AUSTRALIA

SPRUSON & FERGUSON

AUSTRALIA

PATENTS ACT 1990

PATENT REQUEST: STANDARD PATENT

I/We, the Applicant(s)/Nominated Person(s) specified below, request I/We be granted a patent for the invention disclosed in the accompanying standard complete specification.

[70,71] Applicant(s)/Nominated Person(s):

Development Center For Biotechnology, of 81, Chang-hsing Street, Taipei, TAIWAN

[54] Invention Title:

A Method for Preparing an Environmentally Compatible Porous Material

[72] Inventor(s):

Liang-Kuang Chu, Chiao Po Lin, Chien-Ming Shih, Tsae Yueh Lee and Tai-Sen Soong

[74] Address for service in Australia:

Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia (Code **SF**)

DATED this TWENTIETH day of OCTOBER 1997

Development Center For Biotechnology

By: J. Lin

Registered Patent Attorney

IRN: 394966

INSTR CODE: 57261

S080364 20 OCT 1997

AU9742727

(12) PATENT ABSTRACT (11) Document No. AU-A-42727/97
(19) AUSTRALIAN PATENT OFFICE

(54) Title
A METHOD FOR PREPARING AN ENVIRONMENTALLY COMPATIBLE POROUS MATERIAL

(51)[6] International Patent Classification(s)
A01N 063/00

(21) Application No. : **42727/97** (22) Application Date : **20/10/97**

(43) Publication Date : **06/05/99**

(71) Applicant(s)
DEVELOPMENT CENTER FOR BIOTECHNOLOGY

(72) Inventor(s)
LIANG-KUANG CHU; CHIAO PO LIN; CHIEN-MING SHIH; TSAE YUEH LEE; TAI-SEN SOONG

(74) Attorney or Agent
SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001

(57)

The invention relates to a method for preparing an environmentally compatible porous material comprising beneficial nematodes with insecticidal activity, and to the biotic preparations produced therefrom. The environmentally compatible porous material may be directly applied to the desired place to eliminate the recovery procedures and formulation steps in the prior art.

S & F Ref: 394966

AUSTRALIA

PATENTS ACT 1990

COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

Name and Address of Applicant: Development Center For Biotechnology
81, Chang-hsing Street
Taipei
TAIWAN

Actual Inventor(s): Liang-Kuang Chu, Chiao Po Lin, Chien-Ming Shih, Tsae Yueh Lee and Tai-Sen Soong

Address for Service: Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower, 31 Market Street
Sydney, New South Wales, 2000, Australia

Invention Title: A Method for Preparing an Environmentally Compatible Porous Material

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

A Method for Preparing an Environmentally Compatible Porous Material

Field of the invention

The invention relates to a method for preparing an environmentally compatible porous material comprising beneficial nematodes with pesticidal activity, and to the biotic preparations produced therefrom and to the use for controlling pests.

Background of the invention

Nematode-based pesticide is not a nematicide but a kind of novel pesticide, and a biotic preparation which comprises beneficial nematodes associated with the symbiotic bacteria thereof for controlling pests.

Nematode-based pesticides differ from chemical pesticides For instance, general chemical pesticides are highly toxic, pesticide poisonous, and easy to result in environmental pollution; they affect natural predators, destroy ecological balance, and often cause the resistance against the targeted pests.

The beneficial nematodes which are often used in nematode-based pesticides include *Rhabditida*, in particular, *Rhabditidae*, *Steinernematidae* and *Heterorhabditidae*. Similar to most of nematodes, they have a simple life cycle comprising egg, 4 larvae stages and adult. The infective stage is "stage 3 infective juvenile (J3)", or called "infective juvenile (IJ)". IJs are especially resistant to environmental conditions, and enter into hosts through the original opening on the body of hosts, such as mouth, anus or respiration pores. The insecticidal course is described as follow: the infective nematode will actively penetrate the gut wall or trachea and enter the hemocoel, whereupon the symbiotic bacteria therein, such as *Xenorhabdus,* will be released to the host. The symbiotic bacteria will rapidly in the host body. The host will die from septicemia in about 48 hours After uptaking bacterium cells and tissues of the host, the immature nematode will develop to a adult (1).

