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(54) Title: METHOD OF BIOSYNTHESIZING TETRODOTOXIN

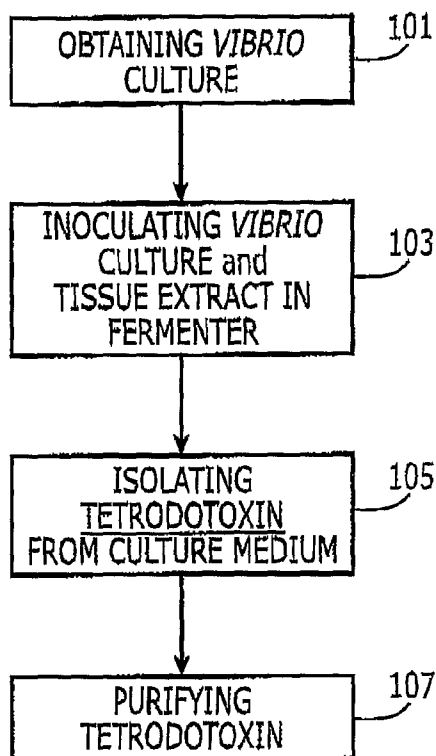


FIG. 1

(57) Abstract: The present invention relates methods for the biosynthesis of tetrodotoxin (TTX) involving the steps of obtaining a culture possessing one or more of a Vibrio species, such as through a seed culture/ inoculating the culture and tissue extract from a tetrodotoxin-bearing organism in a fermenter medium, and isolating and purifying tetrodotoxin from said fermenter, resulting in a yield of TTX of about 0.5 g TTX/L in about 3 to 5 days, such TTX being at least 90% pure.

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METHOD OF BIOSYNTHESIZING TETRODOTOXIN**BACKGROUND**

5 Tetrodotoxin (TTX) was first isolated from the ovaries of the puffer fish. Since then, it has been commercially extracted and purified for many years. However, the extraction rate of TTX is extremely low, about 1g TTX per 100Kg of ovaries, thus making it one of the highest priced
10 natural neurotoxins. Further, the industry is subject to slow down as stocks of puffer fish have been falling due to over production.

 In light of these negatives, many researchers continue to propose new synthesis methodologies. The synthesis of
15 tetrodotoxin presents a significant challenge to the organic chemist (Huang, "Synthetic Approaches to Tetrodotoxin"). For example, some methods include stereoselective methods used to incorporate the guanidine nitrogen at C8a position. Other approaches focus on novel
20 rearrangements. But because of the continued complexity, it is still more economical to extract tetrodotoxin directly from puffer fish ovaries.

 The drive to find more economical routes for TTX production is because of its potential as a novel
25 pharmaceutical drug, being possibly useful in aiding cancer

patients and potential use as a withdrawal formulation for heroin addicts. It is estimated that 1200 g of TTX will be required by 2 million cancer patients in a one-month treatment course, whilst 400 g of TTX will be required by
5 another 2 million drug addicts every 10 days.

It is an object of the present system to overcome the disadvantages and problems in the prior art.

DESCRIPTION

10 The present invention proposes methods for the biosynthesis of tetrodotoxin (TTX) resulting in high yield, highly pure TTX.

The present invention also proposes methods for the isolation and purification of TTX.

15 These and other features, aspects, and advantages of the apparatus and methods of the present invention will become better understood from the following description, appended claims, and accompanying drawings where:

FIG 1 shows a method of biosynthesizing TTX in
20 accordance with the present invention.

FIG 2 shows a method of isolating and purifying TTX in accordance with the present invention.

FIG 3 compares the results of the present invention's method of biosynthesizing TTX with results derived from prior art methods of TTX biosynthesis.

5 The following description of certain exemplary embodiment(s) is merely exemplary in nature and is in no way intended to limit the invention, its application, or uses. Throughout this description, "tetrodotoxin-bearing organism" shall refer to an organism that possesses the
10 compound known as tetrodotoxin, chemical formula $C_{11}H_{17}O_8N_3$, and its analogues, including but limited to anhydrotetrodotoxin, tetrodaminotoxin, methoxytetrodotoxin, ethoxytetrodotoxin, deoxytetrodotoxin, and tetrodonic acid, in their organs either through endogenous means such as
15 self-production, exogenous means such as the food chain, symbiotic relationship, or a combination of such. "Growth factors" shall refer to substances such as proteins that are present in an organisms tissues that allow stabilization of tetrodotoxin structure in the organisms
20 tissues, such that the structure does not decompose or under an analogue modification.

Now, to FIGs 1-3,

FIG 1 is an embodiment for a method of biosynthesizing tetrodotoxin in accordance with the present invention, containing the steps of obtaining a culture possessing one or more *Vibrio* species 101, inoculating the *Vibrio* culture and a tissue extract in a fermenter 103, isolating the resultant tetrodotoxin from the culture medium 105, and purifying the tetrodotoxin 107.

