesses. If desired the mass containing the growth may be ground and then extracted with water by percolation, or otherwise, and the extract thus obtained which contains the enzymes in solution may be concentrated by employing the same extract to percolate successive fresh batches of the mass. If desired, the extract is filtered through suitable filter material, such for example as infusorial earth for the purpose of eliminating any suspended particles, spores, etc., contained in the extract. The extract may then be used as such with a suitable antiseptic added thereto, such as sodium fluoride phenol, cresol, chloroform, toluene, thymol, etc., in sufficient quantity to prevent fermentation, and to preserve and stabilize the extract. If desired, the water extract obtained as above described and without the antiseptic may be evaporated at a low temperature, below 45° C., and under suitable conditions of pressure and aeration, for example, partial vacuum. This concentrated material itself possesses stability and does not ferment even in the absence of an antiseptic.

If desired, a very strong enzymatic substance is obtained by precipitating the water extract without an antiseptic and without evaporation, with a substance having the power of carrying down the enzyme, such, for example, as alcohol, or certain salts, for example, ammonium sulphate. The precipitate is then dried over sulphuric acid in partial vacuo at a temperature of from 30° C. to 40° C. In this case the precipitate thus obtained may be used directly for industrial and other purposes. For food or medicinal purposes it is preferable to dialyze the salt precipitate and then either dry the same in vacuo or precipitate with a small quantity of alcohol and then dry the same. As an industrial example we might point to the process of dissolving the proteins from fibrous materials, and as an example in medicinal use the application as an agent to aid digestion is named, in which case it is taken internally after meals to digest the protein part of foods where individuals suffer from indigestion of proteins.

I have found that when precipitation is effected with ammonium sulphate a product is obtained which possesses a much higher enzymatic power than if precipitation of the water extract is accomplished with alcohol.

The product prepared in any of the ways above described possesses strong proteolytic power although it also contains other enzymes such as starch splitting lipolytic or fat splitting, etc., but to a comparatively

less degree than the proteolytic power, which is the special purpose in view in carrying out my invention.

Having now set forth the object and nature of my invention, and a method of carrying the same into practical operation, what I claim as new and useful and of my own invention and desire to secure by Letters Patent is,—

1. In the manufacture of enzymes, the process of producing an inoculum which consists in developing and growing a culture having strong proteolytic properties capable of producing a growth possessing a high degree of proteolytic power, upon a medium or substrata consisting of wheat bran, bean cake and alfalfa meal.

2. In the manufacture of enzymes, the process of producing an inoculum which consists in mixing together wheat bran, ground bean cake, alfalfa meal and water, then sterilizing the mass, and then inoculating the same with a culture having strong proteolytic properties capable of producing a growth possessing a high degree of proteolytic power.

3. In the manufacture of enzymes, the process of producing an inoculum which consists in mixing together wheat bran, ground bean cake, alfalfa meal and water, then sterilizing the mass, and then inoculating the same with a culture having strong proteolytic properties capable of producing a growth possessing a high degree of proteolytic power, and finally maintaining the inoculated mass at a temperature of about 30° C. for a period of several days.

4. In the manufacture of enzymes, the process which consists in mixing together wheat bran, alfalfa meal and water, then sterilizing the mixture and then inoculating the sterilized mass with a culture having strong proteolytic properties capable of producing a growth possessing a high degree of proteolytic power.

5. In the manufacture of enzymes, the process which consists in mixing together bran, alfalfa meal and water, then sterilizing the mixture and then inoculating the sterilized mass with a culture having strong proteolytic properties capable of producing a growth possessing a high degree of proteolytic power and maintaining the inoculated mass at a temperature of from 28° C. to 32° C. for a period of twenty-four to forty-eight hours.

In testimony whereof I have hereunto set my hand on this 10th day of January, A. D. 1920.

SELMAN A. WAKSMAN.

Patented Sept. 21, 1948

2,449,866

UNITED STATES PATENT OFFICE

2,449,866

STREPTOMYCIN AND PROCESS OF PREPARATION

Selman A. Waksman, New Brunswick, and Albert Schatz, Passaic, N. J., assignors to Rutgers Research and Endowment Foundation, a nonprofit corporation of New Jersey

No Drawing. Application February 9, 1945, Serial No. 577,136

13 Claims. (Cl. 260—236.5)

1

This invention relates generally to antibiotic substances and more particularly to a new and useful antibiotic substance, streptomycin, and the process for preparing the same by cultivation under particular controlled conditions of strains of the microorganism *Actinomyces griseus*. This organism was first isolated from the soil and characterized by one of the present applicants, S. A. Waksman, and is described in his publication in Soil Science 8, 71 (1919).

With the exception of streptothricin, the discovery and characteristics of which were reported by Waksman and Woodruff in Proc. Soc. Biol. Med. 49, 207 (1942) and Jour. of Bact. 46, 299 (1943), most antibiotic substances known at the present time, including penicillin and other mold products as well as gramicidin and actinomycin, act largely upon gram-positive bacteria. Streptothricin is very active against a number of gram-positive and gram-negative bacteria but has very little activity against certain bacteria of both groups, particularly the gram-positive *Bacillus mycoides* and *Bacillus cereus* and the gram-negative *Pseudomonas fluorescens, Pseudomonas aeruginosa,* and *Serratia marcescens.*

It is now discovered, according to the present invention, that upon cultivation of strains of the microorganism *Actinomyces griseus* in a suitable nutrient medium a new substance can be isolated from the resulting culture broth, which substance is thermostable; has the properties of a base; is soluble in water, acid alcohol and dilute acids but is insoluble in ether and chloroform; has a low toxicity to animals; and is strongly active bacteriostatically against many gram-positive and gram-negative bacteria. This substance has been designated as streptomycin. It is in many respects similar to streptothricin but differs from streptothricin as will be apparent from the comparative bacteriostatic spectra in Table I below. In this table the units of activity for streptothricin are based upon purified preparations of streptothricin while the units for streptomycin are based upon the crude and hence less concentrated substance. (A unit of activity is that amount of material which will inhibit the growth of a standard strain of *Escherichia coli* in 1 ml. of a suitable culture medium.) Comparative tests of the two substances, both purified to approximately the same degree, against *E. coli* show

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that they have substantially the same activity against this organism. For a better comparison of the bacteriostatic spectra of streptomycin and streptothricin of the same purity, the units of activity for streptomycin in Table I should therefore be multiplied by 4 in each instance.

TABLE I

Comparative bacteriostatic spectra (based on ash free dry material)

Organism	Gram stain	Units of activity per gram ash-free dry material	
		Streptomycin ×1000	Streptothricin ×1000
B. subtilis 0	+	125	500
B. mycoides 0	+	250	<3
B. mycoides 317-911	+	20	<3
B. cereus	+	30	<3
B. mesentericus	+	15	
B. megatherium	+	100	150
S. aureus	+	15	200
S. lutea	+	100	150
M. phlei	+	100	50
M. tuberculosis	+	30	
Phytomonas pruni	−	100	400
Listerella monocytogenes	−	10	
Shigella gallinarum	−		150
E. coli	−	25	100
S. marcescens	−	25	5
A. aerogenes	−	10	50
P. vulgaris	−	10	50
S. aertrycke	−	2.5	
S. schottmülleri	−		50
Ps. fluorescens	−	2	<3
Ps. aeruginosa	−	1	<3
Cl. butylicum	−	3	<3

It is apparent from a consideration of Table I that streptomycin is more active than streptothricin against certain gram-positive organisms such as *Bacillus mycoides* and *Bacillus cereus* and against certain gram-negative organisms such as *Pseudomonas fluorescens, Pseudomonas aeruginosa,* and *Serratia marcescens.*

A further inherent property of streptomycin is that in comparison with all of the activities listed for it in Table I, it is generally inactive against fungi. Representative fungi, against which streptomycin is relatively ineffective in comparison with the high activity of streptothricin, are listed in Annals of the New York Academy of Sciences, vol. 48, art. 2, page 137.

Regarded in certain of its broader aspects the

novelty in the present invention comprises the antibiotic substance streptomycin and the process for preparing the same by cultivating strains of *Actinomyces griseus*, under either stationary or submerged aerobic (viz., submerged growth with agitation and aeration) conditions, in a nutrient medium containing a growth-promoting factor of the type present in meat extract and corn steep liquor, separating the organism growth from the culture broth, treating the culture broth with activated charcoal to adsorb the active product, eluting the adsorbate with low normality alcoholic mineral acid and recovering streptomycin from the eluate.

For the preparation of streptomycin a culture medium is used comprising an aqueous solution containing approximately 1.0% of carbohydrate such as glucose; 0.5% of complex nitrogenous material such as peptone or tryptone; 0.5% of inorganic salt such as sodium chloride; and a small amount of a complex organic substance containing a specific growth-promoting factor required for satisfactory elaboration of the active product. This growth-promoting factor is present in varying degrees in such complex organic materials as meat extract, corn steep liquor, and the like.

This medium is distributed in appropriate vessels of a depth of 1–2 inches for surface cultivation. For submerged aerobic cultivation, it is placed in deep tanks having suitable means for aeration and agitation of the medium. The medium thus distributed is sterilized at 10 lbs. steam pressure for 35–60 minutes and then cooled.

For inoculation of the culture medium a heavy water-suspension of spores of a strain of *Actinomyces griseus* is prepared by scraping from agar slants or by first growing the organism under submerged aerobic conditions to obtain a heavy mass of growth. Incubation takes place at a temperature of about 22–28° C. Elaboration of the streptomycin is usually complete in 6–12 days in the case of stationary cultures and in 2–4 days when cultivation is under submerged aerobic conditions.

The course of production of streptomycin under submerged and stationary conditions is illustrated in Table II.

TABLE II

Course of production of streptomycin by A. griseus

Submerged cultures			Stationary cultures		
Days	Dilution units	Diffusion units	Days	Dilution units	Diffusion units
2	40	10	3	5	6
3	50	70	5	20	12
4	40	60	7	20	53
7	125	70	9	75	
			12	100	55

The culture broth obtained by either submerged aerobic or stationary cultivation of *Actinomyces griseus* is filtered or centrifuged to remove the growth of the organism. Activated charcoal is then added to the filtered broth and the mixture is stirred for about 5 minutes and then filtered. Alternately, the mixture can be stored for about 8–12 hours at 0–10° C., with stirring at about two-hour intervals and then filtered. The colorless or slightly yellowish filtrate obtained is discarded. The charcoal residue with the adsorbed streptomycin is washed several times with distilled water and finally with 95% ethanol.

The washed material is then suspended in 95% ethanol made approximately 0.15 normal with mineral acid, such as hydrochloric, and the suspension is stirred for several hours and then allowed to stand in the cold for another 6–8 hours with occasional stirring. The suspension is then filtered, the charcoal residue discarded, and the brown to yellow clear filtrate thus obtained is added, with stirring, to approximately an equivalent amount of ether. A brown-colored aqueous layer separates and is drawn off. The alcohol ether solution is washed with additional small amounts of water and the brown aqueous washings are added to the aqueous layer previously drawn off. The aqueous solutions are then neutralized to pH 6–7 and any precipitate formed is filtered off and discarded. A faintly colored aqueous solution containing streptomycin is thus obtained.

When stationary cultivation is employed, the pellicle (or growth of organism) once formed can be utilized again several times. The culture broth after complete elaboration of the active substance, is carefully drained from the pellicle and replaced by an equal amount of fresh culture medium. The containers are again placed in incubation at 22–28° C. and production of streptomycin sets in immediately, reaching a maximum in 3 to 5 days. The broth obtained by re-using the pellicle in this manner is treated as previously described to give a substantially colorless aqueous solution of streptomycin.

The following examples illustrate methods of carrying out the present invention, but it is to be understood that these examples are given by way of illustration and not of limitation.

EXAMPLE I

A medium is prepared having the following composition:

	Per cent
Glucose	1.0
Peptone	0.5
Meat extract	0.3
NaCl	0.5

Tap water.

This medium is distributed in appropriate vessels to a depth of 1–2 inches, sterilized at 10 lbs. steam pressure for 45–50 minutes, and then cooled.

The medium in each vessel is then inoculated with a heavy aqueous suspension of spores of a strain of *Actinomyces griseus*, and the inoculated media are maintained at an incubation temperature of 22–28° C. for 10 days. The growth is then filtered off and the filtrates are combined for further treatment.

To a batch of approximately 10 liters of filtered broth is added 150 gms. of activated charcoal. The mixture is stirred continuously for about five minutes and is then filtered. The slightly yellowish (almost colorless) filtrate is discarded and the charcoal residue is washed several times with distilled water and finally with 95% ethanol. The washed material is then suspended in 1.5 liters of 95% ethanol, made 0.15 normal with hydrochloric acid. The suspension is stirred for about an hour and allowed to stand in the cold for about 10 hours more with occasional stirring. The suspension is then filtered, the charcoal residue discarded, and the yellowish clear filtrate thus obtained is poured into 10 liters of ether, with stirring. A brown-colored aqueous layer separates and is drawn off. The alcohol-ether solution is washed with 100 cc. of water and the brown aqueous layer is drawn off and added to the first aqueous layer. The aqueous solution is

neutralized to pH 6–7 with dilute sodium hydroxide and any precipitate that forms is filtered off and discarded. A faintly colored aqueous solution containing streptomycin is thus obtained.

Example II

A medium is prepared having the following composition:

	Per cent
Glucose	1.0
Peptone	0.5
Sodium chloride	0.5
Corn steep liquor	1.2
Tap water.	