The beneficial nematodes are harmless to plants and safe to human beings and domesticated animals. In 1987, the Environmental Protection Administration of United States had established that biotic preparations comprising *Steinernema* and *Heterorhabditis* and symbiotic bacteria thereof may be not necessary for the statutory requirement of FIFRA Section 25(b)(1). They are commercially available and have not be registered in many countries. They have no effects on the other non-targeted arthropods in soil, and there are no evidences for showing the development of resistance by pests.

US 4 334 498 (2) disclosed a method for rearing nematodes. However, the patent provides the method for cultivating nematodes cultivate and the steps of harvesting the nematodes; but no treatment, storage and application after recovering the nematodes. Therefore, the method cannot be applied to the field to kill pests.

The techniques for rearing nematodes so far in the world are mostly based upon the solid and liquid culture. The most important technique of nematode-based pesticide still

focuses on the technology of the mass production and the formulation of preparations. Because nematodes are multicellular animals, the preparations are hard to attain the purpose of prolonged storage as other microbiotic preparations (such as storage for one year or above). For instance, the methods described in WO 89/04602 (3) and US 4 615 883 (1986) (4) (see, Fig. 1), Biosys Corp. in USA utilise alginate to embed nematodes, and can store the preparations for 3 to 6 months. However, it has to dissolve nematodes by using sodium citrate (taking about 30 minutes), and then be diluted with water before spraying onto crops. Accordingly, they are not convenient for substantial application.

The methods described in US 4 334 498 and WO 88/08668 (5) (see, Fig. 1), used the principle of nematode cryptobiosis, to carry out the dehydration of nematodes under the conditions for decreasing relative humidity stepwise and to make the nematodes into clays. The storage for the clays obtained may be 3 to 6 months under refrigerated conditions. However, it spends too much time and energy in the dehydration procedure, so that the cost required will be relatively high. In addition, the clays easily harm to the lungs of human and have to be treated at high humidity (95% relative humidity) overnight for rehydrating nematodes in the next day. Therefore, the methods are too complicated to be utilised conveniently.

The methods for producing nematode-based insecticide are similar to that for fermenting general microorganisms, except the additional more complicated procedures of cultivating sterile source and the mixed cultivation along with their biosymbiotic bacteria. Because nematodes are multi-cellular animals, the cultivation periods are longer than those of cultivating other microorganisms (for example, it takes 11 and 21 days for the liquid and solid culture, respectively). As illustrated in Fig. 1, the methods used in conventional process need additional steps from recovery to storage. In particular, the recovery step is complicated and labor cost, and cause the loss in nematodes. Moreover, the recovery step always cause the problem of waste water. It presents the same situation in producing the preparation. Besides, the cultivation period of nematode is very long, and the recovery procedure takes lots of work so that the cost for production is relatively high accordingly. Therefore, the costs of nematode-based pesticidal biotic preparations tend to be high, and the price of products are also expensive. Furthermore, the methods for producing nematode-based insecticide used in conventional culture require two separable steps of cultivating symbiotic bacteria and inoculating nematodes in medium before recovery procedure. It also takes lots of work to accomplish the steps, especially for solid culture, in comparison with one-step method for cultivating symbiotic bacteria and inoculating nematodes in medium.

T.N. Wang *et al* in Sugar Research institute (1992) (6) had reported that they utilise sugar dregs to absorb nematode liquid and apply them to the field. (The nematodes may be stored at room temperature for about one month.) However, the use of bagasse as the matrix for culturing nematodes (adding culture medium) is not disclosed in the article. In addition, the bagasse will easily attacked by fungi due to sugar residue.

Georgis. R. p178, Formulation and application technology in "Entomopathogenic Nematodes in Biolcgical Control" (7) only describes that vermiculite and peat can be used as moistened carriers for transporting and storing nematodes. However, the nematodes should be cultured and recovered prior to applying vermiculite and peat. As previously reported, the recovery step is cost-consuming, and cause the loss in nematodes and the problem of waste water. In addition, Georgis did not use vermiculite or peat as a support medium for culturing nematodes.

The disadvantages of the prior art can be minimised by the present invention.