Obtaining a culture possessing one or more *Vibrio* species 101 consists of gathering a culture upon which *Vibrio* species were grown. Gathering can include obtaining a properly stored culture, or a culture obtained from a seed culture. One or more *Vibrio* species in the culture can be selected from the group consisting of *Vibrio nigripulchritudo*, *Vibrio mediterranei*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio salmonicida*, *Vibrio tubiashii*, *Vibrio parahaemolyticus*, *Vibrio campbellii*, *Vibrio natriegens*, *Vibrio nereis*, *Vibrio carchariae*, *Vibrio fluvialis*, *Vibrio fischeri*, *Vibrio vulnificus*, *Vibrio splendidus*, *Vibrio orientalis*, *Vibrio aestuarianus*, *Vibrio pelagius*, *Vibrio wodanis*, *Vibrio furnissii*, *Vibrio proteolyticus*, *Vibrio ichthyoenteri*, *Vibrio pectenocida*, *Vibrio logei*, *Vibrio mimicus*, *Vibrio mytili*, *Vibrio rumoiensis*, *Vibrio anguillarum*, *Vibrio gazogenes*, *Vibrio halioticoli*, *Vibrio hollisae*, *Vibrio ordalii*, and *Vibrio metschnikovii* and *Vibrio cholerae*.

In a preferred embodiment, the *Vibrio* species are one or more of *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio fluvialis*.

Vibrio species samples for culturing can be obtained from the organs of tetrodotoxin-bearing organisms including flatworms, ribbon worms, gastropods, blue-ringed octopuses, starfish, sea urchins, xanthid crabs, horseshoe crabs, gobies, frogs, newts, and puffer fish. In one embodiment, the *Vibrio* species are obtained from puffer fish of the Tetraodontidae, Diodontidae, and Triodontidae families, for example *Arothron hispidus*, *Arothron stellatus*, *Chelonodon patoca*, *Takifugu alboplumbeus*, *Takifugu niphobles*, *Takifugu oblongus*, *Takifugu ocellatus*, *Takifugu xanthopterus*, and *Diodon holocanthus*. In one embodiment, *Vibrio* species are obtained from *Chelonodon patoca*, *Takifugu alboplumbeus*, and *Takifugu niphobles*, specifically the species *Vibrio alginolyticus*. The organs, i.e. tissue, used to provide the *Vibrio* species can be the intestine, liver, ovary, stomach, and skin, used separately or in conjunction with one another. In one embodiment, *Vibrio* species are obtained from the ovary of *Takifugu niphobles*, the skin of *Chelonodon patoca*, and/or the intestine of *Takifugu alboplumbeus*.

In obtaining the *Vibrio* species from the organism tissues, the tissues are firstly excised, preferably aseptically, from the organism. The excised tissue is added to a bioreactor containing a culture medium, for example a shake flask, aeration-agitation bioreactor, percolated

impellor bioreactor, draught tube air-lift bioreactor, draft tube with lasplan Turbine bioreactor, air-lift loop bioreactor, rotating drum bioreactor, and spin filter bioreactor. In one embodiment, a shake flask is used as the bioreactor. The bioreactor can be sized from about 250ml to about 1 Liter. The bioreactor should contain a medium such as sterile seawater, seawater, based media and serum-free medium. In one embodiment, the bioreactor contains sterile seawater prior to the addition of the tissue (batch processing). The tissue in medium can be agitated between about 75rpm to about 250rpm for approximately 5 to 25 minutes.

Following agitations, from about 50ml to 500ml of the bioreactor contents are extracted into a second bioreactor containing a volume of medium, such volume ranging from about 25ml to about 100ml, the medium being either the same as in the first bioreactor, or different. In one embodiment, the medium in the second bioreactor is the same as that used in the first reactor. The final concentration in the second bioreactor should be from about .04g tissue/ml to about .10g tissue/ml.

An extract of about 10% of the previous second bioreactor volume is then taken out and placed onto a suitable substrate. The volume of extract can range from about 2.5ml to about 60ml. The substrate can be made of glass, plastic, ceramic, or a synthetic resin, and contain a growth medium, for example agar. The substrate can include

adherent reactors such as roller bottles, plastic bag, multi-dish, multi-tray, multi-plate, spiral film, glass beads, propagator, and the like. Microcarrier culture methods, such as polymer beads or glass beads, can also be
5 used. Microcarrier can be made of dextran, polyacrylamide, polystyrene cellulose, gelatin, and glass. The microcarriers may optionally be coated with collagen, or negative charge of dimethylanimoethyl, diethylaninopropyl, and trimethyl-z-hydroxyamion propyl groups.

