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(54) Title: A83543 RECOVERY PROCESS

(57) Abstract

Improved process for recovering the useful insecticidal fermentation product A83543, comprising major components A83543A and A83543D and minor components A83543B, A83543C, A83543E, A983543F, A83543G, A83543H and A83543J, which comprises extracting the A83543 from the whole broth into a water miscible solvent such as acetone, filtering to remove the biomass, raising the pH of the filtrate to 10-11 and extracting the A83543 with an immiscible solvent such as ethyl acetate, back extracting with an acidic solution and separating the A83543 by precipitating it with a base.

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Title

A83543 RECOVERY PROCESS

Summary of the Invention

This invention relates to an improved process for recovering fermentation product A83543. A83543, which is comprised of individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J, is a useful insecticide produced by a strain of Saccharopolyspora spinosa sp. nov.

The improved process of this invention comprises:

1) adding an approximately equal volume of a water miscible solvent, such as acetone or acetonitrile, to the whole broth;
2) separating the liquid phase from the biomass;
3) extracting the liquid phase at a pH of about 10 to about 11 with an immiscible solvent such as ethyl acetate or dichloromethane;
4) separating the organic phase from the mostly aqueous phase and concentrating the organic phase to remove the remaining water-miscible solvent;
5) removing the highly colored immiscible aqueous phase by decanting it from the enriched organic phase;
6) back-extracting the organic phase with a dilute solution of an acid, such as tartaric acid; and

7) either i) a) removing the miscible portion of the organic solvent from the aqueous acidic phase, e.g. by vacuum, b) concentrating the enriched aqueous phase approximately 20-fold using reverse osmosis and c) adding a sufficient amount of a base, such as sodium hydroxide, to precipitate the A83543; and separating the A83543 by filtration,

or

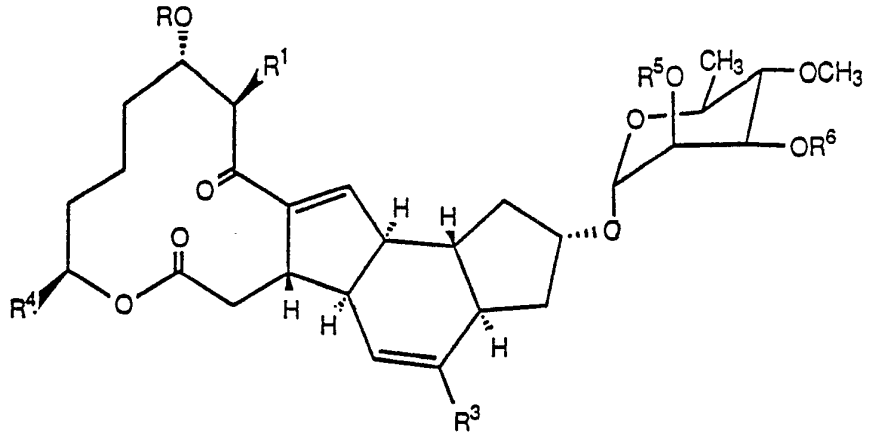
ii) adding a base, such as sodium hydroxide, which contains up to 20% acetone or methanol, in a sufficient amount to precipitate the A83543; and

8) separating the A83543 by filtration.

The advantages of this process are that it permits a quicker recovery of higher yields of A83543 in better form.

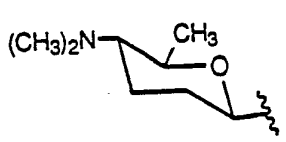
Detailed Description of the Invention

This invention relates to an improved process for recovering a fermentation product which has been designated "A83543". A83543 is comprised of individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H and A83543J, which have the structures shown in formula 1:

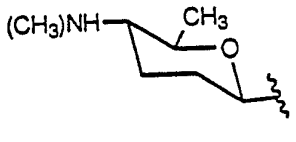


1

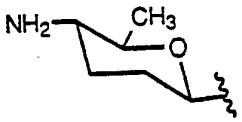
wherein R is:



(a)

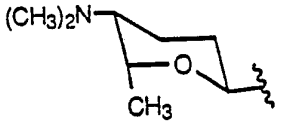


(b)



(c)

or



(d)