This medium is distributed in appropriate deep vessels having suitable means for agitation and aeration of the medium, sterilized at 10 lbs. steam pressure for 45–50 minutes and then cooled.

The medium in each vessel is then inoculated with a heavy suspension of spores of a strain of *Actinomyces griseus*, and the inoculated media are maintained at an incubation temperature of 22–28° C. for 3 days, with constant agitation and aeration. The growth is then removed by centrifuging and the culture broth is combined and further treated as described in Example I to isolate a substantially colorless, clear aqueous solution containing streptomycin.

Example III

The process of Example I is repeated with the exception that at the end of the incubation period instead of removing the broth by filtering it is carefully drained from the pellicle. An amount of fresh medium equivalent to the amount of broth drained from the pellicle is added to each vessel and the fresh media are again placed in incubation at 22–28° C. After 5 days in incubation the broth is again carefully drained from the pellicle and replaced by fresh medium. The broth obtained after each period of incubation is treated as in Example I to obtain a clear, substantially colorless, aqueous solution of streptomycin.

In the foregoing examples it is to be understood that the compositions of the culture media are merely illustrative and can be varied as, for example, by employing tryptone in place of peptone, and by employing meat extract and corn steep liquor alternatively in the several examples.

Modifications may be made in carrying out the present invention without departing from the spirit and scope thereof, and the invention is to be limited only by the appended claims.

What is claimed is:

1. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing material of the group consisting of meat extract and corn steep liquor, at an incubation temperature of 22–28° C. for a time of the order of 6–12 days for stationary cultivation and 2–4 days for submerged aerobic cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth, and recovering the adsorbed streptomycin.

2. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing meat extract, at an incubation temperature of 22–28° C. for a time of the order of 6–12 days for stationary cultivation and 2–4 days for submerged aerobic cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth, and recovering the adsorbed streptomycin.

3. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing corn steep liquor, at an incubation temperature of 22–28° C. for a time of the order of 6–12 days for stationary cultivation and 2–4 days for submerged aerobic cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth, and recovering the adsorbed streptomycin.

4. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing material of the group consisting of meat extract and corn steep liquor, at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, treating the broth with activated carbon to adsorb streptomycin therefrom, and separating streptomycin from the carbon by eluting with acid-alcohol in which streptomycin is soluble.

5. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing material of the group consisting of meat extract and corn steep liquor, to form the latter in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth and recovering the adsorbed streptomycin by elution.

6. In a process for producing streptomycin by growing a culture of a streptomycin-producing organism under conditions favorable to the formation of streptomycin, the steps which comprise separating streptomycin from culture broth containing it, by treating the broth with active carbon, and recovering streptomycin from the carbon by eluting with acid-alcohol in which streptomycin is soluble, said acid-alcohol being of low acid normality.

7. Procedure for recovering streptomycin from a culture broth in which it has been produced, comprising treating the streptomycin-containing broth with activated carbon to adsorb streptomycin therefrom, and separating streptomycin from the carbon by eluting with acid-alcohol in which streptomycin is soluble.

8. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing material of the group consisting of meat extract and corn steep liquor, at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, treating the broth with activated carbon to adsorb streptomycin therefrom, and recovering streptomycin from the carbon.

9. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing meat extract, at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from

the organism growth, and recovering streptomycin from the broth.

10. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* in a medium containing corn steep liquor, at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, and recovering streptomycin from the broth.

11. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth, and recovering the adsorbed streptomycin.

12. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of *Actinomyces griseus* at an incubation temperature of 22–28° C. for a time of the order of 6–12 days for stationary cultivation and 2–4 days for submerged aerobic cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, and recovering streptomycin from the broth.

13. Streptomycin.

SELMAN A. WAKSMAN.
ALBERT SCHATZ.

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Patented Dec. 8, 1925. 1,564,385

UNITED STATES PATENT OFFICE.

SELMAN A. WAKSMAN, OF NEW BRUNSWICK, NEW JERSEY, ASSIGNOR TO JACQUES WOLF & CO., OF PASSAIC, NEW JERSEY, A CORPORATION OF NEW JERSEY.

CONCENTRATED ENZYMIC SUBSTANCE AND METHOD OF PREPARING SAME.

No Drawing. Application filed August 11, 1922, Serial No. 581,236. Renewed February 28, 1925.

To all whom it may concern:

Be it known that I, SELMAN A. WAKSMAN, a citizen of the United States of America, residing at New Brunswick, in the county of Middlesex and State of New Jersey, have invented certain new and useful Improvements in Concentrated Enzymic Substances and Methods of Preparing Same, of which the following is a full, clear, and exact description.

This invention relates to enzymic substances and processes for producing the same and has for one of its principal objects to provide a stable enzymic substance which is more concentrated than those heretofore produced, and a method of producing the same.

Enzymes are unorganized ferments, or chemical substances of vegetable or animal origin that cause certain chemical transformations by their presence. Enzymes are produced by living cells, either animal or vegetable, including various bacteria and molds, for example, by the propagation of mold fungi such as *Aspergillus oryzæ* or other mold fungi of the genera Aspergillus, Penicillium and Mucor upon various substances of plant or animal origin, including bran of various grains. When brought into relation with certain organic compounds, the action of the enzymes tends to decompose such compounds into simpler combinations without themselves undergoing theoretically any change, although practically the enzymes gradually disappear in the course of the reaction. Their precise mode of action is not definitely understood but it is apparently catalytic. Enzymes act only in the presence of water and a small quantity serves to break up or change a large mass of the substance or substances on which it acts. Enzymes have well known properties such as diastatic and proteolytic. By virtue of their diastatic property, they may be employed to convert starch into sugar, this property being predominantly present in saliva which is an enzymic solution. By virtue of their proteolytic property enzymes exert a digestive action upon meat and other proteins, this property being predominantly present in the enzymic digestive juices in the stomach and pancreas of human beings and animals.

The present invention has to do with enzymes of vegetable origin having various properties including diastatic and proteolytic properties. The novel process may be generally described as follows. A suitable mass of fungus-impregnated culture medium is prepared and lixiviated with a suitable liquid such as water to extract the enzymic properties from such mass. A different or separate mass of fungus-impregnated culture medium is then soaked or steeped in the liquid extract. The liquid is then evaporated, i. e., the mixture is dried, leaving the desired enzymic substance.

Preparation of the fungus-impregnated culture medium may be effected in various ways. For example, it may be prepared in the manner disclosed in the now expired United States Patents Nos. 525,820 or 525,823 in which the fungus-impregnated mass is given the name of taka-koji.

Another, and in many respects preferable method of producing the fungus-impregnated mass is substantially as follows. Wheat bran, or other equivalent material; apple pomace, or another ventilating agent; and water are mixed, preferably in the following proportions: apple pomace—120 to 200 pounds; bran—enough to weigh with the pomace—about 1000 pounds; water—about 50 gallons. The mixture so formed is then sterilized by steaming for about one hour and then cooled down to a temperature of about 30° C., about one hour being consumed in the cooling operation. To this mixture so prepared is added about 35 gallons of water containing the seed spores. These seed spores are preferably those formed by mold fungi belonging to the genera Aspergillus, Penicillium or Mucor, such as the species *Aspergillus oryzæ* (or *Eurotium oryzæ*) or Aspergillus flavus which are readily procurable. The mass or mixture of bran, pomace, water and spores is then placed in trays and maintained at a temperature of 30° C. for about 40 to 48 hours, at which time the growth is arrested and if desirable the prepared mass is dried. For convenience I shall refer to this so-prepared dried or undried mass of fungus-impregnated material as protozyme.

Preparation of the liquid extract.

A dried, or undried, mass of fungus-impregnated culture medium is lixiviated with water. This may be done by permitting

water or other suitable liquid to percolate through the mass, or by steeping the mass in the liquid. If desired, the strength of this solution may be increased by repeatedly treating a fresh quantity of the mass therewith. The extract may then be filtered or strained giving the desired liquid extract.

Another, and preferred, method of preparing the liquid extract is to place undried or preferably dried mass of the fungus-impregnated medium (for example—protozyme as described above) in a percolator, into which liquid is poured, allowing it to stand for about one or two hours. The liquid may then be drained off through a thick cloth strainer and this liquid, either strained or unstrained, constitutes the desired extract. Water is added to the same batch of culture medium and drained off to be employed as the liquid for lixiviating a succeeding batch of fresh culture medium.

The next step is to soak or steep a fresh mass of culture medium, prepared as above described, in the liquid extract, prepared as above described. The mixture is allowed to soak or steep for one or two hours and then dried, i. e., the liquid is evaporated off, until the moisture content is reduced to 5 per cent or less. The resultant novel substance is conveniently referred to as Protozyme concentrated. Further concentration of the desired product may be secured by steeping or soaking the Protozyme concentrated, produced as described above, in a fresh quantity of liquid extract, again drying it, and if desirable again repeating this process as many times as desired.

Summarizing a preferred method of carrying out the process: two or more lots of Protozyme are prepared, each in the manner above described. One lot is allowed to dry. The other lot or lots are lixiviated to form a liquid extract, as above described. The lot of dry Protozyme is then soaked in the liquid extract and is then dried. The last mentioned dry product may then be steeped or soaked in a fresh batch of liquid extract, again dried, and this process repeated as often as desired, depending upon the degree of concentration desired. The drying may be effected by an air current in a partial vacuum, or by exposure to the sun on a dry day, or by simply spreading the mixture out in a dry place. In any case, the drying is continued until the moisture content is reduced to 5 per cent or less. The resultant dry product is Protozyme concentrated.

This resultant product not only possesses the stability of dry Protozyme or dry taka-koji but in its enzymic properties (diastatic and proteolytic and others) it is more than twice as concentrated as either of its constituents—Protozyme and liquid extract of Protozyme.

It has been proposed by various workers in the enzymic art (see pages 769 and 770 of "Allegemeine Mikrobiologie", Kruse 1910, published at Leipzig, Germany) to preserve enzymic solutions by employing antiseptics of various kinds. In my process I employ no antiseptic whatsoever, yet the resultant product does not deteriorate because it is in dry form.

The novel product resulting from the above described novel process may be described as a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate (i. e., a product of a process of concentration by drying or evaporation) of a liquid extract of a fungus-impregnated culture medium. This product is powerful in its enzymic properties, though of small bulk. Being dry, i. e., of reduced moisture content, it will keep and preserve its enzymic properties practically indefinitely.

The novel product may be employed to great advantage in the clarification of fruit juices, as a medicinal preparation against dyspepsia and in various other fields, arts and industries.

What I claim is:

1. The process of preparing an enzymic substance which comprises lixiviating a fungus-impregnated culture medium, soaking in the liquid extract a fresh mass of fungus-impregnated culture medim, and drying the mixture.

2. The process of preparing an enzymic substance which comprises preparing two lots of fungus-impregnated culture medium, drying out one lot, lixiviating the other lot to form an aqueous extract, soaking the dried lot in the extract formed from the other lot, and drying the mixture.

3. The process of preparing an enzymic substance which comprises propagating a fungus on a culture medium, drying one lot of such medium, lixiviating another lot of such medium with a liquid to which a third lot of culture medium has been subjected, soaking the dried lot of culture medium in the extract, and reducing the moisture content of the mixture until not more than 5 per cent moisture remains.

4. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate of a liquid extract of a fungus-impregnated culture medium.

5. The process of preparing an enzymic substance which comprises lixiviating a fungus-impregnated culture medium, soaking in the liquid extract a fresh mass of fungus-impregnated culture medium, drying the mixture, soaking the dried product in a fresh quantity of liquid extract of fungus-impregnated culture medium, and drying the mixture.

6. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate of a liquid extract of different lots of fungus-impregnated culture medium.

7. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrates of liquid extracts of a fungus-impregnated culture medium.

In testimony whereof I hereto affix my signature.

SELMAN A. WAKSMAN.

(19) Canadian Intellectual Property Office / Office de la Propriété Intellectuelle du Canada

An Agency of Industry Canada / Un organisme d'industrie Canada

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(54) FUMARIC ACID PRODUCTION

(54) PRODUCTION D'ACIDE FUMARIQUE

(57) Abstract:

This invention relates to processes for the production of fumaric acid from various carbohydrates and carbohydrate-containing materials, by means of certain strains of fungi.

The production of fumaric acid by certain fungi, particularly by Rhizopus nigricans, is known. However, the reported yields were either variable or insufficient for the utilization of the processes for industrial

purposes. These variations were due not only to a lack of a realization of the existence of a specific fungus capable of producing fumaric acid to the substantial exclusion of other acids but also to an insufficient recognition of the fact that an economic production of fumaric acid depends strictly on a knowledge of the physiology of the specific organism and on certain conditions of culture.

Though the importance of the specific strains of fungi, from a standpoint of merely a tendency to form fumaric acid, has hitherto been recognized, it has not been previously perceived that the ability to form fumaric acid is not a property characteristic of all strains of Rhizopus; that there is not only a quantitative difference but also a qualitative difference in acid-forming capacity between strains of Rhizopus; and that the various morphologically identical strains of Rhizopus nigricans, as well as the male and female races of even the same strain of this species, may differ strikingly in their acid-forming capacities.

I have now invented processes for the production of fumaric acid which give consistently high yields of fumaric acid under standardized specified conditions of fungal growth, treatment, and control.

My processes are carried out in two distinct ways - the one whereby the solution in which the fungal growth takes place is removed and is replaced by one or more solutions in which the fermentation for the production of fumaric acid predominates; the other whereby the

growth of the fungus and the entire production of the fumaric acid take place in the same solution. Aseptic technique is practised throughout.