Summary of the invention

It is an object of the present invention to provide a biotic preparation for controlling insect comprising an environmentally compatible porous material containing beneficial nematodes with pesticidal activity in which the nematodes are obtained by incubating symbiotic bacteria and the nematode source in the porous material containing production medium

It is an other object of the present invention to provide a method for preparing an environmentally compatible porous material containing entomogenous beneficial with pesticidal activity comprising inoculating symbiotic bacteria and the nematode source in the porous material containing production medium.

It is a further object of the present invention to provide a method for controlling pests comprising treating the desired objectives to be kept free from the pests with an environmentally compatible porous material containing beneficial nematodes with pesticidal activity.

Brief description of figures

Fig. 1 describes the schemes of the methods used by conventional liquid culture and solid culture and the invention for preparing pesticidal preparations.

Detailed description of the invention

Definitions

The following terms are used in illustrating the description, examples and claims of the invention.

The term "beneficial nematodes" refers to nematodes including Rhabditida, in particular, *Rhabditidae*, *Steinernematidae* and *Heterorhabditidae*, which kill hosts by its association with the symbiotic bacteria.

The term "environmentally compatible" refers to "without causing environmental contamination or poison, and agreeable to the relative regulations of environmental conservation."

The term "biodecompositable" refers to "decompositable or destructible by microorganisms or organisms occurring in the nature."

The term "porous material(s)" refers to material(s) with many pores. The porous materials used herein include environmentally compatible porous material (for example, vermiculite) or biodecompositable porous material (for example, sponge, loofah, etc.), both of which may be useful in mass production of entomogenous nematodes and make no pollution to the public.

"controlling pests" includes repulsion and destruction. Repulsion refers to the procedure that insects or slugs will escape far away from the desired place without causing any destructive effect. Destruction refers to the procedure that nematodes will kill the insect hosts or slugs thereof by its association with the symbiotic bacteria.

For more detailed description of the technology and characteristic of the present invention, it is provided by following examples to illustrate the invention.

Materials and Methods

Tested nematodes: *Steinernema carpocapsae* (SC) strain A11 (SCA). [After the *in vivo* culture in silkworm or tobacco cutworm, it was isolated and stored at 10°C.] The monoxenic nematodes were obtained by the method disclosed in Edward J. and Irene Popiel in Journal of Nematology 21(4):500-504, 1989 (8).

Tested bacteria: the symbiotic bacteria isolated from the intestine tract of SCA, and which was identified by Food Science & Technology Research Institute in Taiwan as *Xenorhabdus nematophilus*. It was stored at -70°C.

The composition of TSA (Tryptic Soy Agar) is listed as follows:

TSA (Difco)	40g
distilled water	1L

The composition of YS is listed as follows:

K_2HPO_4 (Merck)	0.5g
$NH_4H_2PO_4$ (Sigma)	0.5g
$MgSO_4\ 7H_2O$ (Merck)	0.2g
NaCl (Merck)	5.0g
yeast extract (Merck)	5.0g
distilled water	1L

The composition of production medium (PM) is listed as follows:

egg (ordinary supermarket)	500g
yeast powder (Taiwan Sugar Corp.)	12g
Salad oil (Taiwan Sugar Corp.)	60g
distilled water	1L

The sequential procedure of cultivating the symbiotic bacteria was described as follows: incubating the bacteria at –70°C in TSA; at 28°C for 24 hours; in YS liquid medium for 16 hours (at 25°C, 150rpm, in 30mL/125mL flask).

The culture is thoroughly mixed with monoxenic nematode source to form a uniform mixture. Then, the mixture is inoculated into environmentally compatible porous material including production medium autoclaved at 121°C for 30min in a sealable vessels, for a period sufficient to the nematodes populated in the porous material (such as 15-21 days) at

25°C to produce environmentally compatible porous material comprising the nematodes populated therein.

The environmentally compatible porous material comprising the nematodes can be directly applied to the desired place such as field, grass, etc.) for controlling pests.

Insect for Bioassay: 4th instar Tobacco cutworm *(Spodoptera litura).*

Method for Bioassay: Petri-disk and filter paper method disclosed in Jennifer L. Woodring *et al*, 1988 (1). The ratio of nematodes to insects = 25:1.

The plastic sponge (2 x 2 x 2cm^3) used in the tests was made of polyether polyurethane, which might be autoclaved and purchased from Jih-Hung Sponge Inc.