;

Component	R	R^1	R^3	R^4	R^5	R^6
A	(a)	Me	H	Et	Me	Me
B	(b)	Me	H	Et	Me	Me
C	(c)	Me	H	Et	Me	Me
D	(a)	Me	Me	Et	Me	Me
E	(a)	Me	H	Me	Me	Me
F	(a)	H	H	Et	Me	Me
G	(d)	Me	H	Et	Me	Me
H	(a)	Me	H	Et	H	Me
J	(a)	Me	H	Et	Me	H

The aminosugar in A83543A has been shown to be β-D-forosamine; and the neutral sugar in A83543A is α-2,3,4-tri-O-methylrhamnose.

A83543 and the individual A83543 components are useful for the control of insects, particularly Lepidoptera species such as Southern armyworm, Diptera species such as blow fly, stable fly and mosquito, and Homoptera species such as cotton aphids and leaf-hoppers.

A83543 is produced by culturing an A83543-producing strain of Saccharopolyspora spinosa sp. nov. under submerged aerobic conditions by methods disclosed in the copending U.S. patent application of Boeck, Chio, Eaton, Godfrey, Michel, Nakatsukasa and Yao, Serial No. 07/429,441, filed 30 October, 1989, which is incorporated herein by reference, in a suitable culture medium until a recoverable amount of A83543 is produced.

Four A83543-producing Saccharopolyspora spinosa cultures, NRRL 18395, NRRL 18537, NRRL 18538 or NRRL 18539, have been deposited and made part of the stock culture collection of the Midwest Area Northern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604, from which they are available to the public under the specified NRRL accession numbers.

The culture medium used to grow these Saccharopolyspora spinosa cultures can be any one of a number of media.

Preferred carbon sources in large-scale fermentation are glucose and maltose, although ribose, xylose, fructose, galactose, mannose, mannitol, soluble starch, potato dextrin, methyl oleate, oils such as soybean oil and the like can also be used.

Preferred nitrogen sources are cottonseed flour, peptionized milk and digested soybean meal, although fish meal, corn steep liquor, yeast extract, enzyme-hydrolyzed casein, beef extract, and the like can also be used.

Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding zinc, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate, nitrate and like ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other substituents of the medium in amounts sufficient to meet the growth requirements of the organism.

Usually, if foaming is a problem, small amounts (i.e., 0.2 ml/L) of an antifoam agent such as polypropylene glycol) may be added to large-scale fermentation media. In the case of the A83543-producing cultures, however, conventional defoamers inhibit A83543 production. Foaming can be controlled by including soybean oil or pluronic L-101 (BASF) in the medium (1-3%). Additional oil may be added if foaming develops.

The ratio of the components in A83543 will vary, depending upon the fermentation conditions used to produce it. In general, A83543 contains about 85-90% A83543A, about 10-15% A83543D and minor amounts of A83543B, C, E, F, G, H and J.

The percentage of a particular A83543 component may be varied by media changes. For example, adding valine or isobutyric or propionic acids increases the percentage of A83543D produced.

For production of substantial quantities of A83543, submerged aerobic fermentation in stirred bioreactors is preferred. Small quantities of A83543 may be obtained by shake-flask culture. Because of the time lag in production commonly associated with inoculation of large bioreactors with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or myceliall fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger bioreactor. The vegetative inoculum medium can be the same as that used for larger fermentations, but other

media are also suitable.

A83543 is produced by the A83543-producing organisms when grown at temperatures between about 24° and about 33°C. Optimum temperatures for A83543 production appear to be about 28-30°C.

As is customary in submerged aerobic culture processes, sterile air is blown into the vessel from the bottom while the medium is stirred with conventional turbine impellors. In general, the aeration rate and agitation rate should be sufficient to maintain the level of dissolved oxygen at or above 35%, and preferably at or above 50%, of air saturation with an internal vessel pressure of 0.34 atmospheres.

Production of the A83543 components can be followed during the fermentation by testing extracts of the broth. HPLC, using a system as described in Example 1, is a useful assay for this purpose.