10 Following colony formation on the substrate, the colonies may be optionally purified by methods including concentration of suspended particles, extraction, adsorption ion exchange, and the like.

The resultant pure cultures of the *Vibrio* species are
15 then placed into a medium used as a seed culture in subsequent steps. Transfer may occur by fitting a hose and tank coupling device to the bioreactor. In another embodiment, the culture may be transferred first to a sterile metal container which is attached to the seed
20 fermenter. The medium in the seed culture may consist of amounts of sugars, carbon, nitrogen, water, minerals, salts, polyols, and other elements required for growth. Natural ingredients, such as soy peptones, oatmeal, and the like can also be used. In one embodiment, the medium is comprised of
25 a soy peptone in an amount of from about .25g to about 1g, yeast extract in an amount of from about .25g to about 1g, glucose in an amount of from about .25 to about 1g,

distilled water, an agent for pH adjustment, at a pH of between 7.0 to 7.7. Incubation of the seed culture may occur between 20-30°C for 24 to 48 hours, the seed medium may be from 150mL to 1000mL in volume.

5 The number of successive steps involving growth in the seed medium can depend on the scale of the production of TTX in the final phase. From stage to stage, there can be a tenfold volume increase in inoculum volume. However, in some embodiment, the inoculum volume can be larger than a tenth
10 of the fermenter volume.

 The culture may then be transferred to the fermenter. In one embodiment, transfer is accomplished by air pressure. The medium in the fermenter can be comprised of soy protein, yeast extract, sugars, distilled water, and the like. In one
15 embodiment, soy protein is used in an amount from about .25g to about 1.0g, yeast extract is an amount from about .25g to 1.0g, sucrose from about 2.5g to about 10g, distilled water to level, and a buffer to adjust pH to about 7.1 to about 7.7, for example 2M ammonia or 2M hydrochloric acid. The
20 fermenter chamber has a temperature between 28°C to 35°C, and the medium is supplied with sterile air and agitation.

 To the fermenter medium, a growth tissue extract is added simultaneously with the culture. Simultaneously does not refer to both the culture and the growth tissue extract
25 being added at the same precise moment, rather it refers to the culture and the growth tissue extract being in the fermenter, specifically the fermenter medium, at the same

time. The growth tissue extract or the culture may be added one before the other and vice versa. The growth tissue extract and the culture may inoculate in the medium for the same period of time, or one slightly less time than the other. Modifications in the length of times of either remaining with the medium is within the scope of this invention and is not a derivation therefrom. As will be discussed later, the growth tissue extract is called such because of its possession of growth factor elements.

The growth tissue extract can be taken from the organism used in obtaining the culture 101. In another embodiment, the growth tissue extract may be taken from a different organism or species provided such organism provides growth factors and TTX synthesis is exhibited in its tissue. The growth tissue extract may be obtained from the skin, intestine, liver, or ovary of the organism. In one embodiment, the extract is obtained from the liver or ovary. Following excising of the growth tissue extract, the extract is homogenized with a saline solution, and sterilized by filtration. The extract, prior to use, may be stored at between about -80°C to about -60°C . The extract can be inoculated with the fermenter medium at a concentration of between about .5g/L to about 1.5g/L.

Through the addition of the extract to the fermentation medium with the culture, it is believed growth of TTX will be synergistic as compared with the culture in the medium by

itself. Such synergistic growth is likely brought about through growth factors added via the tissue extract.

Fermentation is allowed to continue for at least 60 hours, or until there is an initial drop in observed cell density due to cell lysis. In isolating tetrodotoxin from the fermenter medium, as will be discussed later, various steps may be used in conjunction, for example centrifuging, filtration, solvent extraction, ion exchange, adsorption, elution, pH-adjustment, heating, evaporation, drying, and other steps as deemed necessary to obtain highly pure, high yield tetrodotoxin (TTX). In one embodiment, a yield of at least 90% pure TTX is obtained in an amount of at least 0.5mg TTX/L in a maximum of 3 to 5 days. In another embodiment, a yield of over 95% pure TTX in an amount of 1mg TTX/L in a maximum of 5 to 7 days is obtained.

FIG 2 is an embodiment of the method of isolating and purifying TTX from the culture in accordance with the instant invention.

In the method, the fermentation medium is firstly pH adjusted to between 3.0 and about 4.0. In one embodiment, this is done with an acid such as hydrochloric acid. Additionally, the medium is heated to between 90°C and 100°C for approximately 8 to 12 minutes. The medium is then centrifuged, such as by a tubular-bank centrifuge, disk centrifuge, filtering centrifuge, vertical basket centrifuge, and the like. The medium may be centrifuged at a

speed of 2000 to 9000 rpm for a period of between 10 minutes to 20 minutes at a temperature of 1°C to 10°C. The supernatant liquid is then retained 205 and pH-adjusted to around 6.