In my processes I preferably use certain selected strains of species of fungi belonging to the genus Rhizopus. These are strains of Rhizopus nigricans. These strains are selected as possessing in particular an abundance of the enzyme mechanism by which they are able, when suitably grown and treated according to my processes, to produce fumaric acid in industrially important quantities. However, though the abundant production of fumaric acid by fungi is limited largely to species of the genus Rhizopus and is even still further narrowed by economic considerations to certain strains of the species Rhizopus nigricans possessing a distinctive physiological specificity, I have found that other organisms belonging to the order Mucorales are also capable of forming fumaric acid in industrially important quantities according to my processes herein described.

The isolation and identification of the fumaric acid producing strain of Rhizopus nigricans can be accomplished as follows:

Various natural materials such as soil, decaying organic material, spoiled bread and other spoiled foodstuffs, can be used as a source of the organism. This material is plated out on certain bacteriological media, especially those favorable to the development of fungi. The fungus medium of Waksman can be used for this purpose.

This medium contains, per liter, 5 gms. peptone, 10 gms. glucose, 1 gm. KH_2PO_4, 1/2 gm. $MgSO_4 \cdot 7H_2O$ and 25 gms. agar. The reaction of this medium is adjusted to pH 4.0. The medium is sterilized at 10 pounds pressure for 30 minutes. The natural material is plated out in this medium in various dilutions and allowed to incubate at 25°C. for a period of 2 to 3 days. The colonies of Rhizopus, which can easily be recognized by the trained observer, are then picked and transferred, either to the same medium in test tubes or to any other medium suitable for the growth of this fungus. Pure cultures are thus obtained. These cultures are now grown on liquid media having the composition and under the conditions outlined in this specification. The medium is tested for fumaric acid by known chemical methods after various intervals of incubation at 25°C. to 37°C. Those strains, which, under conditions comparable to those of my processes, give rapid and abundant growth and show a strong fumaric acid producing capacity, to the practical exclusion of undesirable acids, are selected for the purposes of my processes.

Suitable strains of other members of the Mucorales, such as certain species of Mucor, Cunninghamella, and Circinella, can be similarly selected. These strains and those of Rhizopus nigricans will be generally referred to as fumaric acid forming strains.

I have found that the carbohydrate: nitrogen ratio present in the nutrient culture medium is of great importance

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in the fumaric acid producing capacity of the described strains of Rhizopus nigricans as well as other Mucorale which were found to be capable of producing fumaric acid. In general the lower the carbohydrate:nitrogen ratio the more unfavorable are the conditions of fumaric acid accumulation, and conversely, up to a certain maximum, a higher ratio is more favorable for the conditions of fumaric acid accumulation. These carbohydrate (as carbon): nitrogen ratios have been ascertained to range between about 25:1 and 300:1, respectively. However, it has been found further that these conditions become considerably modified by the presence (or addition) of zinc ion to the medium so that in general this element greatly reduces the yields of fumaric acid especially during the period of active growth. This effect is much reduced at higher carbohydrate-nitrogen ratios, as described more fully later.

The most favorable sources of nitrogen have been found to be ammonium sulfate and other salts of ammonia, as well as urea and other compounds of nitrogen, though the influence of urea on fumaric acid formation is less favorable than that of ammonium sulfate.

As it is highly important to balance the nitrogen concentration in the culture medium, since an excessive amount of nitrogen will considerably delay the production of fumaric acid and will reduce or even prevent the yield of fumaric acid, I have determined that, for example, the presence of 200 mgs. to 500 mgs. of nitrogen per liter

of medium containing 50 to 150 gms. of carbohydrate gives favorable results. Maximum mycelial growth is attained, accompanied and followed by the production of fumaric acid.

The other constituents of the basal medium, besides carbohydrate, as a source of carbon, comprise, in certain cases, to be described, nutrient mineral salts, and a neutralizing agent such as calcium carbonate.

These nutrient mineral salts are used by me in various combinations and concentrations, substantially the following representing a typical composition: $(NH_4)_2SO_4$, 0.2%; $MgSO_4.7H_2O$, 0.05%; $K_2H\ PO_4$, 0.05%. In the case of crude carbohydrate materials these required salt concentrations may be less.

I have further found that the presence of certain catalytic or trace elements in the culture medium markedly modifies the physiology of Rhizopus. These elements have a decided effect both upon the growth of the fungus and upon the production of fumaric acid.

Specifically, zinc has a decided stimulating effect upon mycelial growth of Rhizopus at the expense of the accumulation of fumaric acid, whereas iron has a decided stimulating effect upon the production of fumaric acid by Rhizopus with a repressive effect upon its mycelial growth.

It has also been found that it is necessary to balance the nutrient culture medium in such a way as to produce the optimum mycelial growth by accelerating

such growth through the presence of zinc, so that this growth will be accompanied and immediately followed by the maximum formation of fumaric acid.

In attaining this balance, I add a salt of zinc, such as zinc sulfate, $ZnSO_4.7H_2O$, to the culture medium in concentrations of about 1 to 10 mgs. per liter of medium containing 50 to 150 gms. of carbohydrate, and balance the effect of the zinc with salt of iron, such as ferric sulfate, $Fe_2(SO_4)_3$, in concentrations of about 1 to 20 mgs. per liter of medium containing 50 to 150 gms. of carbohydrate. Optimum conditions - insofar as they are inducible by these trace elements - are thus obtained for both the mycelial growth and the fumaric acid production steps, which will be described in the examples to follow.

It is to be emphasized that the stated amounts of zinc and iron salts may not all have to be added to the medium and that somewhat higher concentrations of zinc and iron salts are not eliminated by the figures here given. The minute amounts of these elements which are required render it quite likely that they may be contained in larger or smaller quantities as impurities in some or all of the other major constituents of the medium. The fact remains, however, that zinc and iron are important elements in my processes.

The stated amounts of these elements, zinc and iron, are intended to apply when chemically pure nutrient salts are used together with a crystalline carbohydrate of a high degree of purity.

Analyses of the materials for contents of zinc, iron, and other heavy metals, and supplementary additions thereof, if the contents of them are deficient, provide accurate control of the critical ratio of zinc to carbohydrate in the growth state, as well as control of the factors involving the requirements of iron and other heavy metals.

I have found that up to 20 mgs. of $Fe_2(SO4)_3$ per liter of culture solution, depending on the carbohydrate: nitrogen ratio, provides a satisfactory concentration of iron for the acid-forming capacity of my selected strains of Rhizopus.

It has further been found that, on the one hand, the associative nature, or, on the other hand, the antagonistic nature of these two trace elements, zinc and iron, depends on the ratio in which they are present in the culture medium, their associative nature being manifested when the zinc and the iron exist in a ratio of substantially 1 to 2 of their stated salts.

In addition to iron, trace amounts of manganese and copper may be introduced in order to accentuate the described specific effect of zinc. Such traces do not require such rigid control as do those of zinc and iron.

It is to be especially noted that the addition of zinc as well as manganese and copper and minute quantities of iron may be highly desirable in order to obtain as rapidly as possible an abundant mass of cell substance, especially when a pure carbohydrate is used. This cell

substance would be formed without the simultaneous high yields of fumaric acid, since the presence of these heavy metals promotes the conversion of the carbon of the consumed carbohydrate into cell substance rather than allowing the carbon to accumulate in the form of fumaric acid. Such desirability depends upon the economic interest. Such addition may be particularly desirable when the mature growth substance is supplied with replacement sugar solutions. The cell substance will convert the sugar under such conditions into fumaric acid at a high rate. This procedure is described below.

Moreover, the carbohydrate concentration of the medium influences the intensity of the effects of zinc and iron, and I have determined that thenet effect of zinc and iron on fumaric acid production is associative when the initial carbohydrate concentration of the medium is high (i.e. 20 to 30 percent), but that the net effect of zinc and iron on fumaric acid production is antagonistic when the initial carbohydrate concentration of the medium is low (i.e. below 20 per cent).

I have found that small amounts of certain inorganic salts, namely, phosphates, and sulfates, accelerate both the process of fumaric acid production and the yield of fumaric acid if these salts are added to the culture liquid during the second and later stages of the fumaric acid production step, and that they serve as efficiently as iron in this respect and may be substituted therefor. For purposes of this acid-forming acceleration I may therefore

use instead of iron, for example, dipotassium phosphate, K_2HPO_4, and magnesium sulfate, $MgSO_4.7H_2O$, in concentrations of about 0.3 to 1 gm. each per liter of solution containing about 200 gms. of carbohydrate. Very small amounts of nitrogen may also be added.

I have further found that the mycelial mass of Rhizopus can produce free fumaric acid in large amounts even in the complete absence of a neutralizing agent. The free acid accumulates as such in the culture medium and eventually attains concentrations sufficient to crystallize out in the culture as free acid. This accumulation of fumaric acid has been found to lower the pH of the medium to about 2.4, at which point the crystallization of the acid occurs. The capacity of Rhizopus to produce fumaric acid at this high hydrogen ion concentration is noteworthy, as this pH range is much below that of the activity of most living systems.

However, the removal of the free fumaric acid, by its neutralization with calcium carbonate or other such agent, is decidedly conducive to an abundant mycelial growth especially in the presence of zinc. I, therefore, use a suitable neutralizing agent for this purpose as an essential step toward the attainment of optimum mycelial growth.

In view of the fact that an alkaline environment has a depressive effect on the germination of Rhizopus spores, calcium carbonate is added to the culture after mycelial growth has fairly well advanced, i.e., shortly after spore germination.

The accumulation of free acid soon tends to retard both the total amount and the rate of fumaric acid formation.

Therefore, it is desirable to neutralize this acidity. I have found that calcium carbonate or magnesium carbonate serves very satisfactorily to neutralize this acid and results in a more rapid conversion of sugar to fumaric acid and at a higher rate. This is true during both the original growth phase and during the subsequent replacement phase. The nature of these phases will become evident from the examples to follow.

I have found further that the soluble calcium fumarate salt reaches a concentration which exceeds its solubility and it crystallizes out abundantly throughout the medium and throughout the cell mass until the whole culture presents a solid mass of crystalline material. Under these conditions the conversion of sugar to acid ceases. It is, therefore, desirable to neutralize the acid with alkalies whose fumarate salts are more soluble and hence would not crystallize so readily. I have found that KOH or NaOH can be used quite effectively to obviate this difficulty. Care should be exercised when using the soluble alkalies not to add enough to render the pH above 7.0. It is preferable to adjust the pH periodically at frequent intervals to approximately 5.0 to 6.5.

I have further found that I can readily substitute for pure sugar or other pure carbohydrate a cheap crude material, such as molasses or starch materials, even in the first or growth-phase of my processes. In this way, fumaric acid yields are sacrificed in order to obtain a rapid and abundant cell substance for use in the replacement phase.

Extensive experiments in respect to the use of submerged growths of Rhizopus have been made, and I have determined that the utilization of the fermentation processes by means of this fungus for fumaric acid production is industrially practicable.

Selected strains of Rhizopus nigricans can be grown rapidly and abundantly, in a submerged condition, in large containers corresponding to tank conditions, with the aid of rapid aeration or under air pressure and adequate agitation, with or without calcium carbonate or other neutralizing agents. The rate of growth under these conditions is greatly accelerated (twenty-four to forty-eight hours as compared to five to seven days for stationary suface growth) and the yields of fumaric acid obtained have practical importance. The advantages of a deep-tank fungus fermentation process over shallow-pan processes are obvious. The original nutrient culture solution may be drained off through the false bottom used for aeration or otherwise filtered off, leaving the mass of fungal mycelium in the container, which is then recharged with carbohydrate solution and, if desired, with calcium carbonate, or neutralized periodically with soluble alkalies. The fermentation of this replaced sugar by the preformed mycelium begins immediately at a high rate, resulting in considerable economy of time. The mass of fungal mycelium still possesses a high fermentation capacity through several sugar replacements although with gradually diminishing vigor.

If during submerged fermentation some interference with the process should tend to arise from the crystallization and the solidification of the culture solution as a result

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of the limited solubility of calcium fumarate, such interference can be readily overcome by the use of lower concentrations of carbohydrate in the replacement solutions, or by the use of soluble alkalies, such as KOH or NaOH.

The use of pure sugar solutions in submerged fermentation results in the occurrence of a product of a high degree of purity, and the relative insolubility of fumaric acid permits the liberation of the free fumaric acid directly from the culture solution merely by concentration and by addition of a mineral acid, such as HNO_3 or HCl.

The temperature optimum for mycelial growth, with complete disregard for acid production, is about 35°C. However, the optimum for fumaric acid production during the replacement phase is about 28°C. It is consequently advisable to conduct growth at about 28-35°C; fermentation is best conducted at about 28°C.

The maximum growth of the fungus can be empirically observed to have occurred by the extent of the proliferation; or it can be determined from the nitrogen consumed; or it can be roughly ascertained by the removal from the process container and the weighing of a given area of the pellicle in the case of stationary cultures, or by the removal of aliquot portions of the contents of the container and the weighing of the fungus material contained therein in the case of submerged growths.

The several successive process steps which I have found to be, by virtue of their specific combination and sequence, valuable for the successful and economic production of fumaric acid will now be described.

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EXAMPLE I.

A culture solution, containing glucose or another carbohydrate or a mixture of carbohydrates, in concentrations of about 5 to 15 per cent total carbohydrate, is supplemented with the described nutrient salts and salts of catalytic or trace elements, and is sterilized by heat.