This invention relates to an improved process for recovering A83543 from the fermentation medium in which it is produced. The A83543 produced during fermentation of the A83543-producing organism occurs in both the mycelial mass (the biomass) and the broth. A83543 appears to be lipophilic. In addition, traditional defoaming agents inhibit A83543 production. When A83543 is produced in bioreactors, foaming is a problem, and a substantial amount of oil has to be used to control it. Later, separation of the lipophilic A83543 from the medium involved a simultaneous separation of the oil. Thus, complete recovery of A83543 required additional separations to remove the oil.

Prior to this invention, therefore, A83543 was isolated by:

1) filtering the whole broth to separate the biomass;
2) extracting the biomass with methanol;
3) concentrating the methanol extract;
4) adding the concentrate to a nonfunctional resin, such as HP20;
5) washing impurities from the resin with a water/methanol solution;
6) eluting the product from the nonfunctional resin;
7) concentrating the eluting solution to give the product as a crude oil;
8) purifying the product over multiple columns of ionic sephadex such as LH-20 to remove the oil;
9) concentrating the eluting solvents to give the product; and
10) crystallizing the product.

Unfortunately this recovery process was time consuming taking approximately three weeks and resulted in large losses of material because it required multiple purifications. Ionic sephadexes such as LH-20 have a low loading capacity; therefore, many columns were required to carry out step 8, resulting in a significant loss of material.

This invention relates to the discovery that the troublesome oil could be removed by extracting the A83543 and oil with an immiscible solvent such as ethyl acetate and then back extracting the A83543 into an aqueous phase by using a dilute solution of an acid, such as tartaric acid. After concentrating the acidic solution to remove the miscible portion of the organic solvent,

A83543 could be precipitated as the base, by adding a base such as sodium hydroxide to a pH of about 10-11. This approach enabled recovery of purified A83543 without the multiple columns required in step 8.

Furthermore, another aspect of the invention was the discovery that this approach could be used on the whole, unfiltered broth, thereby avoiding the product losses involved with steps 2-7.

Thus, the improved process of this invention comprises:

1) adding an approximately equal volume of a water miscible solvent to the whole broth;
2) separating the liquid phase from the biomass;
3) extracting the liquid phase at a pH of about 10 to about 11 with an immiscible solvent;
4) separating the organic phase and concentrating it to remove the remaining water-miscible solvent;
5) removing the highly colored immiscible aqueous phase from the enriched organic phase;
6) back-extracting the organic phase with a dilute solution of an acid; and
7) either i) a) removing the miscible portion of the organic solvent from the aqueous acidic phase, b) concentrating the enriched aqueous phase using reverse osmosis, and c) adding a sufficient amount of a base to precipitate the A83543;

or

ii) adding a base which contains up to 20% acetone or methanol in a sufficient amount to precipitate the A83543; and
8) separating the A83543 by filtration.

Step 2 is preferably accomplished by filtration, using a filter aid. Preferable solvents for step 3 are ethyl acetate or dichloromethane.

In step 5 the aqueous phase is best separated by decanting it. A preferred acid for step 6 is tartaric acid. In step 7(i), separation (a) is most readily accomplished under vacuum. A preferred base for step 7(i)(c) or 7(ii) is sodium hydroxide.

This process is much more practical because it can be completed in a substantially shorter time, e.g. in about three days, whereas the prior process took approximately three weeks. Furthermore, it provides higher yields of A83543, results in a material which is easy to precipitate and gives a product of higher purity.

The A83543 thus recovered can be separated into individual components A83543A, A83543B, A83543C, A83543D, A83543E, A83543F, A83543G, A83543H, and A83543J by chromatographic procedures. A preferred separation procedure involves reverse-phase silica-gel (C_{18} or C_8) chromatography.

In order to illustrate more fully the operation of this invention, the following examples are provided:

EXAMPLE 1

A83543 HPLC Assay Method

The following analytical HPLC method is useful for monitoring the fermentation for production of A83543:

Centrifuge a sample of the whole broth, decant and remove the supernatant. Add enough methanol to the biomass to return the sample to the original volume, mix, and allow the mixture to stand a minimum of fifteen minutes. Centrifuge and filter the supernatant through a 0.45 μ filter.

Alternatively, the whole broth can be extracted with acetonitrile (1:4 broth:solvent) or acetone.