The vermiculite (No.2) and pearlite (No.2) used in the tests were all purchased from Nan-Hei Vermiculite Corp.

The glass containers used in the coincubation steps of nematodes and symbiotic bacteria were equipped by a 125mL flask(Pyrex) with sponge or 2 to 5g porous material described above.

Example 1

The solid culture of SCA in different materials

In the example vermiculite, pearlite and foam sponge (or called sponge) were used, and the addition ratio is listed in Table 1.10% symbiotic bacteria (ie. 2mL) and 10^4 monoxenic SCA were inoculated. After 14 day *(25*°C), the yields and the insecticidal activity were determined, and the results are listed in Table 1.

Table 1.

The effects on solid culture of SCA in different material

material	weight (g)	medium (mL)	yield (10^4/mL)	insecticidal activity
vermiculite	4.0	20	44	95
pearlite	5.0	20	11	70
sponge	2.0	20	49	80

Example 2

The cultivation of beneficial nematodes in environmentally compatible porous material (such as vermiculite)

A 14L vessel charged with 600g of vermiculite and 3000mL of PM was autoclaved for 30 minutes, then were inoculated 300mL of symbiotic bacteria and 2 million monoxenic SCA. After being cultured for 20 days at 25°C, the yield and pesticidal activity were determined. The results are listed in Table 2.

Table 2

vessel volume	yield (10^4/g vermiculate)	insecticidal activity
14L	29.5	100
flask	30.0	95

Example 3

Evaluations of the biological control on larvae of tobacco cutworm by SCA presented in vermiculite preparation in the pot test

Period of test: May 24, 1994 to June 10, 1994

Testing place: the 6th balcony of Developmental Center of Biotechnology (DCB, address: 81 Chang Hsing Street, Taipei, Taiwan).

Tested nematodes: SCA_1 which were cultured in vermiculate and PM medium.

Insects to be tested: the fourth instar of *Spodoptera litura* Fabricius (tobacco cutworm) which was provided by Professor Shih Cheng-jen in Department of Botanical Blight of National Taiwan University.

Grass to be tested: *Cynodon spp.* D.W., strain 419 and strain 328, obtained from the construction site of Green in Lung T'an and Yang-Sneng golf course (Yang Mei) respectively.

Pot: 20.5 x 13.5 x 7.5cm^3, area 276.75cm^3.

Design of test: completely random design (CRE)) was taken and repeated 4 times.

Application dosage: 2.4 billion nematodes/ha., 1.2 billion nematodes/ha., 0.6 billion nematodes/ha.

Each pot of grass was shaven 2 days before applying the worms. Twenty plantlets were examined in each pot. The bitetrace numbers on the top 6 leaves of each plantlet were calculated as the numbers of affected grasses and leaves.

Every pot of grass was irrigated with 400mL running water before the application of the pesticide and worm, and was irrigated with 150mL running water after the application to facilitate the release of nematode SCA from vermiculite. The results are listed in Table 3.

Table 3.

The effects of the biological controlling on tobacco cutworm by SCA cultured in vermiculite in the pot test

Treatment dosage (per ha)	repeat	number of damaged leaves	X±DS%	number of damaged grass	X±DS%
$2.4x10^9$	1	1	4±2.4b3.3	1	2±1.5c10
	2	5		2	
	3	2		1	
	4	6		4	
$1.2x10^9$	1	13	14±3.4b11.7	12	8±2.6b40
	2	10		6	
	3	13		8	
	4	15		7	
$0.6x10^9$	1	43	37±11.2a30.8	13	14±3.7a70
	2	38		14	
	3	21		9	
	4	46		15	
0	1	15	31±12.9a25.8	8	14±3.9a65

	2	44	17	
	3	26	14	
	4	38	15	

Data was analysed by One Way ANOVA and tested by Duncan's Multiple Range test. The different numbers behind means represent the significant difference between treatments (p=0.05).

The numbers of damaged leaves and grasses were calculated by random selection of 20 plantlets and the top 6 leaves of each plantlet, so the total number of leaves to be calculated was 120 leaves/per pot.

Results

(I) As shown in Table 1, a high yield of nematode can be obtained when using porous materials. The yield obtained by using sponge as culture material was the highest.