5 Next, the supernatant liquid is evaporated 207. Evaporation may occur through an evaporation system that consists of one or more evaporators, installed in series. The evaporators may be selected from forced circulation, submerged-tube forced circulation, oslo-type circulation, 10 short-type vertical, propeller calandria, long-tube vertical, reairculating long-tube vertical, falling film, horizontal tube, and the like. The supernatant can be evaporated at a temperature between about 40°C and 55°C, preferably at a reduced pressure. The supernatant is 15 evaporated to between 2% to about 8% of its original volume, resulting in the resultant TTX.

 An adsorption medium, such as activated carbon, is then mixed with the resultant TTX 209. The adsorption medium is preferably used in an equal volume to the resultant toxin 20 extract.

 An elution step on the adsorption medium is then performed 211. In a preferred embodiment, the adsorption medium is eluted at least 2 times, more preferably 3 times. The elution may occur through a 1:20 acetic acid/ ethanol 25 solution.

 An evaporation of the eluant collected may be performed 213. In one embodiment, evaporation may occur between 40 to

50°C in reduced pressure. Evaporation occurs until the eluant is between .5% to 2% of its original volume. In an alternate embodiment, prior to evaporation 213, the eluant may be pH-adjusted to around pH 6.

5 Following evaporating, the resultant extract, TTX, is loaded unto an ion exchange column 215. In a preferred embodiment, the column is packed with weak cation exchange resin beads. The toxic extract is then eluted therefrom 217, with a weak acid, for example 0.5% acetic acid. The extract
10 can then be pH adjusted to around 6, and evaporated 219 between 40°C to 50°C under reduced pressure to about .5% to 2% of its original volume.

 The extract can be dried 221, for example by freeze drying, spray drying, vacuum drying, fluid bed drying, etc.,
15 following by dissolution in water such as distilled water, ddw, and the like. The resultant solution is loaded unto another column 223, such column preferable possessing weak cation exchange resin beads. The column is eluted 225, and the eluant collected. The resultant eluant extract can be pH
20 adjusted to around 6, followed by evaporation 227 between 40°C to 50°C to between .5% and 2% of its volume. The extract can then be dried, such as by freeze drying, spray drying, vacuum drying, fluid drying, etc.

 As stated previously, the resultant TTX is at least 90%
25 pure TTX, and in a preferred embodiment, over 95% pure TTX.

Adjustment to the above methods may be made to meet the needs of scale-up without deviating from the scope and breath of this application. Techniques in scaling up are well known in the art and to those skilled therein.

5

EXAMPLE

Several strains of *Vibrio* species were screened and isolated from the intestines of puffer fish species *Takifugu alboplumbeus*. The strains was stored on ORI agar plates at 4
10 degrees Celsius and subcultured to fresh plates before inoculation to culture media.

All culture media were sterilized by autoclaving at 121 degree Celsius for 20 minutes. Seed medium was prepared from the following components (per 1000ml of medium):

15

TABLE 1	
Phytone-peptone	1.0g
Yeast extract	1.0g
Glucose	1.0g
Distilled water	to 1000 ml
pH	7.5
(adjusted with 1 M NaOH)	

A discrete colony of each *Vibrio* species on an agar plate was transferred aseptically with an inoculation loop and was suspended in 250 ml seed medium in a 500 ml culture flask. The inoculated culture was incubated at 25 degrees
20 Celsius for 24-48 hours in an incubation shaker at 100 rpm.

Takifugu alboplumbeus tissue extract was prepared from the following components and sterilized by filtration through a 0.2 μ m membrane:

TABLE 2	
Ovary (<i>Takifugu alboplumbeus</i>)	100g
Phosphate buffered saline	100ml
pH	7.5
(adjusted with 2M hydrochloric acid)	

A fermentation medium was prepared from the following components (per 1 L of medium):

TABLE 3	
Phytone-peptone	1.0g
Yeast extract	1.0g
Sucrose	10g
Distilled water	to 1000ml
pH	7.5
(adjusted with 2 M ammonia or 2M hydrochloric acid)	

5 200ml of seed culture and 10g of *Takifugu alboplumbeus* tissue extract were used to inoculate 10L fermentation medium aseptically in a 12L fermenter. The fermenter was supplied with sterile air and agitation, and was maintained at a temperature of 30 degrees Celsius and a pH of 7.5 for 3
10 days.