HPLC System:

Column Support: 8-x 100-mm column, silica gel-4μ spherical C_{18} (Nova C18, Waters)

Mobile Phase: $CH_3CN/MeOH/H_2O$ (45/45/10) containing 0.05% ammonium acetate

Flow Rate: 4 mL/min

Detection: UV at 250 nm

Retention Times: A83543A - 3.6 - 3.7 min
A83543D - 4.4 - 4.5 min

EXAMPLE 2

Preparation of A83543 with NRRL 18395

A. Shake-flask Fermentation

The culture Saccharopolyspora spinosa NRRL 18395, either as a lyophilized pellet or as a suspension maintained in liquid nitrogen, is used to inoculate a vegetative medium having composition A or B (medium B is preferred for large scale production):

VEGETATIVE MEDIUM A

Ingredient	Amount (%)
Trypticase soy broth*	3.0
Yeast extract	0.3
$MgSO_4.7H_2O$	0.2
Glucose	0.5
Maltose	0.4
Deionized water	q.s. 1 L

No pH adjustment

*Baltimore Biological Laboratories

VEGETATIVE MEDIUM B

Ingredient	Amount (%)
Enzyme-hydrolyzed casein*	3.0
Yeast extract	0.3
$MgSO_4.7H_2O$	0.2
Glucose	1.0
Deionized water	q.s. 1 L

pH 6.2, adjust to 6.5 with NaOH

*NZ Amine A, Sheffield Products, P.O. Box 638 Norwich, NY 13815

Slants or plates can be prepared by adding 2.5% agar to vegetative seed medium A or B. The inoculated slant is incubated at 30°C. for from about 10 to 14 days. The mature slant culture is scraped with a sterile tool to loosen the spores and remove and macerate the myceliall mat. About one-fourth of the loosened spores and culture growth thus obtained is used to inoculate 50 mL of a first-stage vegetative seed

medium. Alternatively, the first-stage medium may be inoculated from a liquid nitrogen ampoule.

When the culture is maintained in liquid nitrogen, ampoules are prepared using equal volumes of vegetative culture (48-72 hr incubation, 30°C) and suspending medium. The suspending medium contains lactose (100 g), glycerol (200 mL) and deionized water (q.s. to 1 L).

A liquid nitrogen ampoule is used to inoculate 100 mL of vegetative medium in 500-mL Erlenmeyer flasks (or 50 mL medium in 250-mL flasks). The cultures are incubated at 30°C for 48 hours on a shaker orbiting in a two-inch (5.08 cm) circle at 250 rpm.

The incubated culture (5% v/v inoculum) is used to inoculate 100 mL of a production medium having the following composition:

PRODUCTION MEDIUM I

Ingredient	Amount (%)
Glucose	4
Vegetable protein, partially hydrolyzed enzymatically*	1.5-3
Cottonseed flour**	1.0
$CaCO_3$ (reagent or technical grade)	0.3
Soybean oil	1.0
Tap water	q.s. 1 L

(Presterilization pH adjusted to 7.0 with NaOH)

*Sheftone H, Sheffield Products

**Proflo, Traders Protein, P.O. Box 8407, Memphis, TN 38108

The inoculated production medium is incubated in 500-mL Erlenmeyer flasks at 28-30°C. for 6 to 8 days on a shaker orbiting in a two-inch circle at 250 rpm.

B. Stirred Bioreactor Fermentation

In order to provide a larger volume of inoculum, 10 mL of incubated first-stage medium, prepared as described in Section A, is used to inoculate 400 mL of a second-stage vegetative medium having the same composition as that of the first-stage vegetative medium. This second-stage medium is incubated in a 2-L wide-mouth Erlenmeyer flask for about 48 hours at 30°C. on a shaker orbiting in a two-inch circle at 250 rpm.

Incubated second-stage vegetative medium (2 L) thus prepared is used to inoculate 80 to 115 liters of sterile production medium, prepared as described in Section A. Additional soybean oil is added to control foaming, if needed.

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for 5 to 8 days at a temperature of 28°C. The airflow and agitator speed in the stirred vessel are computer controlled to maintain a dissolved oxygen level at or above 50% of air saturation.