(II) As shown in Table 2, the addition of symbiotic bacteria facilitated mass rearing of nematode SCA. The yield of culture was 1.2 billion/vessel. The pesticidal activity at day 5 was 95%.

(III) The effects of the biological controlling on tobacco cutworm by SCA cultured in vermiculite in the pot test were as follows:

As shown in Table 3, the treatment at 2.4 x 10^9/ha and 1.2 x 10^9/ha were significantly better than that at 6 x 10^8/ha and control. For the damage of grass, the controlling effects were 2.4 x 1 0^9/ha > 1.2 x 10^9/ha > 6 x 10^8/ha. Nematode SCA presented in vermiculite preparation demonstrated the effect on preventing tobacco cutworm to destroy lawn. Therefore, the method for culturing nematode of the present invention produced at a high yield, and the insecticides made by the method have high pesticidal activity and preventive effects.

Moreover, the storage for the biotic preparations could be more than 6 months under refrigerated condition.

Although the examples of present invention have been described and illustrated in the specification and figures, but it should be appreciated that all of them are only the exemplification of the concepts and characters of present invention, and not for limiting the scopes of present invention and claims.

The claims defining the invention are as follows:

1. A biotic preparation for controlling insects comprising an environmentally compatible porous material containing beneficial nematodes with pesticidal activity in which the nematodes are obtained by being incubated symbiotic bacteria and the nematode source in the porous material containing production medium.

2. The biotic preparation as defined in claim 1, wherein said environmentally compatible porous material is vermiculite, peat or pearlite.

3. The biotic preparation as defined in claim 1 or claim 2, wherein said environmentally compatible porous material is biodecompositable.

4. The biotic preparation as defined in any one of claims 1 to 3, wherein said environmentally compatible porous material is in the form of granule.

5. The biotic preparation as defined in any one of claims 1 to 4, wherein said symbiotic bacteria is *Xenorhabdus* or *Photorhabdus*.

6. The biotic preparation as defined in any one of claims 1 to 5, wherein said nematode source is monoxenic or sterilised.

7. The biotic preparation as defined in any one of claims 1 to 6, wherein said nematode source is *Steinernema carpocapsae.*

8. A biotic preparation for controlling insects substantially as hereinbefore described with reference to any one of the examples.

9. A method for preparing an environmentally compatible porous material containing entomogenous nematodes with pesticidal activity comprising inoculating symbiotic bacteria and the nematode source in the porous material containing production medium.

10. The method as defined in claim 9, wherein said environmentally compatible porous material is vermiculite or pearlite.

11. The method as defined in claim 9 or claim 10, wherein said environmentally compatible porous material is biodecompositable.

12. The method as defined in any one of claims 9 to 11, further comprising the steps of mixing symbiotic bacteria and the nematode and forming a mixture for inoculation.

13. The method as defined in any one of claims 9 to 12, wherein said symbiotic bacteria is *Xenorhabdus* or *Photorhabdus.*

14. The method as defined in claim 13, wherein said *Xenorhabdus* is *Xenorhabdus nematophilus.*

15. The method as defined in any one of claims 9 to 14, wherein said nematode source is monoxenic or sterilised.

16. The method as defined in any one of claims 9 to 15, wherein said nematode source is *Steinernema carpocapsae.*

17. A method for preparing an environmentally compatible porous material containing entomogenous nematodes with pesticidal activity substantially as hereinbefore described with reference to any one of the examples.

18. A method for controlling pests comprising treating the desired objectives to be kept free from the pests with an environmentally compatible porous material containing beneficial nematodes with pesticidal activity.

19. A method as claimed in claim 18, wherein said environmentally compatible porous material is in the form of granule.

20. A method as claimed in claim 18 or claim 19, wherein said objectives are selected from the group consisting of grass, field, pot and lawn.