The fermentation process was completed after at least 60 hours when initial drop in cell density due to cell lysis observed. Before collecting the fermentation medium from the fermenter chamber, the pH of the medium is adjusted to 3.5
15 with 2M hydrochloric acid and heated to 95°C for 10 minutes. The medium is centrifuged at 8000 rpm at 4°C for 15 minutes to remove microscopic cells. Only the supernatant in each centrifuge tube is retained, which is then adjusted to pH 6 with 2M ammonia solution. The pooled supernatant is
20 evaporated at 45°C under reduced pressure to 5% of its

original volume and then mixed with an equal amount of activated carbon (v/w). The adsorbed toxin in the carbon is eluted with 1% acetic acid in 20% ethanol for three times. The eluant is adjusted to pH 6 with 2M ammonia before
5 evaporation at 45°C in reduced pressure to 1% of its original volume. The toxin extract is loaded onto a column (3x50 cm) of weak cation exchange resin (Bio-Rex® 70 Resin). The toxin is eluted with 0.5% acetic acid and the toxic fractions are monitored with HPLC analyses or mouse
10 bioassay. The toxic factions are pooled together and adjusted to pH 6 with 2M ammonia solution before evaporation at 45°C under reduced pressure to 1% of its original volume. The extract is freeze-dried and then dissolved in distilled water, which is loaded onto another column (1x80 cm) of weak
15 cation exchange resin (Bio-Rex® 70 Resin). The toxin extract is eluted with 0-0.2% acetic acid (linear gradient) and the toxic fractions are monitored with HPLC analyses or mouse bioassay. The toxic factions are pooled together and adjusted to pH 6 with 2M ammonia solution before evaporation
20 at 45°C under reduced pressure to 1% of its original volume. The extract is finally freeze-dried and it gives over 95% pure TTX.

FIG 3 compares the production of TTX in accordance with
25 the present invention 301 against TTX production by prior methods 303, and TTX production by simply growing the *Vibrio* culture in a flask 305. As shown by FIG 3, TTX production

via the present invention 301 is quite rapid, yielding 0.5mg TTX/L within 3 days. The prior art 303 shows 0.4mg TTX/L within 6 days, and less than 0.1mg TTX/L by mere flask culturing 305 in over 10 days.

5

The above system and measurement in accordance with the present invention can be controlled and monitored by instrumentation and control system. Such instrumentation and control system may contain on-line sensors, such as probes
10 including thermometer, thermometer, pH reference electrodes, Ag and Pb probes, conductance probes, capacitance probes, on-line instruments including tachometers, watt-meters, mass flow meters, rotameters, strain gauges, load cells, spring diaphragm, oil filled diaphragm, electro magnetic flow
15 meters, vortex devices, and gas analysers including paramagnetic analyser, mass spectrometers, and infrared analyser. Off line instruments can also be included, such instruments to measure packed cell volume, dry weight, optical density, microscopic observation, Coulter counter,
20 and plate counts. The control systems may also comprise one or more controllers, such controller possessing processors, memory both temporary and permanent, algorithms for operating the production and fermenters, User Interface Devices, including displays, keyboard, mouses, etc.
25 Examples of controllers include computers, pda's, laptop computers, and control panels.

Having described embodiments of the present system with reference to the accompanying drawings, it is to be understood that the present system is not limited to the precise embodiments, and that various changes and
5 modifications may be effected therein by one having ordinary skill in the art without departing from the scope or spirit as defined in the appended claims.

In interpreting the appended claims, it should be understood that:

10 a) the word "comprising" does not exclude the presence of other elements or acts than those listed in the given claim;

b) the word "a" or "an" preceding an element does not exclude the presence of a plurality of such elements;

15 c) any reference signs in the claims do not limit their scope;

d) any of the disclosed devices or portions thereof may be combined together or separated into further portions unless specifically stated otherwise; and

20 e) no specific sequence of acts or steps is intended to be required unless specifically indicated.

CLAIMS

1. A method of biosynthesizing tetrodotoxin, comprising the steps of:

5 -obtaining a culture from a seed culture or storage possessing one or more *Vibrio* species;

 -simultaneously inoculating said culture and a growth tissue extract excised from a tetrodotoxin-bearing organism in a fermenter with a medium; and

10 -isolating and purifying tetrodotoxin from said fermenter medium.

2. The method of biosynthesizing tetrodotoxin in claim 1, further comprising, before obtaining said culture:

15 -excising a tissue extract from a tetrodotoxin-bearing organism;

 -adding said tissue extract to a bioreactor;

 -extracting the bioreactor contents into a second bioreactor;

20 -extracting the second bioreactor contents onto a substrate; and

 -placing the resultant cultures from said substrate into a medium to form the seed culture.