This culture solution is inoculated with a pure culture of selected strains of the fungus Rhizopus nigricans. Inoculation is effected by means of either the fungal spores or a predeveloped suspension of germinated spores, the latter resulting in considerable economy of time. The culture is incubated at about 28°-35°C.

In respect to the first stage, or growth phase, of this process, the culture is now allowed to grow either in a stationary manner, wherein a surface mass of growth or pellicle develops, or in a submerged condition, wherein the culture is gently agitated with air or with a mechanical stirrer, being held at atmospheric pressure or under increased air pressure, for 24 to 60 hours.

A lattice-like or other suitable support for the growing fungus is essential for the rapid development of an abundant mass of mycelial growth into a heavy pellicle in the stationary vessels, especially where a large surface area of liquid is to be covered by the growth. The lattice should preferably be approximately parallel to the liquid surface.

Calcium carbonate or other alkali is added to the culture liquid, stoichiometrically and aseptically, to neutralize the fumaric acid which the fungus has produced from the carbohydrate.

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In stationary growth tanks the liquid is agitated under the culture and care is taken not to injure the thin pellicle of growth which has formed on the surface.

The submerged culture is subjected to aeration either in rotating or in stationary tanks with air passing through the culture or under increased pressure.

When the growth of the fungus has reached its maximum at about 28°-35°C., which is usually within 2 to 7 days, depending on the relative concentration of the nutrient materials, trace elements and conditions of growth, such as temperature, the nutrient culture liquid in the containers in which growth has occurred is removed as completely as possible by drainage, by siphoning or by centrifuging.

This removed culture liquid contains the equivalent of about 10 to 45 per cent of fumaric acid on the basis of the carbohydrate consumed by the fungus, and this liquid is saved for the recovery of the fumaric acid therein.

The cell substance remaining in the containers (such substance being called "pellicles" in the case of the growths in stationary cultures) is then washed with sterile water to remove the last traces of nutrient salts, and this wash solution is drained off. This step is not essential for successful production of fumaric acid.

In respect to the second stage, or the replacement phase, of the process of fumaric acid production, the growth culture liquid is now substituted by the addition to the preformed cell substance of a solution of carbohydrate in concentrations containing up to about 20 per cent of carbohydrate, without supplementary nitrogen or other nutrient mineral salts. Simultaneously, calcium

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carbonate, substantially stoichiometrical to the anticipated fumaric acid yield, which may be up to about 50 per cent of the carbohydrate consumed by the fungus, is added to the fresh culture liquid, or neutralization is effected periodically by KOH or NaOH.

Accelerators of fumaric acid formation are also added at this step, for example: $Fe_2(SO_4)_3$ and $MgSO_4 \cdot 7H_2O$, each in concentrations of 0.1 gm. to 1.0 gm. per liter of solution containing up to 20 per cent of carbohydrate.

The mass of fungal growth, which abundantly provides a strong enzyme mechanism for producing fumaric acid, is now allowed to act upon this carbohydrate solution for a period varying from 1 to 7 days, being aided by agitation and aeration or air pressure similar to that described.

The rates of growth and fumaric acid production are greatly accelerated by the presence of a surface support in the stationary cultures and by aeration, by air pressure and by agitation of the submerged type of growth.

Upon the initiation of the second stage the temperature is kept at about 28°C., which appears to be about optimum for fumaric acid production.

The culture solution is removed, as before, and the replacement of the carbohydrate by new solutions is repeated several times until the enzyme system of the Rhizopus mycelium is substantially exhausted.

The mature cell substance retains for some time its function as an enzyme system under these conditions, converting the carbohydrate and so continuing to produce fumaric acid with gradually reduced vigor, however, to the practically complete exclusion of other acids, from as

many as three or four successive replacements with new carbohydrate solutions.

No vegetative growth, or very little, occurs in the replacement phase since nitrogen and other essential growth elements are lacking.

The addition of calcium carbonate or other neutralizing agent in the replacement phase is not absolutely essential in this process, because a substantially pure fumaric acid is formed and it crystallizes from the culture liquid owing to its low solubility. However, in order to bring about a high rate and an increased yield of fumaric acid formation, calcium carbonate or other neutralizing agents may be stoichiometrically added in this second stage.

Small amounts of other acids may be produced during both stages.

The final removal of the culture liquid containing calcium fumarate or other salts of fumaric acid and of the precipitated crystalline mass produced in the vessel completes the process. The fumaric acid is recovered as described.

EXMAPLE II

The several steps of the first stage or growth phase, of the process described in Example I are followed, but the replacement of the culture medium solution by successive carbohydrate solutions, as described in the second stage of the process of Example I, is avoided by utilizing an excess of carbohydrate (such as 20 to 30 per cent) over nitrogen in the medium in its initial preparation. This excess of carbohydrate over nitrogen functions in the manner already stated. Aeration is effected as previously described.

Thus, this process is continuous up to the point of substantial enzyme-system exhaustion of the fungal mycelium.

As in Example I, the presence of calcium carbonate as a neutralizing agent is not essential, though it or other neutralizing agents may be used in substantially stoichiometrical proportions for the purpose of increasing the yield of fumaric acid.

As also in Example I, removal of the liquid from the substantially exhausted fungal mycelium and removal of the crystalline mass formed in the vessel complete the process.

EXAMPLE III

The several successive steps of the first stage, or growth phase, of the process described in Example I are followed, except that a cheap crude carbohydrate-rich material, such as molasses or starch materials, is used in place of pure sugar and nutrient salts.

This crude material is diluted with water to afford a concentration from 5 to about 30 per cent of carbohydrate content, depending on the nature of the material, and, for reasons stated, it is supplemented with a nitrogen salt and a zinc salt, especially if these are lacking, as shown by analysis of the crude carbohydrate material. Excellent mycelial growth is produced on this medium.

As to the second stage of this process, this growth is now treated for the production of fumaric acid by the addition of a solution of sugar or other carbohydrate, in a concentration up to about 20 per cent of carbohydrate, as well as calcium carbonate or other neutralizing agent previously stated. An iron salt, and potassium phosphates and magnesium sulfate in proportions previously indicated

may also be introduced. Aeration is effected as previously described.

By the introduction of pure sugar solutions in the second stage of this process, a fumaric acid of high purity can be obtained in each of several successive treatments of the mass of fungal mycelium, until the latter's acid-producing activity is substantially exhausted.

As in the process of Example I, the presence of calcium carbonate during the second stage is not essential, as enough slightly soluble fumaric acid goes into solution and attains concentration to crystallize from the culture liquid. A neutralizing agent may be used, however, to cause a considerably increased yield of fumaric acid.

As also in Example I, final removal of the liquid from the fungal mycelium and removal of the crystalline mass formed in the vessel complete the process.

These examples are given by way of illustration and not of limitation, as it is obvious that certain modifications may be made in the steps of these processes, and in the kinds and proportions of the materials employed, without departing from the spirit and scope of the invention and the purview of the claims.

Having regard to the foregoing disclosure, the patent of which this specification forms part confers, subject to the conditions prescribed in the Patent Act, 1935, the exclusive right, privilege and liberty of making, constructing, using and vending to others to be used, the invention as defined in claims submitted by the patentee as follows:

1. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with the said strains, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5-6.5 and conducting the fermentation at about 28° C. to substantial completion.

2. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of Rhizopus, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric-acid-producing strains of Rhizopus, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5-6.5, and conducting the fermentation at about 28° C. to substantial completion.

3. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of Rhizopus nigricans, which comprises the successive steps of inoculating a carbohydrate solution containing nutrient materials with fumaric-acid-producing strains of Rhizopus nigricans, developing maximum fungal growth in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., removing the nutrient culture solution, supplying the developed mycelium with a fresh carbohydrate solution in the presence of an iron salt, adding a neutralizing agent in sufficient quantity to raise the pH value to about 5-6.5, and conducting the fermentation at about 28° C. to substantial completion.

4. Process for the production of fumaric acid by fungal fermentation of carbohydrate material, comprising successive steps of preparing a culture solution of fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates in concentrations up to about 5 to 15 percent total carbohydrate, supplementing such culture solution with nutrient salts and salts of members of the group consisting of manganese, copper and zinc, sterilizing said culture solution by heat, inoculating the sterile culture solution with a pure culture of fumaric-acid-producing strains of species of a genus of the order Mucorales, incubating the resulting inoculated culture solution from about 24 to 60 hours at about 28° to 35°C. until maximum mycelial growth has been attained, meanwhile supplying air under gentle agitation at not less than atmospheric pressure, adding an alkaline agent to neutralize the formed acid and to favor fungal growth, removing the culture solution from the container when fungal growth has reached the optimum growth of mycelium, adding to the fungal growth a solution of carbohydrate in concentrations containing up to about

20 percent of carbohydrate and a neutralizing agent, all in the presence of phosphates and sulfates, allowing the mass of fungal growth to act upon the said renewed carbohydrate solution for a period varying from 1 to 7 days at about 28° C., removing the culture solution and replacing it by successive fresh solutions of carbohydrate material until the fumaric acid forming capacity of the fungal growth is substantially exhausted.

5. Process described in claim 4, with the following modifications: utilizing an excess of fermentable carbohydrate selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates, over nitrogen in the medium in its initial preparation, whereby the replacement of the culture medium solution by successive carbohydrate solutions is avoided and whereby the process of fumaric acid formation is continuous up to the point of substantial enzyme system exhaustion of the mycelial growth.

6. Process described in claim 4, with the following modifications: substituting crude for pure fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates and nutrient salts, diluting such crude material with water, supplementing such solution with a nitrogen salt and a zinc salt, developing substantially maximum fungal growth on this medium, treating this growth for the production of fumaric acid by the addition of a solution of fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates in a concentration up to about 20 percent of carbohydrate.

7. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of a species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient materials and inoculating such solution with such fungi, according to claim 1, the added step which consists in growing the fungi in a submerged condition in deep culture tanks.

8. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing mutrient elements and inoculating such solution with such strains, the step which consists in balancing the nitrogen concentrations in the culture medium so that it will contain substantially 200 mgs. to 500 mgs. of nitrogen per litre of medium containing 50 to 150 gms. of carbohydrate.

9. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements and inoculating such solution with such strains, the step which consists in adding to the culture medium a zinc salt in substantially the concentrations represented by 1 to 10 mgs. of $ZnSO_4 \cdot 7H_2O$ per litre of medium containing 50 to 150 gms. of carbohydrate, whereby mycelial growth is stimulated.

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10. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc salt and inoculating such solution with such strains, the step which consists in adding to the culture medium an iron salt in substantially the concentrations represented by 1 to 20 mgs. of $Fe_2(SO_4)_3$ per litre of medium containing 50 to 150 gms. of carbohydrate, whereby an associative balanced concentration of iron and zinc salts is attained, and whereby fumaric acid formation is stimulated.

11. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc and iron salts and inoculating such solution with such strains, the step which consists in balancing the initial carbohydrate concentration of the medium by providing a carbohydrate content of 20 to 30 percent, whereby the net effect of the zinc and the iron is associative in the medium in concentrations substantially represented by the relation of 1 to 10 mgs. of $ZnSO_4 \cdot 7H_2O$ to 1 to 20 mgs. of $Fe_2(SO_4)_3$ per litre of medium.

B

12. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements together with zinc salt and inoculating such solution with such strains, the step

which consists in adding to the culture medium phosphates and sulfates in substantially the typical concentrations of about 0.3 to 1 gm. of dipotassium phosphate, K_2HPO_4, and about 0.3 to 1 gm. of magnesium sulfate, $MgSO_4 \cdot 7H_2O$, per litre of solution containing about 200 gms. of carbohydrate, whereby the effect of zinc is neutralized and fumaric acid formation is stimulated.

13. In a process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, involving the operations of preparing a carbohydrate solution containing nutrient elements and inoculating such solution with such strains, the step which consists in partially neutralizing the formed fumaric acid with alkalies, whose fumarate salts are more soluble than calcium fumarate, to a pH range of about 5.0 to 6.5, whereby the process of fermentation is not hindered by the occurrence of a solid mass of crystalline material within the container.

14. Process for the production of fumaric acid from fermentable carbohydrates selected from the group consisting of monosaccharides, starch, and materials containing these carbohydrates, by means of fumaric-acid-producing strains of species of a genus of the order Mucorales, which comprises the successive steps of inoculating such a carbohydrate solution containing nutrient materials with the said strains, developing fungal growth in a submerged condition in the culture solution at not less than atmospheric pressure in the presence of a zinc salt at a temperature range of approximately 28° to 35° C., meanwhile subjecting the nutrient culture solution to aeration, removing that solution, supplying the residual fungal mycelium with a fresh solution of such carbohydrate in the presence of an iron salt, conducting the fermentation at about 28°C. to substantial completion, and meanwhile subjecting the renewed solution to similar aeration.