Dated 20 October 1997

DEVELOPMENT CENTER FOR BIOTECHNOLOGY

Patent Attorneys for the Applicant/Nominated Person

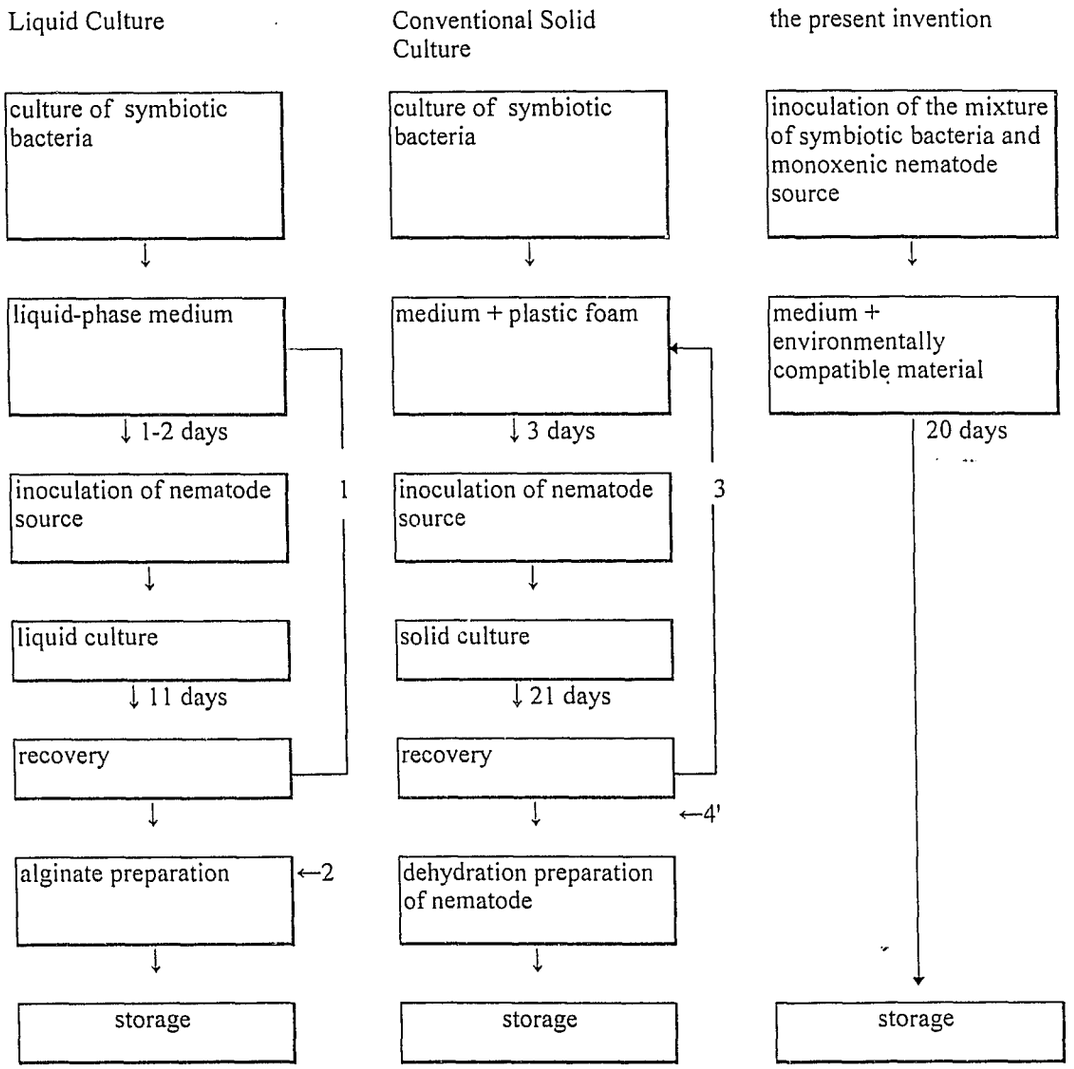
SPRUSON&FERGUSON

A Method for Preparing an Environmentally Compatible Porous Material

Abstract

The invention relates to a method for preparing an environmentally compatible porous material comprising beneficial nematodes with insecticidal activity, and to the biotic preparations produced therefrom. The environmentally compatible porous material may be directly applied to the desired place to eliminate the recovery procedures and formulation steps in the prior art.

Fig. 1



1. International Patent Application WO 89/04602 (1989)

2. US Patent 4,615,883 (1986)

3. US Patent 4,334,498 (1982)

4. International Patent Application WO 88/08668 (1988)

END