3. The method of biosynthesizing tetrodotoxin in claim 1, wherein said one or more *Vibrio* species is selected from

the group consisting of *Vibrio nigripulchritudo*, *Vibrio mediterranei*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio salmonicida*, *Vibrio tubiashii*, *Vibrio parahaemolyticus*, *Vibrio campebellii*, *Vibrio natriegens*, *Vibrio nereis*, *Vibrio*
5 *carchariae*, *Vibrio fluvialis*, *Vibrio fischeri*, *Vibrio vulnificus*, *Vibrio splendidus*, *Vibrio orientalis*, *Vibrio aestuarianus*, *Vibrio pelagius*, *Vibrio wodanis*, *Vibrio furnissii*, *Vibrio proteolyticus*, *Vibrio ichthyocenteris*, *Vibrio pectenocida*, *Vibrio logei*, *Vibrio mimicus*, *Vibrio*
10 *mytili*, *Vibrio rumoiensis*, *Vibrio anguillarum*, *Vibrio gazogenes*, *Vibrio halioticoli*, *Vibrio hollisae*, *Vibrio ordalii*, and *Vibrio metschnikovii*.

4. The method of biosynthesizing tetrodotoxin in claim 2, wherein said one or more *Vibrio* species is obtained from
15 *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio fluvialis*.

5. The method of biosynthesizing tetrodotoxin in claim 2, wherein said tissue extract is taken from organs including intestine, liver, ovary, stomach, or skin.

20 6. The method of biosynthesizing tetrodotoxin in claim 2, wherein said tissue extract is from a tetrodotoxin-bearing organism selected from the group consisting of flatworms, ribbon worms, gastropods, blue-ringed octopuses, starfish, sea urchins, xanthid crabs, horseshoe crabs, gobies, frogs,
25 neuts, and puffer fish.

7. The method of biosynthesizing tetrodotoxin in claim 2, wherein said tissue extract is from puffer fish.

8. The method of biosynthesizing tetrodotoxin in claim 7, wherein said puffer fish can be selected from the group consisting of *Arothron hispidus*, *Arothron stellatus*, *Chelonodon patocoa*, *Takifugu alboplumbeus*, *Takifugu niphobles*, *Takifugu oblongus*, *Takifugu ocellatus*, *Takifugu xanthopterus*, and *Diodon holocanthus*.
9. The method of biosynthesizing tetrodotoxin in claim 1, wherein said growth tissue extract is excised from the organs including liver, intestine, ovary, stomach, or skin.
- 10 10. The method of biosynthesizing tetrodotoxin in claim 1, wherein said growth tissue extract is excised from organs from tetrodotoxin-bearing organisms selected from the group consisting of flatworms, ribbon worms, gastropods, blue-ringed octopuses, starfish, sea urchins, xanthid crabs, horsehoe crabs, gobies, frogs, newts, and puffer fish.
11. The method of biosynthesizing tetrodotoxin in claim 2, wherein said bioreactor is a shake flask.
12. The method of biosynthesizing tetrodotoxin in claim 2, wherein said medium in said seed culture is comprised of soy peptone, yeast extract, glucose, and distilled water.
- 20 13. The method of biosynthesizing tetrodotoxin in claim 1, wherein said fermenter medium comprises soy peptone, yeast extract, sugars, distilled water, and buffers.
14. The method of biosynthesizing tetrodotoxin in claim 13, wherein said fermenter medium is exposed to sterile air and agitation.
- 25

15. The method of biosynthesizing tetrodotoxin in claim 2, wherein said growth tissue extract is excised from a different organism or species than the source of said tissue extract added to said bioreactor.

5 16. The method of biosynthesizing tetrodotoxin in claim 1, wherein said growth tissue extract contains one or more growth factors.

17. The method of biosynthesizing tetrodotoxin in claim 1, wherein isolating and purifying tetrodotoxin from said
10 fermenter comprises the steps:

- adjusting the pH and heating of said fermenter medium;

- centrifuging said medium;

- evaporating supernatant liquid collected from said
15 centrifuged medium;

- mixing an adsorption medium with a resultant toxin;

- eluting said adsorption medium;

- evaporating an eluant collected from said eluted adsorption medium;

- loading a resultant tetrodotoxin extract unto an ion
20 exchange column;

- drying said resultant tetrodotoxin extract;

- loading a resultant tetrodotoxin extract solution
unto a second ion exchange column;

- 25 -eluting said second ion exchange column;

- evaporating an eluant collected therefrom; and
- drying said eluant.

18. The method of biosynthesizing tetrodotoxin in claim 17, whereby said method results in at least 90% tetrodotoxin in an amount of at least 0.5mg TTX/L in a maximum of 3 to 5 days.