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

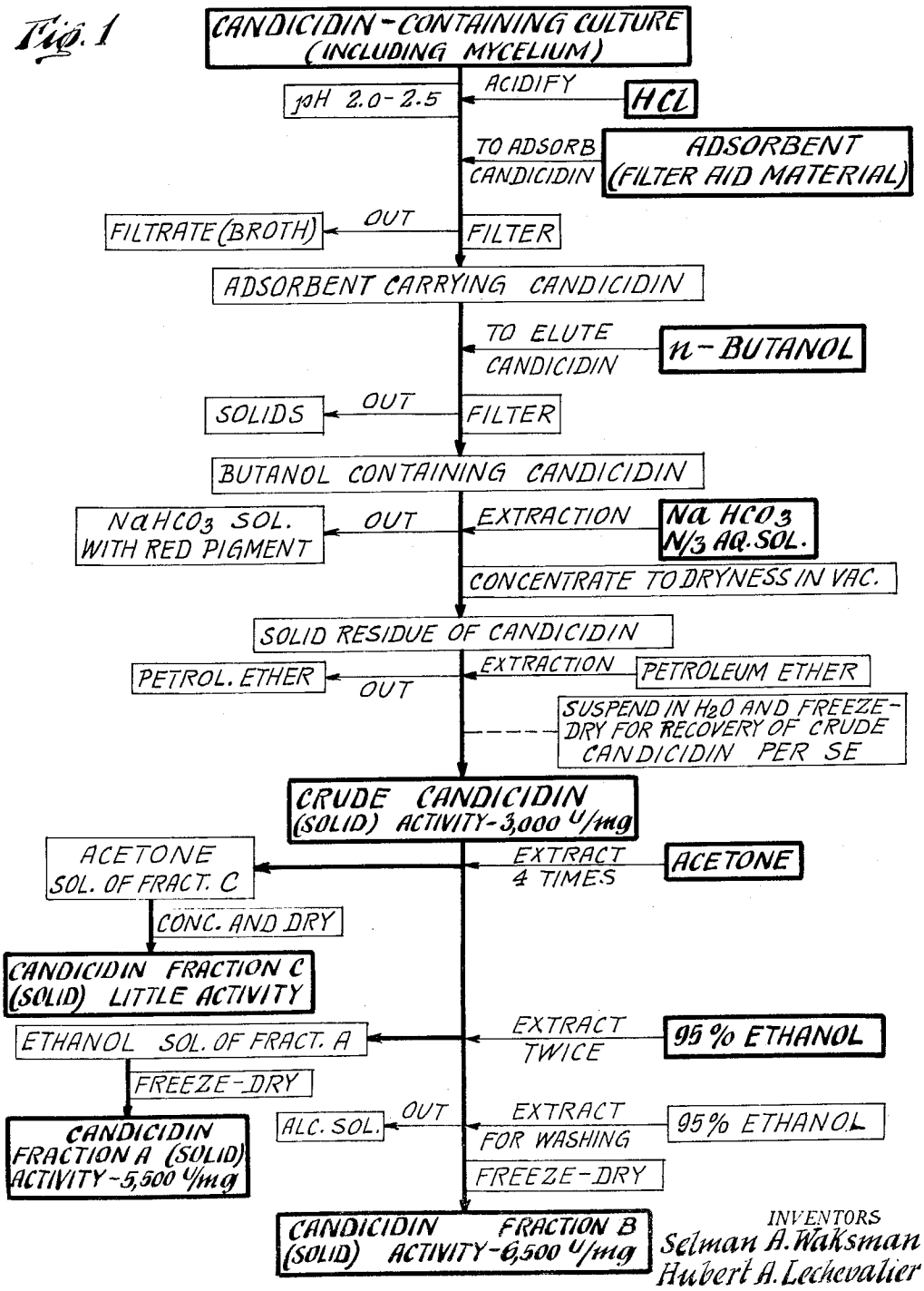
Cette Section est Absente

July 11, 1961 S. A. WAKSMAN ET AL 2,992,162

CANDICIDIN AND PROCESS OF PREPARATION

Filed Sept. 9, 1952 2 Sheets-Sheet 1

Fig. 1



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CANDICIDIN AND PROCESS OF PREPARATION

Filed Sept. 9, 1952 2 Sheets-Sheet 2

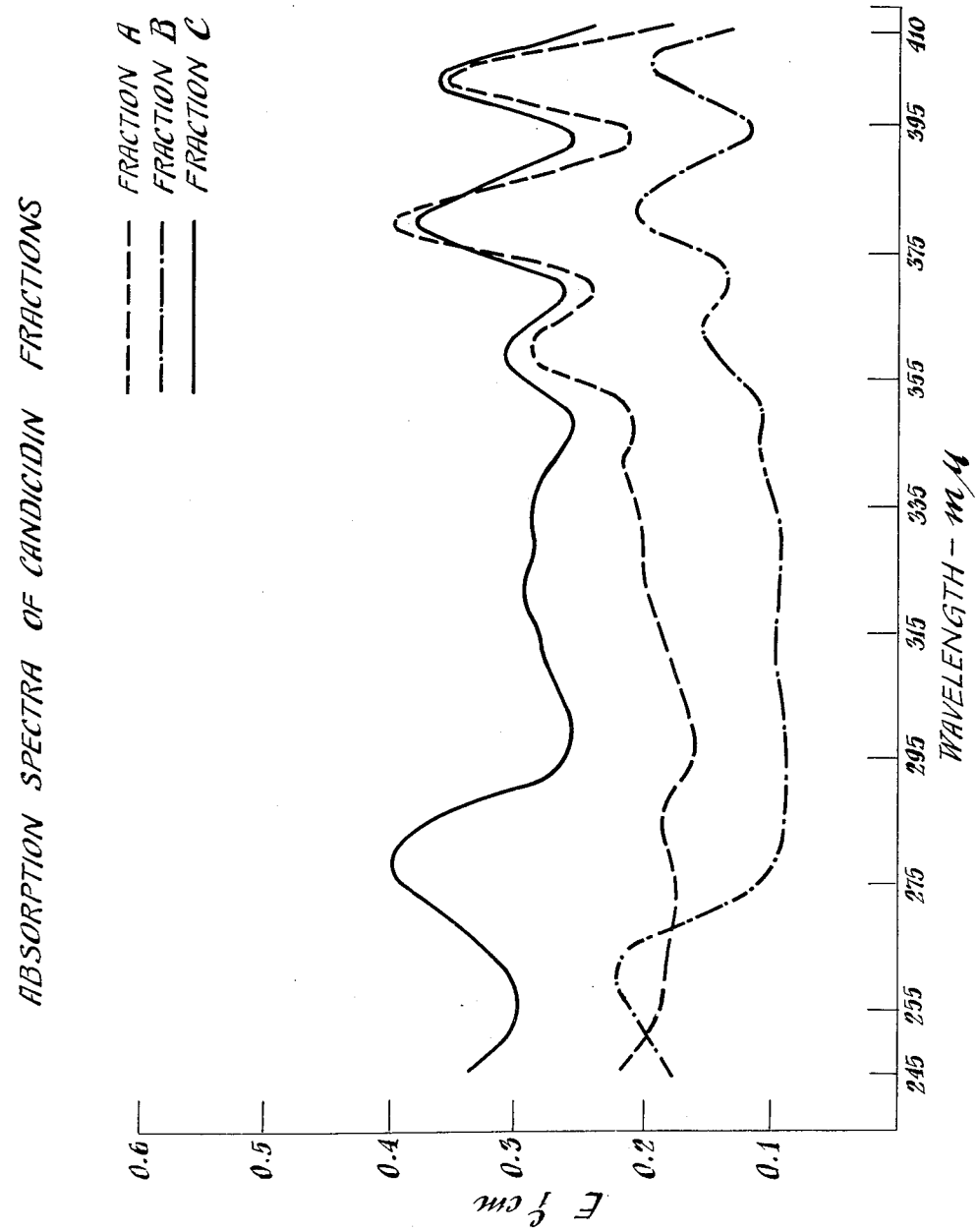

Fig. 2

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CANDICIDIN AND PROCESS OF PREPARATION

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4 Claims. (Cl. 167—65)

This invention relates to new organic chemical substances, viz. antibiotic compositions of the class of Streptomyces antibiotics, and to methods of preparing such substances. More particularly the invention relates to a new antifungal composition or complex and certain fractions of it, which have now been produced by cultivation under artificial conditions of a micro-organism of the genus Streptomyces, specifically a strain of *Streptomyces griseus*. The new antibiotic has been named "candicidin" and may be conveniently so identified herein. It exhibits high antifungal activity against a considerable variety of fungi, being efficiently fungistatic (markedly so against yeast and yeast-like fungi), and also fungicidal toward certan organisms, particularly *Candida albicans*. The novel antibiotic, considered as a complex or mixture, and likewise each of its fractions, particularly those hereinbelow defined as candicidin A and candicidin B, is unquestionably different from known antifungal agents such as actidione, antimycin A, fradicin, fungicidin, rimocidin and actinone. Candicidin, especially with respect to its fractions A and B and mixtures of them, has shown important utility in treatment of fungus infections of animals and plants, with no toxic effects and no detriment to the germination or growth of plant life.

In the accompanying drawings:

FIG. 1 is a combined flow diagram showing procedure for extracting candicidin and for separating its fractions; and

FIG. 2 is a graph of the ultra-violet spectra of the respective fractions.

The micro-organism which was used in the operations discovered (as described below) to product candicidin has been classified as belonging to the species *Streptomyces griseus*, by reason of its cultural and morphological properties. Minor differences distinguish it from

commonly known strains of *S. griseus*, e.g. streptomycin-producing strains and grisein-producing strains. This newly identified strain of *S. griseus* has been deposited in the culture collection of the Department of Microbiology of Rutgers University, the State University of New Jersey, as No. 3570. The following table describes certain morphological and cultural properties of the organism, together with corresponding properties of a streptomycin-producing strain of the same species:

TABLE I

Morphological and cultural properties of S. griseus *and No. 3570*

	S. griseus (streptomycin-producing)	No. 3570
Structure of aerial mycelium on yeast-dextrose and Czapek's agars.	Branching filaments forming tufts of straight sporulating hyphae.	Branching filaments forming tufts of curved sporulating hyphae.
Gelatin stab	Rapid liquefaction. Greenish-yellow or cream colored surface growth with brownish tinge.	Rapid liquefaction. Flaky growth falls to bottom. Faint brownish pigment.
Czapek's agar	Thin, colorless, spreading, becoming olive buff. Aerial mycelium thick powdery, water green.	Poor growth, thin cream colored, with brownish dark tinge in vegetative growth and greenish tinge in aerial mycelium.
Yeast-dextrose agar	Cream colored growth elevated in center, grey-green powdery aerial mycelium.	Cream colored lichnoid growth, grey powdery aerial mycelium.
Nutrient agar	Abundant cream colored, lichnoid growth powdery, white to light grey aerial mycelium.	Cream colored growth thin, powdery, light grey aerial mycelium.
Glucose-asparagine	Thin cream colored growth. Light grey aerial mycelium. No soluble pigment.	Same.
Litmus milk	Cream colored ring. Coagulation with rapid peptonization, becoming aklaline.	Coagulation rapid with peptonization, becoming alkaline.
Potato plug	Brownish, lichnoid growth, covered with white powdery aerial mycelium. No soluble pigment.	Same.
Pigment	Not soluble in medium	Same.
Nitrate reduction	+	+
Optimum temperature	37° C.	37° C.
Sensitivity to phage M-1	Sensitive	Resistant.
Sensitivity to streptomycin	100 mcg./ml.	2 mcg./ml.
Source	Garden soil	Cow manure.

It has thus been discovered that by cultivation of this organism under artificial conditions (e.g. upon inoculating a suitable, sterile medium with spores of the organism or with submerged growth) the new antibiotic or antibiotic complex, candicidin, is produced, including its particularly useful fractions A and B. It may be noted that the substance resulting from such cultivation and from initial stages of separation procedure as described below, usually constitutes a complex of fractions A, B and C, and is sometimes herein identified as crude candicidin; although fractions A and B are of particularly useful antifungal character (both individually and in admixture with each other), the complex or crude candicidin which also includes fraction C has nevertheless been found of distinct utility and because of less complexity in its production, may be deemed to represent a definitely new product of practical value as such. While other culture media may be employed, and likewise other modes of growth (e.g. in shallow stationary cultures), effective results have been obtained by growing the named organism on a yeast extract-containing medium under submerged aerobic conditions. After cultivation for a period of several days at appropriate temperature with shaking as on a conventional shaking machine, crude candicidin may be extracted from the culture, e.g. by suitable adsorption and solvent extraction as explained below. By further treatment, for example involving solvent separation or chromatography, fractions A, B and C are individually recovered.

The crude candicidin, which is not necessarily so styled because it has foreign impurities (from which in some cases it may even be essentially free) but because it contains all three fractions, if found to represent a substantially stable and highly effective antifungal agent, having apparently no action on bacteria, mycobacteria or actinomycetes. As stated above, an important applica-

tion of candicidin, both in its crude or complex state and in the form of either of its fractions A and B, is in controlling fungus infections or disease of plant and animal life.

The following are some examples of procedures for producing and extracting this antibiotic.

In one such process, the described strain of *Streptomyces griseus* (No. 3570) was grown in yeast-dextrose broth, i.e. in shaken culture, for four days, the broth at the end of this time being found to have a pH of 8.1 and to exhibit an activity of 900 candicidin "units" per milliliter. For this and other assay purposes mentioned herein, so-called dilution assays were employed rather than diffusion methods, in that candicidin does not diffuse readily in agar media. Thus using the streak-dilution assay method, one unit of candicidin was defined as the minimum amount of the antibiotic per one milliliter of peptone-glucose agar, which would completely inhibit the growth of *Candida albicans*. The peptone-glucose agar medium utilized for assay purposes consisted of 1% glucose, 0.5% peptone, 0.5% sodium chloride, 0.3% meat extract and 1.5% agar, in tap water, the pH being adjusted to give 7.2 after sterilization. For assay purposes, the inoculum consisted of an 18 hour old culture of *C. albicans* grown on yeast-glucose agar, although it appeared that differences in the age of the *C. albicans* culture used for assays by the streak dilution method did not cause appreciable variation.