EXAMPLE 3

Previous Process for Purification of A83543A and D

Fermentation broth (10 L) was prepared as described in Example 2 Sect. A, except that 1) 200 mL of

production medium was used in 1-L flasks; 2) soybean oil was omitted from the production medium; and 3) incubation was at 30° for 4-6 days. The broth was filtered. The filtrate, containing 4 mcg of A83543A/ mL and no detectable quantities of A83543B, C, or D/mL, was discarded.

The biomass was washed with water and extracted for one hour with methanol. The extract (7 L) contained 72 mcg of A83543A/mL and 7 mcg of A83543D/mL.

The methanol extract was concentrated to a volume of 5 L, and added to HP-20 resin (150 mL, Mitsubishi Chemical Industries, Ltd., Japan) in water (2 L). This mixture was stirred for one hour.

The HP-20 resin mixture was then placed in a glass column. The initial effluent and the eluate using methanol:water (1:1, 1 L) were not active. The second eluate using methanol:water (7:3, 1 L) contained trace quantities of A83543A. The following eluate using methanol (1 L) contained the A83543A and A83543D activity.

The methanol eluate was concentrated and combined with 2 similar fractions from other work-ups and concentrated to dryness. The residue was dissolved in 75 mL of methanol:THF (4:1) and precipitated by addition into 10 volumes of acetonitrile. The mixture was filtered, and the filtrate was concentrated to dryness.

The residue was dissolved in methanol (25 mL) and applied to a 5.5-x 90-cm column of LH-20 Sephadex (Pharmacia LKB Biotechnology Inc., U.S.A.), prepared in methanol, collecting and analyzing 125 25-mL fractions, using the HPLC procedure described in Example 1.

Fractions containing the desired compounds were combined and concentrated. The residue was dissolved in methanol (10 mL) and applied to a 41.1-mm x 25-cm preparative column prepacked with 8μ C-18 reversed phase silica gel (Rainin Dynamax).

The column was conditioned in methanol: acetonitrile:water (37.5:37.5:25). After sample application, the column was developed using a 180-min linear gradient of the following solvents:

Solvent System		
	Amount (mL)	
Solvent	A	B
CH_3OH	37.5	45
CH_3CN	37.5	45
H_2O	25	10

The gradient was run from 100% A to 100% B, collecting 25-mL fractions.

Fractions containing A83543A were pooled, concentrated to dryness, dissolved in t-BuOH (5 mL) and lyophilized to give 778 mg of pure A83543A.

Fractions containing A83543D were combined with D-containing fractions from 6 similar separations and were concentrated and chromatographed as described supra, using the same column but different solvents. The column was conditioned in methanol:acetonitrile: water(40:40:20). The solvent systems used to develop the column in a 180-min linear gradient operation were:

Solvent Systems

Solvent	Amount (mL) A	B
CH_3OH	40	95
CH_3CN	40	95
H_2O	20	10

Fractions containing A83543D were combined and concentrated. The residue was dissolved in t-BuOH (5 mL) and lyophilized to give 212 mg of A83543D.

EXAMPLE 4

Preparation of A83543 with NRRL 18537

A. Shake-flask Fermentation

Using procedures like those of Example 2, Section A, the culture Saccharopolyspora spinosa NRRL 18537 is cultivated in shake flasks, but using vegetative medium C as follows:

VEGETATIVE MEDIUM C

Ingredient	Amount
Enzyme-hydrolyzed casein*	30.0 g
Yeast extract	3.0 g
$MgSO_4.7H_2O$	0.2 g
Glucose	10.0 g
Glycerol	20 mL
Deionized water	q.s. 1 L

pH 6.6, adjust to 7.0 with NaOH

*NZ Amine A

B. Stirred Bioreactor Fermentation

Liquid nitrogen ampoules of the culture are prepared as described in Example 2, using the general procedures of Sect. B. One ampoule is used to inoculate a first-stage vegetative culture (50 mL of medium C in 250-mL flasks), which is incubated for about 48-72 hours. Incubated first-stage culture is used to inoculate (10-mL inoculum) a second-stage culture (400 mL of medium C in 2-L flasks), which is incubated for about 48 hrs. The incubated second-stage culture (5 L) is used to inoculate a production medium (115 L) having the following composition:

PRODUCTION MEDIUM II

Ingredient	Amount (g/L)
Glucose	80
Peptonized milk*	20
Cottonseed flour**	20
$CaCO_3$	5
Methyl oleate	30 mL/L
Tap water	q.s. to 1 L

* Peptonized Milk Nutrient, Sheffield Products; additional continuous feed, beginning the fourth day, at a rate of 5 mg/mL/day, may be used.
** Proflo (Presterilization pH adjusted to 7.0 with NaOH)

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for about 8 to 10 days, or longer, at a temperature of 30°C. Dissolved oxygen (DO) levels are regulated by computerized systems

set to maintain the DO level above 50% of air saturation as described in Example 2, Section B.

EXAMPLE 5

Preparation of A83543 with NRRL 18539

A. Shake-flask Fermentation

Using procedures like those of Example 2, Section A, the culture Saccharopolyspora spinosa NRRL 18539 is cultivated in shake flasks, using vegetative medium B.

B. Stirred Bioreactor Fermentation

Liquid nitrogen ampoules of the culture are prepared as described in Example 2, using the general procedures of Sect. B. One ampoule is used to inoculate a first-stage vegetative culture (50 mL of medium B in 250-mL flasks), which is incubated for 48 to 72 hours. Incubated first-stage culture is used to inoculate (10-mL inoculum) a second-stage culture (400 mL of medium B in 2-L flasks), which is incubated for about 48 hr. The incubated second-stage culture (2 L) is used to inoculate a production medium (115 L) having one of the following compositions:

PRODUCTION MEDIUM III

Ingredient	Amount (g/L)
Glucose	80
Vegetable protein, partially hydrolyzed enzymatically*	20
Cottonseed flour**	10
$CaCO_3$	5
Methyl oleate	30 mL/L Tap
water	q.s. to 1 L

* Sheftone H

** Proflo (Presterilization pH adjusted to 7.0 with NH_4OH)

PRODUCTION MEDIUM IV

Ingredient	Amount (Percent)
Glucose	8
Cottonseed flour*	3
Peptionized Milk**	2
Corn steep liquor	1
$CaCO_3$ (tech. grade)	0.5
Methyl oleate	3.0
Tap water	q.s. to 1 L

* Proflo

** Peptionized Milk Nutrient (Presterilization pH adjusted to 7.0 with NaOH)

The inoculated production medium is allowed to ferment in a 165-L stirred bioreactor for about 8 to 10 days, or longer, at a temperature of 30°C. DO levels are regulated as described in Example 4.

EXAMPLE 6

Preparation of A83543 with NRRL 18538

Using procedures like those of Examples 2 and 5, the culture Saccharopolyspora spinosa NRRL 18538 is cultivated but using vegetative medium B and production medium III.

EXAMPLE 7

Separation of A83543 by the New Process

Fermentation broth is prepared as described in Example 6. A83543 is separated from the broth as follows:

1. Add an equal volume of acetone to the broth and filter, using a ceramic filter or filter aid with a filter press.

2. Adjust the filtrate broth to pH 10.

3. Add ethyl acetate (¼-½ the volume of the broth).

4. Recover the ethyl acetate extract by decanting off the immiscible aqueous portion; and concentrate the ethyl acetate extract to ½ volume by vacuum.

5. Extract the concentrated ethyl acetate solution with aqueous 0.1 M tartaric acid (½ volume); and separate the phases.

6. Remove the soluble ethyl acetate from the aqueous phase by vacuum (about 5%) evaporation. Concentrate the aqueous solution, using a reverse osmosis operation.

7. Adjust the concentrated aqueous solution to pH 10-11 with sodium hydroxide.

8. Separate the precipitate by filtration; wash with water; and dry under vacuum to give A83543.

EXAMPLE 8

Recovery of A83543 A and D from Fermentation Broth by the New Process

Whole fermentation broth is prepared by a procedure similar to that described in Example 2. The A83543-producing culture used was a progeny of the NRRL 18395 culture, but an ancestor of the NRRL 18538 and 18539 strains. The strain used produces approximately six-fold lower antibiotic titers than the NRRL 18538 and 18539 strains produce. Vegative Medium B was used, and the production medium was as follows:

PRODUCTION MEDIUM V

Ingredient	Amount (g/L)
Glucose	2.0
Vegetable protein, partially hydrolyzed enzymatically*	20.0
Cottonseed flour*	10.0
Sodium acetate	0.2
Glycerol	5.0
Soybean Oil	10.0
Tap water	q.s. to 1 L

The production medium was incubated at 30°C for about eight days.