19. A method of biosynthesizing tetrodotoxin, comprising:

- obtaining a culture from a seed medium possessing one or more *Vibrio* species, said seed medium created through the steps of a) through d);

a) excising a tissue extract from a tetrodotoxin-bearing organism;

b) adding said tissue extract to a bioreactor;

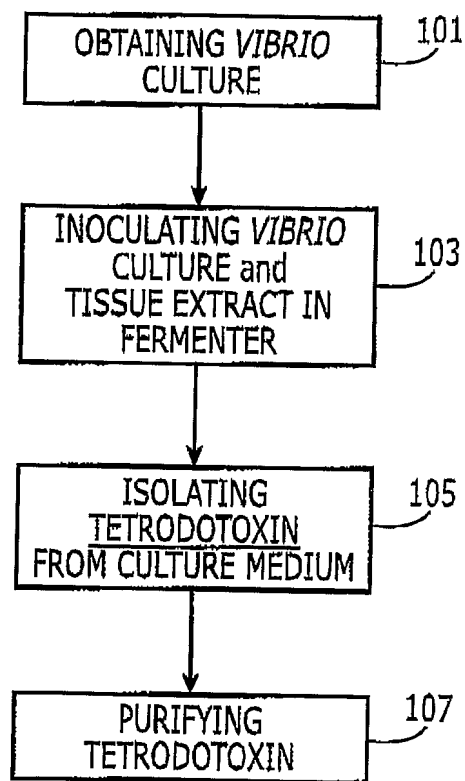
c) extracting said bioreactor contents onto a substrate; and

d) placing the resultant cultures from said substrate into a medium of a seed culture.

-simultaneously inoculating said culture and a growth tissue extract excised from a tetrodotoxin-bearing organism in a fermenter with a medium; and

-isolating and purifying tetrodotoxin from said fermenter medium.