It will be understood, therefore, that references herein to the activity or potency of candicidin preparations in the above units (i.e. "dilution units") are generally to be taken as meaning the number of the defined minimum quantities of candicidin per milliliter of the preparation in question.

For extraction of crude candicidin from the yeast-dextrose broth cultivated as described above, one procedure was as follows: the broth, after separation of the mycelium by filtration, was extracted three times with one-sixth of its volume of n-butanol. The aqueous layer, which had no appreciable activity, was discarded, and the butanol extract was concentrated in vacuo and then freeze dried. The resulting solid preparation of crude candicidin was found to have a strength of 900 units per milligram. The yield by this method of operation represented 20 to 40% of the activity in the culture broth.

Another and particularly effective mode of producing candicidin comprised the cultivation of the stated organism on yeast-glucose medium. A satisfactory, specific medium of this character consisted of bacto yeast extract 1%, cerelose (commercial glucose) 1%, and tap water; before sterilization the pH was adjusted to 7.5 with 20% sodium hydroxide solution, and the medium was then sterilized in a conventional manner. For effective production in substantial quantity with this medium, the following steps were found useful. A primary inoculum of *S. griseus* No. 3570 was first prepared by transferring spores from agar slants into 250 ml. flasks each containing 100 ml. of the described yeast-glucose medium. These flasks were shaken for 24 hours at 28° C. and the cultures were used to inoculate a series of similar flasks, which were identically incubated for a further 24 hours, to provide a secondary inoculum. Finally, large flasks, i.e. 2000 ml. flasks, each containing 300 ml. of the same medium, were then each inoculated with one of the cultures of the second inoculum and were shaken at 28° C. for four days. At the end of that time the large flasks were found to contain broths having candicidin potencies of 1000 to 5000 units per ml., the cultivation thus being very effective for production of the antibiotic, i.e. in its crude or complex state.

Using the same method of sterilization, inoculation and incubation, the following medium gave even higher yields (10,000 to 20,000 units per ml.) than the yeast-glucose medium: a soya peptone-glucose medium, for

example consisting of 2% soya peptone (De Lamar-Hendrey) and 1% cerelose, in tap water.

Yet another cultivation process for making candicidin involved the use of a glutamic acid-glucose medium. One effective medium of this character contained the following (parts being given in grams or milliliters):

K_2HPO_4	g--	0.5
$MgSO_4 \cdot 7H_2O$	g--	0.2
$Fe(SO_4)_3 \cdot 9H_2O$	g--	0.01
$ZnSO_4 \cdot 7H_2O$	g--	0.01
Anhydrous glucose	g--	10.
L-glutamic acid	g--	10.
Distilled water	ml--	1000

The pH of the medium was adjusted to 7.3 (with NaOH) before sterilization in a conventional manner, e.g. sterilization by autoclaving at 15 pounds for 15 minutes. In this case, 250 milliliter flasks each containing 100 ml. of the medium were inoculated with a washed one-day-old yeast-glucose shake culture of *S. griseus* No. 3570. So inoculated, the culture medium was incubated by maintaining the flasks on a shaking machine at 28° C., for five days. Broths having candicidin potencies of 3000 to 5000 units were obtained by such operation.

An improved and particularly effective extraction procedure, which has been utilized for the yeast-glucose and glutamic acid-glucose broths (it being understood nevertheless that each of the various extraction procedures described herein can be used with any of the cultivation methods) was the following, illustrated in the upper part of FIG. 1. As the result of growth of the organism under the conditions outlined above, e.g. growth on the yeast-glucose medium, the culture attained a pH value of 7.5 to 8.5. The entire culture, including the mycelium, was treated with hydrochloric acid to give a pH of 2.5. The acidified broth was then treated with material to adsorb or retain the active material, a suitable adsorbent being filter aid material such as diatomaceous earth. Thus specifically, the acidified culture, still including the mycelium, was stirred for 10 minutes with 0.5% Hyflo Super-Cel, viz. a filter aid substance of the type described. The resulting Super-Cel pad was filtered off and was found to retain, at least in substantial measure, the antibiotic present both in the broth and in the mycelium.

A suspension was then made by mixing equal volumes of the wet Super-Cel and n-butanol. The solvent, i.e the butanol, took on a deep red color and was found to contain the active substance. It was accordingly filtered, and the insoluble material was discarded. The butanol solution was then treated by extracting it exhaustively with 2% of its volume of one-third normal sodium bicarbonate solution; by such extraction the color was substantially removed, the $NaHCO_3$ extraction thus being understood to separate the pigment. The residual, more or less decolorized butanol was then concentrated to dryness in vacuo and the dry residue was extracted with petroleum ether. It appeared that most or all of the activity remained in the residue which was insoluble in the petroleum ether, such solvent (which presumably removed further impurities) being correspondingly discarded. The residue from the petroleum ether extraction was then suspended in water and freeze dried, to produce a preparation of crude candicidin which contained approximately 3000 units per milligram.

The extraction thus resulted in a relatively potent candicidin product, the actual yield (measured against the cultivated broth) being from 40 to 60%. It conclusively appeared that this product represented the same antibiotic (crude candicidin or the candicidin complex) as was obtained by the extraction procedure described above, except that the product of the more complete procedure was considerably purer. As explained, it was further found that crude candicidin essentially consists of three fractions, herein called A, B and C. Fractions A and B had almost equivalent biological activity and

do not appear, from present experiments, to be differentiated by their anti-microbial spectra. The third fraction, i.e. fraction C, was found to have negligible activity, although as a component of crude candicidin, fraction C does not seem deleterious.

It was discovered that the three fractions could be separated by a variety of procedures, one convenient mode being with organic solvents. For instance, crude candicidin may be extracted first with acetone, which is found to dissolve the fraction C, leaving a solid residue which contains both fractions A and B. Upon extracting this residue with alcohol (i.e. ethanol) candicidin A is separated in solution, the remaining alcohol-insoluble solid then constituting candicidin B.

By way of specific example of this solvent method (as shown in the lower part of FIG. 1) for separating the fractions: 1.9 grams of crude candicidin obtained (as described above) by the more complete extraction process from an incubated yeast-glucose broth and having an activity of 3000 units per mg., were extracted four times with acetone, using 25 milliliters each time. The acetone fractions were combined and found to contain fraction C of the crude material, such fraction being isolated by a suitable process such as evaporation in vacuo and appropriate freeze drying. As explained, fraction C has no significant biological activity.

The solid residue from the butanol extraction in itself constitutes a very useful antibiotic composition, being a concentrate of fractions A and B, although a somewhat purer preparation of such character may be obtained by later re-mixing the ultimately separated and dried fractions. For effecting such separation the above-described solid residue (from butanol extraction) was extracted twice with 25 ml. of 95% ethanol. Combining the quantities of ethanol (separated from the solid) the alcohol solution was then freeze dried to yield 500 milligrams of a solid product, identified as fraction A and having an activity of 5500 candicidin units per milligram. The solid residue left from the ethanol extraction was then extracted four more times with 25 milliliters of ethanol, the further alcohol solutions being discarded. After such washing, the remaining solid (upon appropriate drying) was found to represent fraction B, having a weight of 420 mg. and an activity of 6500 candicidin units per milligram. It will thus be seen that candicidin A and candicidin B are distinguished by insolubility in acetone, in which fraction C is soluble. At the same time, fraction A is soluble in ethanol, while fraction B is not.

Crude candicidin has also been purified, and specifically separated into its fractions, by chromatography, for example on a chromatographic column composed of cellulose powder. The column was prepared by pouring a water suspension of cellulose powder into a glass tube; while still moist, it was washed successively with equal amounts of ethylene glycol monomethyl ether, ethanol, and chloroform. The column (2.5 cm. in diameter and 70 cm. high) was then loaded with a chloroform suspension of crude candicidin, e.g. the product of the more complete extraction process described above, having an activity of 3000 units per mg. The chloroform suspension, with which the column was loaded, contained approximately 40 million units of candicidin.

Successive elutions were effected, i.e. by using a total series of 18 solvent cuts in a conventional manner, for recovery of the several fractions. The first elution was carried out with 95% ethanol and yielded a reddish brown eluate, fraction C. Further elution was accomplished by the addition of an equal mixture of ethylene glycol monomethyl ether and ethanol. This mixture eluted first a reddish brown compound, fraction A, followed by a greenish compound, fraction B. As far as could be determined from the comparison of their biological spectra, solubility properties, ultra violet adsorption spectra, and R*f*-value upon paper chromatography, these three fractions A, B and C were identical with the corresponding fractions obtained by solvent extraction.

Referring now more specifically to the properties of candicidin, it is apparent, especially since fraction C exhibited little or no antibiotic activity, that the activity of crude candicidin is due to the action of fractions A and B. The following table represents the antifungal spectrum of crude candicidin (i.e. the candicidin complex) extended through a considerable number of fungus organisms, including particularly yeasts and yeast-like fungi. In attaining this spectrum, the various organisms were tested on peptone-glucose medium, with incubation in each case for 2 to 5 days at 28° C. The readings are expressed in micrograms of crude candicidin (being the 3000 unit material) per milliliter of medium for complete inhibition; thus the lower the value of the reading, the greater or more potent the activity against a given organism.

TABLE II

Antifungal spectrum of crude candicidin

	Microgram/per ml. for complete inhibition
Acrostalagmus sp.	>10
Alternaria sp.	0.6–10
Aspergillus niger	5–70
Candida albicans	0.3–0.5
Cercospora kikuchii	8–15
Ceratostomella ulmi (P. strain)	0.15–0.3
C. ulmi (H. strain)	3–5
Botrytis sp.	16–50
Diaporthe sp.	13–70
Epicoccun sp.	30–50
Fusarium sp.	66–>100
Helminthosporium sp.	13–20
Hormodendron sp.	30–50
Isaria sp.	>100
Oospora sp.	>100
Mucor sp.	3–10
Penicillium notatum	5–100
Pestalizzia sp.	0.6
Phoma sp.	66–100
Polyporus sulphureus	8–20
Pullularia sp.	1–3
Pythium sp.	66–100
Saccharomyces cerevisiae	<0.15–0.3
Sclerotium rolfsii	>100
Spicaria sp.	>100
Stemphylium sp.	>85
Stysanus sp.	>100
Trichoderma sp.	20–30
Ustilago zeae	1–20
Verticillium sp.	0.6–>100

The spectrum of crude candicidin was also extended through a considerable range of other types of organisms, such as bacteria (including mycobacteria) and actinomycetes, but no activity was found against any organisms of these kinds, despite the test of a considerable variety. Thus even with concentrations of crude candicidin equaling from 85 to 100 micrograms per milliliter of a nutrient agar medium, effective inhibition was not obtained (in streak dilution tests involving incubation at 37° C. for 18 to 48 hours), with any of the following organisms:

Escherichia coli
Aerobacter aerogenes
Serratia marcescens
Pseudomonas fluorescens
Bodenheimer's organism
Staphylococcus aureus
Bacillus subtilis
Mycobacterium phlei
M. avium
Mycobacterium sp. 607
Streptomyces fradiae

The antifungal spectrum of fraction B, taken with a considerable number of different organisms is represented in the following table, wherein tests were made on peptone-glucose agar as in the case of Table II, above, with results given, again, in micrograms per ml. for complete inhibition:

TABLE III

Antifungal spectrum of fraction B

Organism	Days of Incubation	
	1	5
Aspergillus niger [1]	2	>10
Candida albicans [2]	0.22	1.1
C. brumptii [2]	3.3	>10
C. kruseii [2]	1	2.2
C. neoformans [2]		0.5
C. pseudotropicalis [2]	1.7	>10
C. stellatoides [2]	0.14	1.1
C. tropicalis [2]	2	>10
Ceratostomella ulmi (P. strain) [1]		1.1
Cryptococcus neoformans [2]		0.5
Fusarium sp.[2]		10
Hormodendrum pedrosoi [1]		>10
Mucor sp.[1]	0.1	>10
Penicillium notatum [1]		1.1
Phialophora verrucosa [1]		>10
Rhizopus nigricans [1]	>10	>10
Saccharomyces cerevisiae [2]	0.06	0.14
Sporotrichum schenkii [1]		>10
Trichophyton gypsum [1]		>10
T. mentagrophytes [1]		>10

[1] Incubated at 28° C.
[2] Incubated at 37° C.

Tests also showed that the antibiotic spectra of fractions A and B differed only in degree of inhibition of the test organisms, a representative comparison being shown in the following table, where assays were made on the peptone-glucose medium, but wherein the results are expressed in candicidin dilution units per milligram (for inhibition), such that the larger figures represent greater activity.

TABLE IV

Comparison of antifungal activities of fractions A and B

	Dilution units per mg. Fractions	
	A	B
Candida albicans	2,000	3,000
Ceratostomella ulmi	>10,000	>10,000
Trichoderma sp	90	250
Penicillium notatum	<90	<250
Aspergillus niger	200	200
Rhizopus nigricans	<90	<250

In addition to its fungistatic effect, candicidin was found to have substantial fungicidal activity. Thus the crude material was applied to heavy suspensions of cells *Candida albicans*; complete sterilization of a suspension of resting cells was effected in three hours with a concentration of 5 micrograms per ml., while 1 microgram per ml. was found sufficient to sterilize a culture of growing cells in three hours. When *C. ulmi* and *A. niger* were tested in a similar manner only little fungicidal activity was obtained for candicidin.