After incubation, the broth, contained 141 mcg/mL A83543A and 13 mcg/mL A83543D (total of 16.94 grams of A83543A and A83543D). An equal volume of acetone (110 liters) was added. The broth/acetone was filtered through a plate and frame press to remove the mycelial solids.

The recovered filtrate (200 L.) was adjusted from pH 7.6 to pH 10 with 5 N sodium hydroxide. Then, ethyl acetate (60 L) was added to extract the activity. After stirring, the phases were separated, and the lower phase (100 L.) was discarded. The upper phase (>50% ethyl acetate) was concentrated from 160 L. to 80 L. to remove acetone.

The aqueous highly colored phase (20 L.) was then removed by decantation, and the enriched, concentrated ethyl acetate phase (60 L.) was back-extracted, using an equal volume of 0.1N tartaric acid in water. The enriched aqueous tartaric acid phase (70 L.) was recovered, concentrated under vacuum to remove miscible ethyl acetate and then concentrated to a volume of 4 L. (20 fold), using a Millipore ProLab reverse osmosis unit.

The concentrated aqueous tartaric acid solution (4 L.) was adjusted from pH 3 to pH 10 with sodium hydroxide, slowly allowing a precipitate to form. The precipitate was filtered, washed with water and dried under vacuum at 40°C to give 13.4 grams of dry precipitate with a potency of 815 mcg/mg of A83543A and 67 mcg/mg of A83543D (by HPLC). These yields represent an overall recovery of 11.8 grams A83543A and A83543D which is a 69.6% yield from whole broth.

I CLAIM:

1. A process for recovering fermentation product A83543, comprising A83543A and A83543D, which comprises:

1) adding an approximately equal volume of a water miscible solvent to the whole broth;

2) separating the liquid phase from the biomass;

3) extracting the liquid phase at a pH of about 10 to about 11 with an immiscible solvent;

4) separating the organic phase and concentrating it to remove the remaining water-miscible solvent;

5) removing the highly colored immiscible aqueous phase from the enriched organic phase;

6) back-extracting the organic phase with a dilute solution of an acid; and

7) either i) a) removing the miscible portion of the organic solvent from the aqueous acidic phase, b) concentrating the enriched aqueous phase using reverse osmosis, and c) adding a sufficient amount of a base to precipitate the A83543;

or

ii) adding a base which contains up to 20% acetone or methanol in a sufficient amount to precipitate the A83543; and

8) separating the A83543 by filtration.

2. A process of claim 1 wherein step 7 (i) is used.

3. A process of claim 1 wherein step 7 (ii) is used.

4. A process of claim 1 wherein the water miscible solvent in step 1 is acetone.

5. A process of claim 1 wherein the solvent used in step 3 is ethyl acetate.

6. A process of claim 1 wherein the acid used in step 6 is tartaric acid.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06187

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07H 1/06, 1/08, 17/08

U.S. Cl: 536/7.1, 16.9, 127

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	536/7.1, 16.9, 127

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 2,881,162 (WALASEK) 07 April 1959, see column 5, lines 40-57.	1-6
Y	Chemical Abstracts, vol. 87, no. 24, issued 12 December 1977, R.Datta et al., "Concentration of antibiotics by reverse osmosis," see page 310, see the abstract No. 189423a, Biotechnol. Bioeng. 1977, vol. 19, no. 10, pages 1419-1429.	1-2,4-6
Y	US, A, 4,213,966 (LILL ET AL.) 22 July 1980, see column 4, lines 30-54.	1-6
Y	US, A, 4,366,308 (SOMA ET AL.) 28 December 1982, see column 6, lines 15-32.	1-6
Y	US, A, 4,764,602 (KUMAGAI ET AL.) 16 August 1988, see column 4, lines 11-29.	1-6

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
31 December 1990	08 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Elli Peselev Elli Peselev