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**FIG. 1**

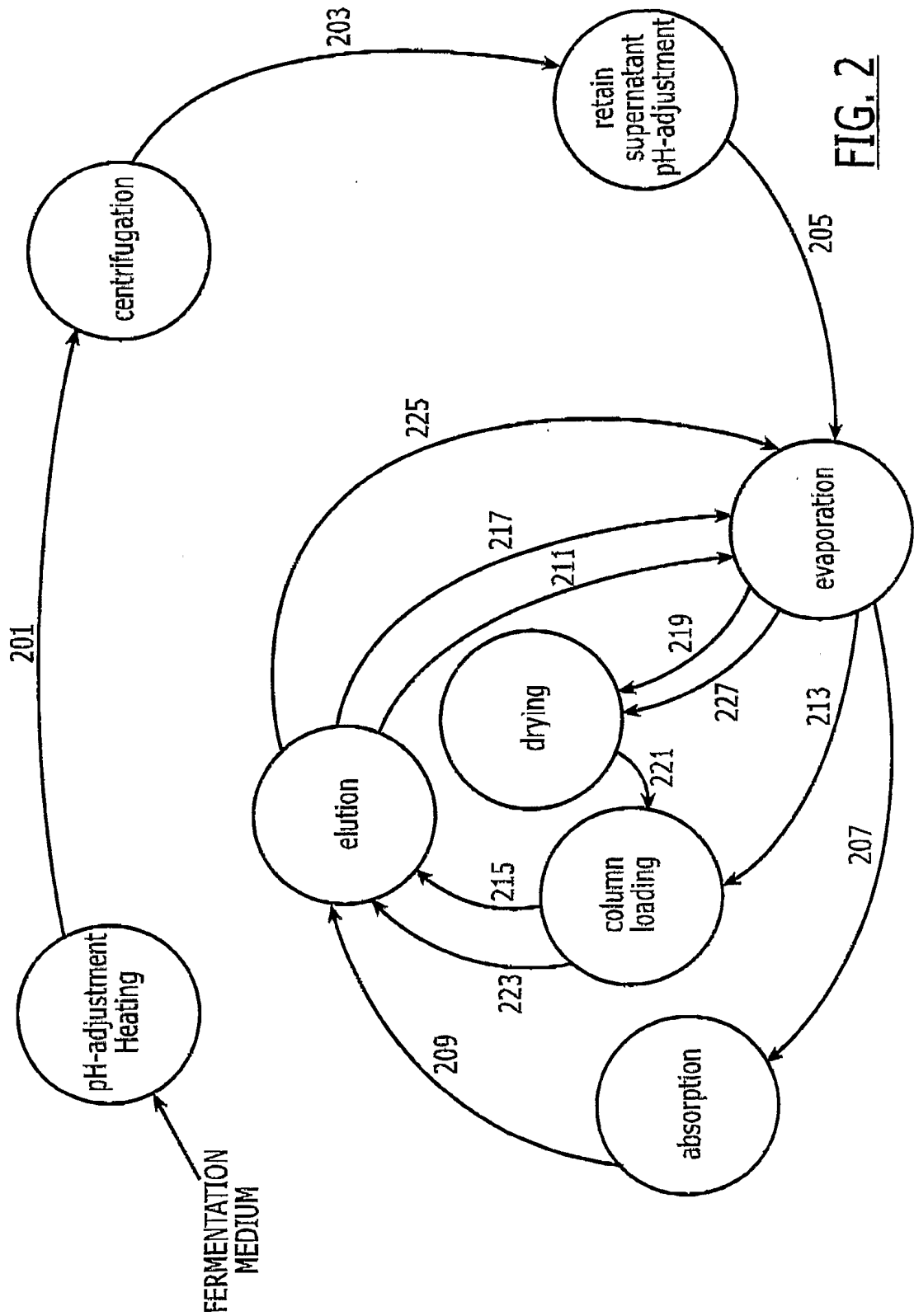


FIG. 2

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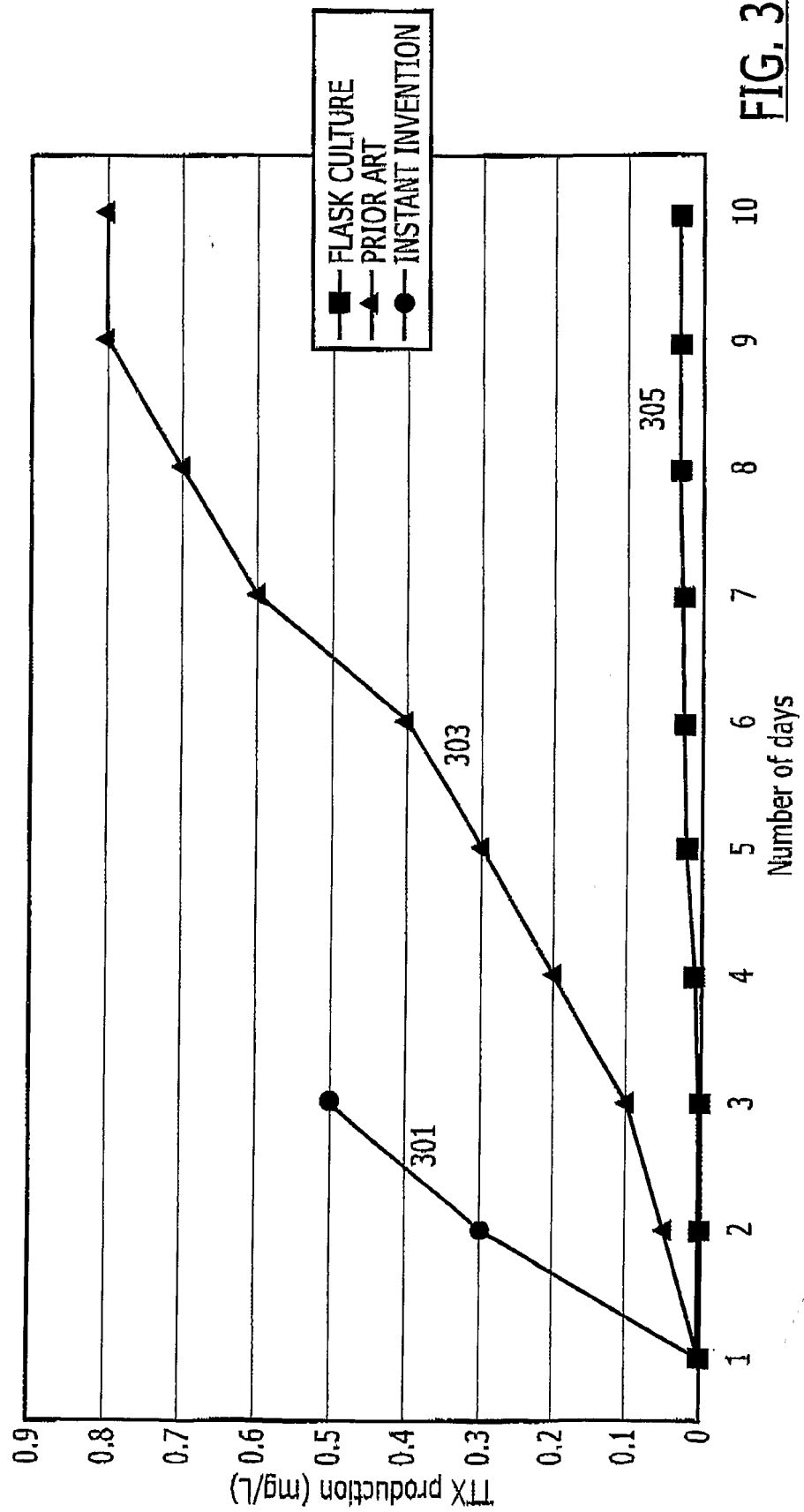


FIG. 3