Crude candicidin, i.e. the complex containing fractions A, B and C, was found to be soluble in the higher alcohols (such as glycerol and benzyl alcohol) but insoluble in benzene, petroleum ether, carbon tetrachloride, xylol, carbon disulfide, ethylene dichloride, ether, and ethyl acetate. Each of the fractions is therefore likewise soluble in the higher alcohols and insoluble in each of the last-named group of organic liquids. Further data on solubility of the crude or complex material and the fractions are set forth in the following table, wherein plus signs indicate solubility, minus signs indicate that the material is insoluble and wherein a combination of plus and minus signs indicates partial solubility:

TABLE V

Solubility of candicidin (complex and fractions)

	Crude candicidin	Fraction		
		A	B	C
Water	+—	+	—	—
Ethanol	+—	+	—	+
Butanol	+	+	+*	+
Ethylene glycol (EG)	+	+	+*	+
EG monomethyl ether	+	+	+*	+
Acetone	+—	—	—	+

The solubility indicated by plus signs represents generally a high solubility, except in the instances marked with an asterisk, where there was somewhat less, but nevertheless substantial, solubility. That is, the fraction B solid preparations appeared to have a solubility in these solvents of about 100 micrograms per ml. It will be noted that only fraction A is soluble in water, and that only fraction C is soluble in acetone and that there is likewise a difference among the fractions with respect to solubility in ethanol.

Candicidin appeared to have reasonably good stability, both at room and other temperatures, concentrated solutions being more stable than dilute ones. At neutrality, each of fractions A and B effectively withstood heating for 10 minutes at 60° C., and in one set of tests was kept for a week at 4° C. without appreciable loss of activity. Thus it would appear that the antibiotic, especially its active fractions, can be effectively stored under low temperature conditions for relatively long times. Each of fractions A and B showed progressive loss of activity upon standing at room temperature, i.e. when dissolved in ethylene glycol monomethyl ether.

The three fractions of candicidin had very similar ultra violet absorption spectra as shown in FIG. 2. These spectra were attained with solutions of the respective substances, i.e. fractions A, B and C, in absolute methanol, by conventional procedure using a Beckmann spectrophotometer. The concentration of fraction A was 0.04 milligram per milliliter, of fraction B 0.02 mg. per ml., and of fraction C 0.1 mg. per ml. In the graph shown, wave lengths are measured along the horizontal axis and spectral intensity is indicated on the vertical axis by specific extinction, a conventional representation of intensity duly correlated with the actual concentration of the substance in its solution. Specifically, significant peaks were obtained for the three substances as follows, the wave length being measured in millimicrons in each instance:

Fraction A ---------- 360, 380, 403 mμ
Fraction B ---------- 362, 381, 404 mμ
Fraction C ---------- 358, 379. 402 mμ

The sets of three peaks noted respectively for the three fractions constitute distinctive characterizations of them, distinguishing them from other antibiotic substances of this class.

It was also found that crude candicidin did not dialyze through a cellophane membrane, it being correspondingly apparent that none of the fractions would so dialyze.

Elementary analysis of fraction A gave the following values on an ash free basis: H=9.6%, C=62.9% and N=4.7%. Fraction B was somewhat different: H=9.9%, C=57.8% and N=7.3%. Some sulfur could also be detected in both fractions but it is very possible that it was present as an impurity in the ash.

As stated above, candicidin (and thus its active fractions A and B) has exhibited utility for controlling fungus infections in plants, with no deleterious effects on germination, growth, foliage or other factors of importance in agriculture. For example, in one set of tests crude candicidin was found to have no ill effect upon the germination of pea seeds, i.e. in concentrations (of candicidin complex) of 125 micrograms per milliliter or

less, and even at somewhat higher concentrations the effects were not very serious. In making these tests, 2 ml. of the candicidin solution (i.e. the 3000 unit material described above) were added to 5.5 centimeter petri dishes each containing 5 to 10 pea seeds. These dishes were placed inside regular size (9.0 cm.) dishes containing an absorbent pad saturated with water. The seeds were incubated for 5 to 6 days at 20° C., and both germination and root growth were measured, relative to controls which were similarly incubated but without the candicidin. As stated, no ill effects were observed in either respect, up to a rather large concentration of the antibiotic.

In one set of practical tests relative to the utility of the antibiotic for control of fungus infections, it was found that upon spraying young bean plants once a week with an aqueous suspension of crude candicidin (660 micrograms per ml.) there resulted an effective and useful decrease of a powdery mildew infection. The spraying was started about 5 days before the mildew infection was apparent on the plant leaves, it being known that the plants had been or would become infected with the organism causing the infection. Although a single spraying before the appearance of the mildew did not reveal therapeutic value, the use of a plurality of sprayings, e.g. at the indicated intervals, was effective. No toxic reactions, e.g. in damage to foliage or otherwise, were noted in the tests.

It will be appreciated that upon comparison with other antibiotics that are produced by actinomycetes and that are antifungal and lack antibacterial activity, candicidin is clearly distinguished in a number of ways, including its various properties and characteristics outlined above. Critical distinction is afforded by the ultra violet absorption spectra since such spectra for the candicidin fractions were distinctly different from the spectra for actidione, antimycin A, fradicin, fungicidin and rimocidin. Although the substance actinone was not available for the making of spectra for comparison, clear distinction was evident in other respects; thus known data indicate that actinone is less active in vitro than candicidin and that the solubility properties and the nitrogen content of the two substances are clearly dissimilar.

Although by way of example reference has been made above to *S. griseus* No. 3570, other strains of *S. griseus* have been isolated from soil which have been found to have the capacity to produce the candicidin complex. Likewise, still other Streptomyces strains (different from *S. griseus*) such as a culture identified as No. 3633 in the above-mentioned Rutgers University culture collection, have the capacity to produce selectively fraction A (the water soluble fraction) of candicidin.

It is to be understood that the invention is not limited to the specific preparations and procedures hereinabove described but may be carried out in other ways without departure from its spirit.

We claim:

1. Candicidin, an organic antifungal antibiotic composition: which is soluble in methanol, butanol, glycerol, benzyl alcohol, ethylene glycol, and monomethyl ether of ethylene glycol; which is insoluble in acetone, benzene, petroleum ether, carbon tetrachloride, xylol, carbon disulfide, ethylene dichloride, ether and ethyl acetate; which in methanolic solution displays absorption peaks of ultraviolet light at approximately the following wave lengths expressed in millimicrons, 360–62, 380–81 and 403–4; which is strongly active against fungi, including yeasts, yeast-like fungi and plant-pathogenic fungi; which has substantial fungistatic effect upon

Candida albicans
Ceratostomella ulmi
Saccharomyces cerevisiae

which is relatively inactive against bacteria, mycobacteria and actinomycetes; which has substantial fungicidal effect on *Candida albincans*; which is capable of embodiment in water-soluble form A and in water-insoluble form B, each of said forms having all of the aforesaid properties; and which is produced by the process of growing a culture of *Streptomyces griseus* No. 3570 in a nutrient medium at a suitable incubation temperature and for a suitable period of cultivation to form the composition in the culture, and then recovering the so produced composition from the culture.

2. A process for producing the antibiotic composition candicidin, which is defined in claim 1, comprising growing a culture of *Streptomyces griseus* No. 3570 in an aqueous, sugar-containing nutrient medium at a suitable incubation temperature and for a suitable period of cultivation to form candicidin in the culture, and separating candicidin from the culture medium.

3. In a process for producing the antibiotic substance candicidin, which is defined in claim 1, the steps of establishing a material containing candicidin by growing a culture of *Streptomyces griseus* No. 3570 in an aqueous nutrient medium, separating the candicidin from said material by treatment with butanol to dissolve the candicidin, separating the butanol solution from said material, and separating candicidin from the butanol.

4. A process for producing the antibiotic composition candicidin, which is defined in claim 1, comprising growing a culture of *Streptomyces griseus* No. 3570 in a nutrient medium at a suitable incubation temperature and for a suitable period of cultivation to form candicidin in said culture, and then recovering the so produced candicidin from the culture.

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(54) CHEMICAL COMPOUNDS AND PROCESSES FOR PREPARING THE SAME

(54) COMPOSES CHIMIQUES ET PROCEDES POUR LEUR PREPARATION

(57) **Abstract:**

This invention relates generally to antibiotic substances and more particularly to a new and useful antibiotic substance, streptomycin, and the process for preparing the same by cultivation under particular controlled conditions of strains of the microorganism Actinomyces griseus. This organism was first isolated from the soil and characterized by one of the present applicants, S.A. Waksman, and is described in his publication in Soil Science 8. 71 (1919).

With the exception of streptothricin, the discovery and characteristics of which were reported by Waksman and Woodruff in Proc. Soc. Biol. Med. 49, 207 (1942) and Jour. of Bact. 46, 299 (1943), most antibiotic substances known at the present time, including penicillin and other mold products as well as gramicidin and actinomycin, act largely upon gram-positive bacteria. Streptothricin is very active against a number of gram-positive and gram-negative bacteria but has very little activity against certain bacteria of both groups, particularly the gram-positive Bacillus mycoides and Bacillus cereus and the gram-negative Pseudomonas fluorescens, Pseudomones aeruginosa, and Serratia marcescens.

It is now discovered, according to the present invention, that upon cultivation of strains of the micro-organism Actinomyces griseus in a suitable nutrient medium a new substance can be isolated from the resulting culture

broth, which substance is thermostable; has the properties of a base; is soluble in water, acid alcohol and dilute acids but is insoluble in ether and chloroform; has a low toxicity to animals; and is strongly active bacteriostatically against many gram-positive and gram-negative bacteria. This substance has been designated as streptomycin. It is in many respects similar to streptothricin but differs from streptothricin as will be apparent from the comparative bacteriostatic spectra in Table I below. In this table the units of activity for streptothricin are based upon purified preparations of streptothricin while the units for streptomycin are based upon the crude and hence less concentrated substance. (A unit of activity is that amount of material which will inhibit the growth of a standard strain of Escherichia coli in 1 ml. of a suitable culture medium). Comparative tests of the two substances, both purified to approximately the same degree, against E. coli show that they have substantially the same activity against this organism. For a better comparison of the bacteriostatic spectra of streptomycin and streptothricin of the same purity, the units of activity for streptomycin in Table I should therefore be multiplied by 4 in each instance.

TABLE I

Comparative Bacteriostatic Spectra
(based on ash free dry material)

Organism	Gram Stain	Units of activity per gram ash-free dry material: Streptomycin X 1000	Streptothricin X 1000
B. subtilis 0	+	125	500
B. mycoides 0	+	250	3
B. mycoides 317-911	+	20	3
B. cereus	+	30	3
B. mesentericus	+	15	—
B. megatherium	+	100	150
S. aureus	+	15	200
S. lutea	+	100	150
M. phlei	+	100	50
M. tuberculosis	+	30	—
Phytomonas pruni	—	100	400
Listerella monocytogenes	—	10	—
Shigella gallinarum	—		150
E. Coli	—	25	100
S. marcescens	—	25	5
A. aerogenes	—	10	50
P. vulgaris	—	10	50
S. aertrycke	—	2.5	—
S. schottmülleri	—	—	50
Ps. fluorescens	—	2	3
Ps. aeruginosa	—	1	3
Cl. butylicum	—	3	3

It is apparent from a consideration of Table I that streptomycin is more active than streptothricin against certain gram-positive organisms such as Bacillus mycoides and Bacillus cereus and against certain gram-negative organisms such as Pseudomonas fluorescens, Pseudomonas aeruginosa, and Serratia marcescens.

Regarded in certain of its broader aspects the novelty in the present invention comprises the antibiotic substance streptomycin and the process for preparing the same by cultivating strains of Actinomyces griseus, under

either stationary or submerged aerobic (viz., submerged growth with agitation and aeration) conditions, in a nutrient medium containing a growth-promoting factor of the type present in meat extract and corn steep liquor, separating the organism growth from the culture broth, treating the culture broth with activated charcoal to adsorb the active product, eluting the adsorbate with low normality alcoholic mineral acid and recovering streptomycin from the eluate.

For the preparation of streptomycin a culture medium is used comprising an aqueous solution containing approximately 1.0% of carbohydrate such as glucose; 0.5% of complex nitrogenous material such as peptone or tryptone; 0.5% of inorganic salt such as sodium chloride; and a small amount of a complex organic substance containing a specific growth-promoting factor required for satisfactory elaboration of the active product. This growth-promoting factor is present in varying degrees in such complex organic materials as meat extract, corn steep liquor, and the like.

This medium is distributed in appropriate vessels of a depth of 1 - 2 inches for surface cultivation. For submerged aerobic cultivation, it is placed in deep tanks having suitable means for aeration and agitation of the medium. The medium thus distributed is sterilized at 10 lbs. steam pressure for 35 - 60 minutes and then cooled.

For inoculation of the culture medium a heavy water-suspension of spores of a strain of Actinomyces griseus is prepared by scraping from agar slants or by first growing the organism under submerged aerobic conditions to obtain a heavy mass of growth. Incubation takes place at a temperature

of about 22 - 28°C. Elaboration of the streptomycin is usually complete in 6 - 12 days in the case of stationary cultures and in 2 - 4 days when cultivation is under submerged aerobic conditions.

The course of production of streptomycin under submerged and stationary conditions is illustrated in Table II.

TABLE II

Course of Production of Streptomycin by A. griseus

Submerged cultures			Stationary cultures		
Days	Dilution Units	Diffusion units	Days	Dilution units	Diffusion units
2	40	10	3	5	6
3	50	70	5	20	12
4	40	60	7	20	53
7	125	70	9	75	
			12	100	55

The culture broth obtained by either submerged aerobic or stationary cultivation of Actinomyces griseus is filtered or centrifuged to remove the growth of the organism. Activated charcoal is then added to the filtered broth and the mixture is stirred for about 5 minutes and then filtered. Alternately, the mixture can be stored for about 8 - 12 hours at 0 - 10°C, with stirring at about two-hour intervals and then filtered. The colorless or slightly yellowish filtrate obtained is discarded. The charcoal residue with the adsorbed streptomycin is washed several times with distilled water and finally with 95% ethanol.

The washed material is then suspended in 95% ethanol made approximately 0.15 normal with mineral acid, such as hydrochloric, and the suspension is stirred for several hours and then allowed to stand in the cold for another 6 - 8 hours with occasional stirring. The suspension is then filtered, the charcoal residue discarded, and the brown to yellow clear filtrate thus obtained is added, with stirring, to approximately an equivalent amount of ether. A brown-colored aqueous layer separates and is drawn off. The alcohol ether solution is washed with additional small amounts of water and the brown aqueous washings are added to the aqueous layer previously drawn off. The aqueous solutions are then neutralized to pH 6 - 7 and any precipitate formed is filtered off and discarded. A faintly colored aqueous solution containing streptomycin is thus obtained.

When stationary cultivation is employed, the pellicle (or growth of organism) once formed can be utilized again several times. The culture broth after complete elaboration of the active substance, is carefully drained from the pellicle and replaced by an equal amount of fresh culture medium. The containers are again placed in incubation at 22 - 28°C and production of streptomycin sets in immediately, reaching a maximum in 3 to 5 days. The broth obtained by re-using the pellicle in this manner is treated as previously described to give a substantially colorless aqueous solution of streptomycin.

The following examples illustrate methods of carrying out the present invention, but it is to be understood that these examples are given by way of illustration and not of limitation.

Example I

A medium is prepared having the following composition:

1.0% glucose
0.5% peptone
0.3% meat extract
0.5% NaCl
tap water

This medium is distributed in appropriate vessels to a depth of 1 - 2 inches, sterilized at 10 lbs. steam pressure for 45 - 50 minutes, and then cooled.

The medium in each vessel is then inoculated with a heavy aqueous suspension of spores of a strain of Actinomyces griseus, and the inoculated media are maintained at an incubation temperature of 22 - 28°C for 10 days. The growth is then filtered off and the filtrates are combined for further treatment.

To a batch of approximately 10 liters of filtered broth is added 150 gms. of activated charcoal. The mixture is stirred continuously for about five minutes and is then filtered. The slightly yellowish (almost colorless) filtrate is discarded and the charcoal residue is washed several times with distilled water and finally with 95% ethanol. The washed material is then suspended in 1.5 liters of 95% ethanol, made 0.15 normal with hydrochloric acid. The suspension is stirred for about an hour and allowed to stand in the cold for about 10 hours more with occasional stirring. The suspension is then filtered, the charcoal residue discarded, and the yellowish clear filtrate thus obtained is poured into 10 liters of ether, with stirring. A brown-colored aqueous layer separates and is drawn off. The alcohol-ether solution is washed with 100 cc. of water and the brown aqueous layer is drawn off and added to the first aqueous layer. The aqueous solution is neutralized to pH 6 - 7 with dilute sodium hydroxide and any precipitate that forms is filtered off and discarded. A faintly colored aqueous solution containing streptomycin is thus obtained.

Example II

A medium is prepared having the following composition:

1.0% glucose
0.5% peptone
0.5% sodium chloride
1.2% corn steep liquor
tap water

This medium is distributed in appropriate deep vessels having suitable means for agitation and aeration of the medium, sterilized at 10 lbs. steam pressure for 45 - 50 minutes and then cooled.

The medium in each vessel is then inoculated with a heavy suspension of spores of a strain of Actinomyces griseus, and the inoculated media are maintained at an incubation temperature of 22 - 28°C for 3 days, with constant agitation and aeration. The growth is then removed by centrifuging and the culture broth is combined and further treated as described in Example I to isolate a substantially colorless, clear aqueous solution containing streptomycin.

Example III

The process of Example I is repeated with the exception that at the end of the incubation period instead of removing the broth by filtering it is carefully drained from the pellicle. An amount of fresh medium equivalent to the amount of broth drained from the pellicle is added to each vessel and the fresh media are again placed in incubation at 22 - 28°C. After 5 days in incubation the broth is again carefully drained from the pellicle and replaced by fresh medium. The broth obtained after each period of incubation is treated as in Example I to obtain a clear, substantially colorless, aqueous solution of streptomycin.

In the foregoing examples it is to be understood that the compositions of the culture media are merely illustrative and can be varied as, for example, by employing tryptone in place of peptone, and by employing meat extract and corn steep liquor alternately in the several examples.

Modifications may be made in carrying out the present invention without departing from the spirit and scope thereof, and the invention is to be limited only by the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A process for the production of streptomycin which comprises growing a culture of a streptomycin-producing organism in a liquid culture medium under conditions favourable to the promotion of streptomycin, separating the streptomycin-containing culture broth from the organism growth, admixing to the culture broth an adsorbent on which streptomycin is adsorbed, separating the adsorbent containing the streptomycin from the mixture, and eluting streptomycin from the adsorbent.

2. The process defined in claim 1 wherein the elution is effected with acid-alcohol in which streptomycin is soluble.

3. The process as defined in claim 1 or 2 wherein the adsorbent is activated carbon.

4. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of Actinomyces griseus at a suitable incubation temperature and for a suitable period of cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth, and recovering the adsorbed streptomycin.

5. A process for the production of streptomycin that comprises growing a culture of a streptomycin-producing strain of Actinomyces griseus at an incubation temperature of 22-28°C for a time of the order of 6-12 days for stationary cultivation and 2-4 days for submerged aerobic cultivation, to form streptomycin in the culture broth, separating the culture broth from the organism growth, adsorbing streptomycin from the broth and recovering the adsorbed streptomycin.

6. Streptomycin.

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SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente

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(54) CONCENTRATED ENZYMIC SUBSTANCE

(54) SUBSTANCE ENZYMIQUE CONCENTREE

(57) Abstract:

255812

This invention relates to enzymic substances and processes for producing the same and has for one of its principal objects to provide a stable enzymic substance which is more concentrated than those heretofore produced, and a method of producing the same.

Enzymes are unorganized ferments, or chemical substances of vegetable or animal origin that cause certain chemical transformations by their presence. Enzymes are produced by living cells, either animal or vegetable, including various bacteria and molds, for example, by the propagation of mold fungi such as Aspergillus oryzae or other mold fungi of the genera Aspergillus, Penicillium and Mucor upon various substances of plant or animal origin, including bran of various grains. When brought into relation with certain organic compounds, the action of the enzymes tends to decompose such compounds into simpler combinations without themselves undergoing theoretically any change, although practically the enzymes gradually disappear in the course of the reaction. Their precise mode of action is not definitely understood but it is apparently catalytic. Enzymes act only in the presence of water and a small quantity serves to break up or change a large mass of the substance or substances on which it acts. Enzymeshave well known properties such as diastatic and proteolytic. By virtue of their diastatic property, they may be employed to convert starch into sugar, this property being predominantly present in saliva which is an enzymic solution. By virtue of their proteolytic property enzymes exert a digestive action upon meat and other proteins, this property being predominantly present in the

enzymic digestive juices in the stomach and pancreas of human beings and animals.

The present invention has to do with enzymes of vegetable origin having various properties including diastatic and proteolytic properties. The novel process may be generally described as follows. A suitable mass of fungus-impregnated culture medium is prepared and lixiviated with a suitable liquid such as water to extract the enzymic properties from such mass. A different or separate mass of fungus-impregnated culture medium is then soaked or steeped in the liquid extract. The liquid is then evaporated, i.e., the mixture is dried, leaving the desired enzymic substance.

Preparation of the Fungus-Impregnated Culture Medium may be effected in various ways. For example, it may be prepared in the manner disclosed in the now expired United States patents Nos. 525,820 or 525,823 in which the fungus-impregnated mass is given the name of taka-koji.

Another, and in many respects preferable method of producing the fungus-impregnated mass is substantially as follows. Wheat bran, or other equivalent material; apple pomace, or another ventilating agent; and water are mixed, preferably in the following proportions: apple pomace, 120 to 200 pounds; bran, enough to weigh with the pomace, about 1000 pounds; water, about 50 gallons. The mixture so formed is then sterilized by steaming for about one hour and then cooled down to a temperature of about 30° C., about one hour being consumed in the cooling operation. To this mixture so prepared is added about 35 gallons of water containing the seed spores. These seed

spores are preferably those formed by mold fungi belonging to the genera Aspergillus, Penicillium or Mucor, such as the species Aspergillus oryzae (or Eurotium oryzae) or Aspergillus flavus which are readily procurable. The mass or mixture of bran, pomace, water and spores is then placed in trays and maintained at a temperature of 30° C. for about 40 to 48 hours, at which time the growth is arrested and if desirable the prepared mass is dried. For convenience I shall refer to this so-prepared dried or undried mass of fungus-impregnated material as Protozyme.

Preparation of the Liquid Extract.

A dried, or undried, mass of fungus-impregnated culture medium is lixiviated with water. This may be done by permitting water or other suitable liquid to percolate through the mass, or by steeping the mass in the liquid. If desired, the strength of this solution may be increased by repeatedly treating a fresh quantity of the mass therewith. The extract may then be filtered or strained giving the desired liquid extract.

Another, and preferred, method of preparing the liquid extract is to place undried or preferably dried mass of the fungus-impregnated medium (for example, Protozyme as described above) in a percolator, into which liquid is poured, allowing it to stand for about one or two hours. The liquid may then be drained off through a thick cloth strainer and this liquid, either strained or unstrained, constitutes the desired extract. Water is added to the same batch of culture medium and drained off to be employed as the liquid for lixiviating a succeeding batch of fresh culture medium.

The next step is to soak or steep a fresh mass of culture medium, prepared as above described, in the liquid extract, prepared as above described. The mixture is allowed to soak or steep for one or two hours and then dried, i.e., the liquid is evaporated off, until the moisture content is reduced to 5 per cent or less. The resultant novel substance is conveniently referred to as Protozyme Concentrated. Further concentration of the desired product may be secured by steeping or soaking the Protozyme Concentrated, produced as described above, in a fresh quantity of liquid extract, again drying it, and if desirable again repeating this process as many times as desired.

Summarizing a preferred method of carrying out the process: two or more lots of Protozyme are prepared, each in the manner above described. One lot is allowed to dry. The other lot or lots are lixiviated to form a liquid extract, as above described. The lot of dry Protozyme is then soaked in the liquid extract and is then dried. The last mentioned dry product may then be steeped or soaked in a fresh batch of liquid extract, again dried, and this process repeated as often as desired, depending upon the degree of concentration desired. The drying may be effected by an air current in a partial vacuum, or by exposure to the sun on a dry day, or by simply spreading the mixture out in a dry place. In any case, the drying is continued until the moisture content is reduced to 5 per cent or less. The resultant dry product is Protozyme Concentrated.

This resultant product not only possesses the stability of dry Protozyme or dry taka-koji but in its enzymic properties (diastatic and proteolytic and others)

it is more than twice as concentrated as either of its constituents, Protozyme and liquid extract of Protozyme.

It has been proposed by various workers in the enzymic art (see pages 769 and 770 of "Allegemeine Mikrobiologie", Kruse 1910, published at Leipzig, Germany) to preserve enzymic solutions by employing antiseptics of various kinds. In the present process no antiseptic whatsoever is employed, yet the resultant product does not deteriorate because it is in dry form.

The novel product resulting from the above described novel process may be described as a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate (i.e., a product of a process of concentration by drying or evaporation) of a liquid extract of a fungus-impregnated culture medium. This product is powerful in its enzymic properties, though of small bulk. Being dry, i.e., of reduced moisture content, it will keep and preserve its enzymic properties practically indefinitely.

The novel product may be employed to great advantage in the clarification of fruit juices, as a medicinal preparation against dyspepsia and in various other fields, arts and industries.

What is claimed is:

1. The process of preparing an enzymic substance which comprises lixiviating a fungus-impregnated culture medium, soaking in the liquid extract a fresh mass of fungus-impregnated culture medium, and drying the mixture.

2. The process of preparing an enzymic substance which comppises preparing two lots of fungus-impregnated culture medium, drying out one lot, lixiviating the other lot to form an aqueous extract, soaking the dried lot in the extract formed from the other lot, and drying the mixture.

3. The process of preparing an enzymic substance which comprises propagating a fungus on a culture medium, drying one lot of such medium, lixiviating another lot of such medium with a liquid to which a third lot of culture medium has been subjected, soaking the dried lot of culture medium in the extract, and reducing the moisture content of the mixture until not more than 5 per cent moisture remains.

4. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate of a liquid extract of a fungus-impregnated culture medium.

5. The process of preparing an enzymic substance which comprises lixiviating a fungus-impregnated culture medium, soaking in the liquid extract a fresh mass of fungus-impregnated culture medium, drying the mixture, soaking the dried product in a fresh quantity of liquid extract of fungus-impregnated culture medium, and drying the mixture.

6. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrate of a liquid extract of different lots of fungus-impregnated culture medium.

7. As a new article of manufacture, a dry enzymic substance comprising a fungus-impregnated culture medium and the concentrates of liquid extracts of a fungus-impregnated culture medium.

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SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente